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Enzymatic Modification of Rice Glutelin (*Oryza sativa* L. japonica Group) for the Development of ACE-Inhibitory Bioactive Peptides

Ainaya Isnainun
Graduate School of Biotechnology,
University of Jember, Jember, Indonesia

Lailla Nur Safitri
Graduate School of Biotechnology,
University of Jember, Jember, Indonesia

Eka Putri Mawaddatum Khoiroh
Graduate School of Biotechnology,
University of Jember, Jember, Indonesia

Ari Satia Nugraha
Graduate School of Biotechnology, University of Jember, Jember,
Indonesia and Drug Utilisation and Discovery Research Group,
Faculty of Pharmacy, Universitas Jember, Jember, Indonesia

Tri Agus Siswoyo*
Graduate School of Biotechnology, University of Jember, Jember,
Indonesia and Department of Agronomy, Faculty of Agriculture,
Universityof Jember, Jember, Indonesia

ABSTRACT

Bioactive peptides derived from plant seeds have been shown to have various biological activities, particularly in nutraceutical and pharmaceutical applications. The fact that glutelin contributes approximately 80% of total protein content indicates that rice has strong potential to generate a wide variety of bioactive peptides through protein hydrolysis, including the ability to inhibit angiotensin I-converting enzyme (ACE), making it a potential candidate for managing hypertension. In this study, rice glutelin protein was hydrolyzed using three main proteases: pepsin, trypsin, alcalase, and a combination of pepsin and trypsin for five hours. Degree of hydrolysis (DH), $ABTS^{\bullet+}$ radical scavenging and ACE-inhibitory activity of the hydrolysates were measured. The results indicated the highest DH was obtained with pepsin- and alcalase-based hydrolysates with 23%. Additionally, the $ABTS^{\bullet+}$ radical scavenging activity of the hydrolysate produced by pepsin+trypsin combination (27.64 ± 0.09 µg/mL) the ACE-inhibitory activity of the hydrolysate produced by alcalase was the highest among the other proteases (5.85 ± 0.01 µg/mL).

Keywords: rice-glutelin protein, degree of hydrolysis, enzymatic hydrolysis, angiotensin I-converting enzyme (ACE)-inhibitory peptides, antioxidant peptides.

INTRODUCTION

Rice-derived bioactive peptides (BAPs) are increasingly recognized as important ingredients in the development of functional foods and nutraceutical formulations [1]. Bioactive peptides are short chains of amino acids that are once released from precursor proteins and can provide various health benefits and are commonly sourced from plant-based foods such as fruits, vegetables, grains, and legumes [2]. Seeds are rich sources of proteins, lipids, carbohydrates, minerals, and other essential micronutrients. Bioactive peptides derived from plant seeds have been shown to exhibit a wide range of biological activities, from antimicrobial effects to physiological benefits in humans [3]. Bioactive peptides act as antioxidants by neutralizing free radicals and reactive molecules to prevent cellular damage in the body [4]. Therefore, bioactive peptides play an important role in the mechanisms of various degenerative diseases, including cancer and hypertension [2], [4].

The storage proteins, which represent the second-largest component in rice, are classified into four major fractions: albumin, globulin, prolamin, and glutelin [5]. Glutelin in rice seeds is a high-quality plant protein that is rich in essential amino acids and is easily digested and absorbed by the body [6]. The fact that glutelin contributes approximately 80% [7], [8] of total protein content indicates that rice has strong potential to generate a wide variety of bioactive peptides through protein hydrolysis (protease). Its complex amino acid composition enables glutelin to generate bioactive peptides and may provide physiological health benefits [9]. Protease works by breaking down proteins and peptides through hydrolysis reactions involving water, and differences in substrate specificity for each protease lead to variations in the effectiveness of enzymatic hydrolysis [10], [11]. The generation of bioactive peptides is commonly achieved through enzymatic hydrolysis using proteases such as pepsin, trypsin, and alcalase. These enzymatically released peptides often exhibit functional activities, including the ability to inhibit angiotensin-converting enzyme (ACE), making them potential candidates for managing hypertension [12], [13], [14], [15].

ACE is a key enzyme in the renin-angiotensin system that helps regulate peripheral blood pressure by converting angiotensin-I into the vasoconstrictor angiotensin-II and degrading the vasodilator bradykinin, these actions can contribute to hypertension[13]. Although ACE inhibitors effectively reduce blood pressure and protect against related organ damage, they are associated with side effects like hypotension, impaired kidney function, angioedema, etc. As an alternative, naturally derived ACE-inhibitory peptides from rice are considered safer and tend to produce fewer adverse effects than synthetic drugs. Given that glutelin represents the major storage protein in rice grains, accounting for the largest proportion of the total seed protein, we assumed that its enzymatic hydrolysis would yield a broader range and higher abundance of bioactive peptides compared to unhydrolyzed proteins.

MATERIALS AND METHODS

Materials

Rice grain (*Oryza sativa* L japonica Group), Pepsin, Trypsin, Alkalase, ACE, 2,4,6-trinitrobenzene sulfonic acid TNBS (Wako, Japan), hippuryl-L-histidyl-L-leucine (HHL) (Bachem, Japan), and Captopril (Wako, Japan), and other reagents like N-Hexane, NaCl, Ethanol, NaOH, Sodium sulfite (Na_2SO_3), 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{\bullet+}$) were purchased from Merck (Germany).

Extraction of Rice Glutelin

The rice glutelin was extracted following method by Ju et al. [16] with slight modification. Rice seeds were ground into a powder and sieved 80-mesh screen to obtain a fine rice powder. N-Hexane was then added at a ratio of 1:15 (w/v) to remove the lipids from the rice grains, and the mixture was allowed to stand for 6 h. Then the precipitate was left to dry overnight. In consequence, the defatted samples were mixed with distilled water with ratio 1:5 (w/v) and stirred for 1 h and then centrifuged at 10.000 xg at 4°C for 15 min. The precipitate was collected and added to 0.5 M NaCl solution for 1 h prior centrifuged 10.000 xg at 4°C for 15 min to remove globulin. The precipitate was dispersed in a 70% ethanol solution, stirred for 1 h prior to centrifuging at 10.000 xg at 4°C for 15 min to remove prolamin. The collected precipitate was dispersed in 0.1 M NaOH solution for stirring 1 h prior to being centrifuged at 10.000 xg at 4°C for 15 min. The clear supernatant was collected as rice glutelin protein and stored at -20°C for further study after lyophilization.

Protein Hydrolysis

The glutelin was hydrolyzed using three proteases, namely pepsin, trypsin, and alcalase. The hydrolysis of rice glutelin using pepsin was carried out following the method by Vanvi & Tsopmo[17]. Glutelin was rehydrated with distilled water (1:12), the pH was adjusted to 4.0 using 1M HCl, followed by the addition of pepsin (0.2%). The mixture was homogenized, and the pH was further adjusted to 2.0. The sample was incubated at 37° C and 150rpm for 5 h. The enzyme was then inactivated by adjusting the pH to 7.0 using 1 M NaOH. The mixture was centrifuged at 5000 rpm for 10 min, and the supernatant containing the enzyme hydrolysate and the non-hydrolyzed protein fraction was collected. Hydrolysis with trypsin was performed according to the method of Shu et al. [13]. The protein sample (10 mg/mL) was hydrolyzed by adding 20 µL of trypsin prior incubated at 37° C for 5 h. The reaction was terminated by heating at 95° C for 10 min, and cooling on ice. The mixture was then centrifuged at 5000 rpm for 10 min, and the supernatant was collected. Hydrolysis with alcalase was conducted following the method of Awuor et al. [18]. Sampel protein (10 mg/mL) was hydrolyzed with 10 µL of alcalase and incubated at 50° C for 5 h. The reaction was stopped by heating at 95° C for 20 min. The mixture was centrifuged at 5000 rpm for 10 min, and the supernatant was collected.

Degree of Hydrolysis

The degree of hydrolysis was determined using the TNBS method[19], based on the reaction between free amino groups and trinitro-benzene-sulfonic acid (TNBS) reagent. A 125 µL sample was mixed with 2 mL 200 mM phosphate buffer (pH 8.2) and 1 mL 0.1% TNBS, and then incubated at 50° C for 30 min. The reaction was terminated by adding 2 mL 0.1 M Na2SO3, and the mixture was allowed to cool at room temperature for 15 min. The absorbance was measured at 420 nm. An L-leucine standard curve was used to determine the amino acid concentration. The percentage of degree of hydrolysis (DH) was calculated using the following equation:

$$DH = \frac{\mathrm{AN2-AN1}}{Npb} x\ 100\% \quad (1)$$

Where AN1 is the amino acid group content before hydrolysis, AN2 is the amino acid group content after hydrolysis, and Npb is the total peptide bond content in the substrate.

Profile Hydrolysis Protein

The profiling of glutelin hydrolysate from each protease was determined using SDS-PAGE electrophoresis with the following method by Laemmli [20]. From each sample (10 µg protein) was loaded into 15% and 4% polyacrylamide concentrations were used for the separating and stacking gel, respectively. After the running process was finished, the gel was removed. The gel was stained in 0.025% CBB R-250 dye solutions, including 7% acetic acid, 40% methanol, and distilled water. The gel was taken from the dye after being overnight-stained and washed in de-staining solution (10% acetic acid, 50% methanol, and 40% distilled water).

$ABTS^{\bullet+}$ Radical Scavenging Activity Assay

The free radical scavenging activity was performed based on the $ABTS^{\bullet+}$ activity[21]. The radical cations were prepared by mixing ABTS solution (7 mM, 1 mL) with 1 mL of 2,45 mM potassium persulfate, followed by incubation for 12–16 h in a dark place. The ABTS working solution was diluted with 0.2 M phosphate buffer saline pH 7 to produce an absorbance of 0.700–0.750 at 734 nm. To conduct the experiment, 950 µL ABTS solution and samples with varying concentrations (0, 10, 20, 40 µg/mL) were mixed prior to incubation for 5 min and measured their absorbance at 734 nm. The antioxidant activity of the tested samples was calculated by using the following equation:

$$\% \text{ inhibition} = \frac{blank\ absorbance - sample\ absorbance}{sample\ absorbance} \times 100\% \quad (2)$$

Angiotensin-I- Converting Enzyme Inhibitor Activity Assay

ACE-inhibitory activity was measured using methods by Mugurama et al.[22] with slight modifications. Sample solution (12.5 µL) with varying concentrations (0, 10, 20, 40 µg/mL), 12.5 µL of 0.1 M borate buffer containing 1,2 M NaCl at pH 8.3 and 12.5 µL 1 U/mL ACE was preincubated in a water bath at 37 °C for 5 minutes. Distilled water was used instead of the sample. The substrate, consisting of 12.5 µL 20 mM HHL in 0.1 M borate buffer pH 8.3, was added and incubated again at 37 °C for 30 minutes. The enzymic reaction was terminated by the addition of 31.25 µL of 1 N HCl; the HCL was added before the enzyme in zero-time control assays. After vortexing, 500 µL of ethyl acetate was added to dissolve the hippuric acid (HA) produced by the enzymatic reaction. The HA phase from the upper layer was taken (400 µL), then centrifuged at 800xg for 10 minutes. Evaporate the solution at 35 °C for 15 minutes to remove the ethyl acetate. Resuspend the HA in 500 µL of Aquadest and measure its absorbance at 228 nm. The inhibition of the ACE inhibitor follows equation 2. IC_{50} was defined as the concentration of the rice glutelin protein hydrolysate that inhibited 50% of the ACE activity.

Statistical Analysis

All values are expressed as the mean of three replicates ± standard deviations (SD). One-way analysis of variance (ANOVA) with a significance level of $p<0.05$ was used to assess the significance of the results obtained.

RESULTS AND DISCUSSION

Enzymatic hydrolysis is widely recognized as an effective protein-modification strategy for generating bioactive peptides [23], [24]. The results of enzymatic hydrolysis in this study

showed an increasing trend from the lowest to the highest activity: trypsin, a combination of trypsin and pepsin, a combination of pepsin and trypsin, and alcalase/pepsin.

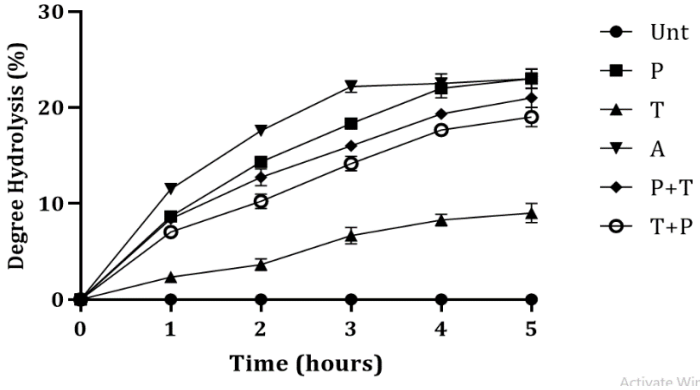

Figure 1: The degree of hydrolysis of rice glutelin protein during hydrolysis using proteases. Unt: Glutelin untreatment; P: Pepsin; T: Trypsin; A: Alcalase; P+T: Pepsin+Trypsin; T+P: Trypsin+Pepsin

The degree of hydrolysis (DH) indicates the cleavage level of peptide bonds, resulting in free amino acids and lower molecular weight peptides [25]. The differences observed among the various proteases can be attributed to their distinct substrate specificities and cleavage sites [10]. In this study, the highest DH was obtained with pepsin- and alcalase-based hydrolysis, with 23% and the lowest was 9% by trypsin-based hydrolysis. Similar findings have been reported in several studies, which consistently demonstrate that alcalase and pepsin with same incubation time exhibits the strongest hydrolytic activity compared with trypsin and another, thereby making it one of the most effective enzyme for producing peptide hydrolysates [12], [26], [27], [28]. Alcalase is an alkaline endopeptidase, and its ability hydrolyze glutelin is greater than that of neutral proteases, because glutelin is soluble under alkaline condition, it can be more readily degraded by alkaline proteases such as alcalase [25].

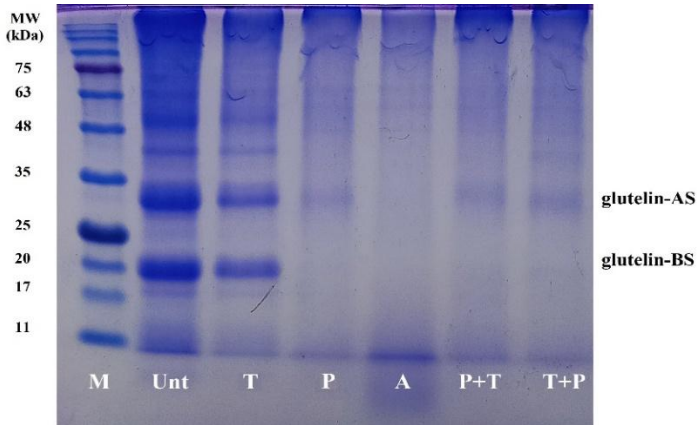

Figure 2: **SDS-PAGE profile of rice glutelin hydrolysate by proteases. M: Marker: MW: molecular weight; Unt: Glutelin untreatment; T: trypsin; P: pepsin; A: alcalase; P+T: pepsin+trypsin; T+P: trypsin+pepsin; AS: acid subunits; BS: basic subunits.**

The SDS-PAGE profiling (Figure 2) confirmed the degradation of rice glutelin protein, as shown by the difference in protein band patterns after hydrolysis by different proteases. In the

untreated glutelin protein, which represents the non-hydrolyzed protein, the protein band appeared clearly and intensely, indicating that the glutelin was intact prior to enzymatic treatment. The glutelin band observed in our SDS-PAGE profile was consistent with previous reports, indicating that rice glutelin was primarily composed of polypeptide subunits with molecular weight between 30-40 kDa (α or acidic subunits) and 19-23 kDa (β or basic subunits) and 57 kDa precursor [29], [30]. In contrast, the protein band in the lanes corresponding to trypsin (T), pepsin (P), alcalase (A), pepsin (P+T), and trypsin+pepsin (T+P) was mostly absent following hydrolysis. This disappearance of the band indicates that the protein was broken down into low-molecular weight peptides and free amino acids[31], [32]. Trypsin-treated glutelin showed substantial degradation, as reflected by the fading of bands compared with the untreated. For the rest of the treatments, most of the bands had fully disappeared, especially for alcalase-treated glutelin.

A growing number of studies have reported that rice bran-derived protein hydrolysates possess strong potential as natural antioxidants. As reported in Figure 3, results suggest that rice glutelin protein hydrolyzed by pepsin, trypsin, alcalase, combination of pepsin+trypsin and combination of trypsin+pepsin reduced the ABTS radical concentration by IC_{50} value of 45.85 ± 0.20, 31.92 ± 0.13, 33.45 ± 0.17, 28.71 ± 0.08, 27.64 ± 0.09, 31.55 ± 0.11 µg/mL respectively with the control was performed using Glutathione (GSH) that has $ABTS^{\bullet+}$ antioxidant capacity significantly higher than protein hydrolysates. Moreover, bioactive peptides are known to exhibit various physiological functions, among which their antihypertensive activity is one of the most widely recognized. This trend is consistent with the degree of hydrolysis results, in which alcalase hydrolysis generated the highest DH value. A similar trend was observed for ACE-inhibitory activity, suggesting that the more intensive proteolytic action of alcalase promotes the release of smaller peptides with structural features that favor ACE inhibition. This supports the notion that the extent and specificity of enzymatic hydrolysis play critical roles in determining the bioactivity of the resulting peptide fractions.

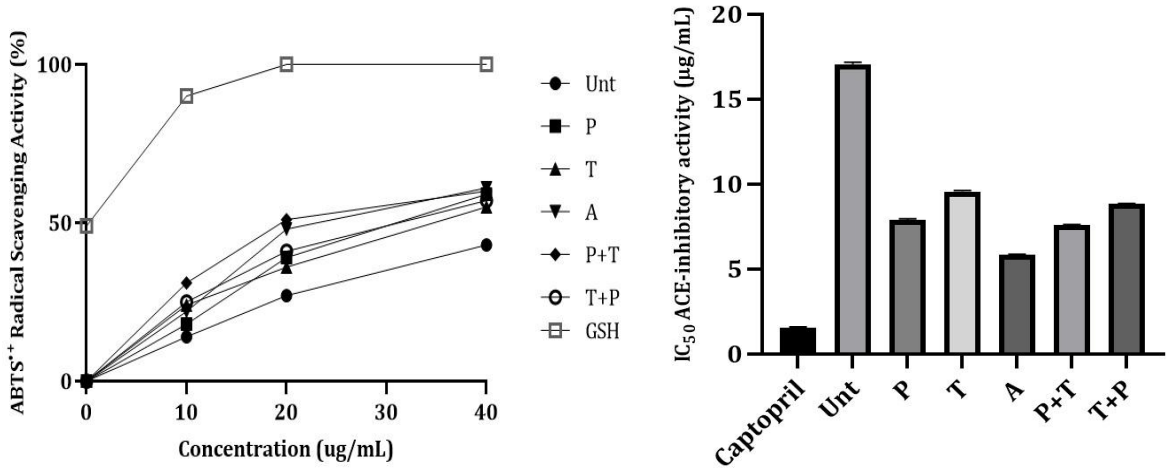

Figure 3: $ABTS^{\bullet+}$ and ACE-inhibitory activity of enzymatic hydrolysates from rice glutelin protein from each protease. Unt: Glutelin untreatment; T: trypsin; P: pepsin; A: alcalase; P+T: pepsin+trypsin; T+P: trypsin+pepsin; GSH: Glutathione.

As shown in Figure 3, the unhydrolyzed rice glutelin protein had a low IC50 ACE-inhibitory activity of 17.06 ± 0.12 μg/mL, while that which had been hydrolyzed using protease, particularly alcalase, produced an IC_{50} value of 5.85 ± 0.01 μg/mL for ACE activity inhibition. Although its value remains lower than captopril as positive drug control, as mentioned by Durak, bioactive peptides are natural and healthier alternatives to synthetic ACE inhibitors without side effects [33]. Protein hydrolysis itself resulted in simpler proteins yet increased protein solubility [34], along with an increase in ACE inhibitory activity.

The mechanism of ACE inhibition peptides from hydrolysate protein can donate their electron to bind with the ACE active site helping via hydrogen bonds, hydrophobic interactions, hydrophilic interactions, electrostatic interactions, and Zn^{2+}binding [35]. Other studies have also reported that the ACE inhibitory activity of protein hydrolysate was more effective from pepsin hydrolysis [36], as mentioned in Jayaprakash et al. [37]. ACE-inhibitory activity by alcalase-based hydrolyzed rice protein suppresses and has strong activities as antihypertensive vasodilator bradykinin. The differences observed between $ABTS^{\bullet+}$ and ACE inhibition among the hydrolysates are likely due to the distinct peptide profile generated by each enzymatic treatment, as reported by several studies. Specific peptide compositions, including variations in amino acid sequence, hydrophobicity and molecular size, can strongly influence the type and intensity of bioactive produced. Several amino acids are known to contribute to the bioactivity of ACE-inhibitory peptides. Hydrophobic amino acids such as valine (V), leucine (L) and isoleucine play an important role in enhancing ACE-inhibitory activity, while proline (P) is associated with improving stability and effectiveness. Aromatic amino acids are known to enhance the ACE-inhibitory activity of peptides, thereby amplifying their antihypertensive effects. Moreover, tyrosine (Y) and methionine (M) are reported to be part of numerous sequences of peptides with antioxidant activity. In addition, the importance of peptide size is linked to the absorption potential into the bloodstream, as well as to the ability to interact with targeted enzymes responsible for regulating blood pressure [27].

CONCLUSION

In summary, enzymatic hydrolysis as a selected protein modification technique yields better results than without hydrolysis, especially since alcalase has a better hydrolysis function for rice glutelin protein than other proteases, because alkaline condition extracts more glutelin as the major protein in rice. In relation to the degree of hydrolysis, bioactive functions as an antioxidant and an ACE inhibitor were also found in rice glutelin protein hydrolysate using alcalase. These results can be utilized as a source and modification technique to obtain bioactive peptides with the ability to scavenge free radicals to inhibit ACE activity. However, this research needs to be continued to identify the specific peptides obtained to prove which specific amino acids work as ACE inhibitors in this study.

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