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Research article

Antioxidant potential of hydrolyzed proteins from Thai rice varieties and docking studies of novel peptides with free radicals

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Abstract: Five native rice varieties from Thailand were used to extract seed storage proteins. The most prevalent proteins were glutelin, globulin, albumin, and prolamin. Significant scavenging activity of crude glutelin was seen in all samples, and this activity was further improved in hydrolysates made with pepsin. Using PeptideCutter (https://web.expasy.org/peptide_cutter/), 99 peptides were produced by simulating the gastrointestinal digestion of rice glutelin (*Oryza sativa* Indica Group; GenBank: AGT59178.1). AnOxPePred-1.0 (https://services.healthtech.dtu.dk/services/AnOxPePred-1.0/) was used to predict the antioxidant potential of many peptides. The top five peptides with high ABTS•+ and DPPH• scavenging activities were examined for molecular docking. The results indicate that, when compared to glutathione, the positive control, the octapeptide TNTPGVVY had the lowest binding affinity with DPPH• (-4.26 kcal/mol), and the 13-amino acid peptide, TQQQEQAQAQDQY, had the lowest binding affinity with ABTS•+ (-3.70 kcal/mol). The antioxidant properties of both synthetic peptides were confirmed by in vitro assays. Neither peptide exhibited cytotoxic effects on human cell lines. This research indicates the value of Thai red glutinous rice and its potential to be developed into health food products for those who love eating sticky rice.

Keywords: Thai indigenous rice; seed storage proteins; antioxidant activity; protein hydrolysates;

molecular docking

1. Introduction

Rice (*Oryza sativa* L.) is a staple food for over half of the world's population and is vital to the economies and diets of many nations, particularly in Asia [1]. Besides offering a significant quantity of calories, rice is an excellent provider of vital nutrients, including proteins, which account for approximately 7%–8% of the grain's weight [2]. Glutelin, prolamin, globulin, and albumin are the primary rice seed storage proteins; each has a distinct solubility and set of functional properties [3–5]. Plant growth and development depend on seed storage proteins because they provide sulfur and nitrogen during seed germination [6]. Roughly 80% of rice's total protein content is made up of glutelin, which is followed by globulins, albumins, and prolamins. These proteins are important for human nutrition in addition to supporting plant development [3,4,7]. Enzymatic hydrolysis of rice proteins can produce peptides or protein hydrolysates with possible bioactive characteristics, such as antioxidant activity [8,9]. Antioxidants are essential for combating free radicals connected to oxidative stress and several chronic illnesses, including heart disease and cancer [4,10].

The effective scavenging of free radicals by rice-derived antioxidant peptides has been drawing recent attention [11,12]. These peptides are known to have antioxidant qualities because of certain amino acid sequences that interact with and neutralize free radicals. These peptides are generally formed by the enzymatic hydrolysis of rice proteins, especially glutelin [8,13]. Peptides from rice protein hydrolysates have been shown in studies to have strong antioxidant properties, making them attractive natural antioxidants for use in a variety of food and medicinal applications [10].

Thai native rice cultivars are widely available and have distinct nutritional profiles and bioactive components [14]. These are excellent research subjects since they are frequently high in phytochemicals, antioxidants, and bioactive peptides. Bioactive peptides, derived from rice proteins, have shown a range of functional properties, including antifungal, antibacterial, and antioxidant activities [15,16].

These peptides can be released through enzymatic hydrolysis, enhancing their bioavailability and therapeutic potential. Despite the potential of bioactive peptides from Thai rice, research on these cultivars is still lacking compared to strains that are regularly grown, which highlights the need for additional studies [14]. The binding orientation and affinity of bioactive compounds and their target molecules can be predicted computationally using a method called molecular docking [17]. This method contributes to our comprehension of the molecular processes that underlie antioxidant and bioactive peptide activity [18]. We used molecular docking analysis and the ABTS radical cation scavenging experiment to assess the antioxidant activity of protein hydrolysate from Thai indigenous rice. Based on these findings, rice protein hydrolysate has a lot of potential as a naturally occurring antioxidant that may be applied to functional foods and nutraceuticals.

2. Materials and methods

2.1. Rice storage protein extraction

Brown rice samples from five different Thai indigenous rice varieties, including Red glutinous

rice (Niew-Daeng), Red jasmine (Mali-Daeng), Hom Nin, Tubtim Chumphae, and Hom Baitoey, were harvested in 2022 from Maha Sarakham Province. Rice grains were finely ground into rice powder and passed through an 80-mesh sieve to obtain a fine powder. To remove fat, the rice powder was treated with hexane at a ratio of 1:15 (rice flour to hexane, g/mL) for 3 h. After extraction, the mixture was filtered through filter paper number 1, and hexane was evaporated from the rice powder for 24 h. The fat-extracted rice powder was then stored at 4 ℃ for subsequent protein extraction.

2.2. Extraction of rice storage proteins

Four types of rice storage proteins (albumin, globulin, prolamin, and glutelin) were sequentially extracted from the fat-extracted rice powder. For albumin extraction, 8 g of rice powder sample was mixed with 40 mL of distilled water and stirred for 1 h. The mixture was then centrifuged at 10,000× g at 4 ℃ for 30 min, and the clear supernatant was collected as the first extract of crude albumin protein. The precipitate was re-extracted, and both extracts were freeze-dried, stored at −20 ℃, and labeled for further analysis.

To extract globulin proteins, the remaining precipitate from the albumin extraction was stirred with 0.5 M NaCl solution for 1 h and then centrifuged at 10,000× g at 4 ℃ for 30 min. The clear supernatant was collected as the first crude globulin protein extract. The precipitate was re-extracted to obtain a second extract. Both crude globulin extracts were desalted by dialysis against distilled water using a 10 kDa MWCO membrane before freeze-drying and storage.

The remaining precipitate from the globulin extraction was used for prolamin extraction. It was mixed with 70% (v/v) ethanol solution, agitated for 1 h, and then centrifuged to collect the clear solution as the first crude prolamin protein extract. The precipitate was re-extracted to obtain a second extract. Both crude prolamin extracts were subjected to ethanol removal by evaporation before freeze-drying and storage.

Finally, the residual precipitate from the prolamin extraction was treated with 0.1 M NaOH for 1 h and centrifuged at 10,000×g at 4 ℃ for 30 min, and the clear supernatant was collected as the first crude glutelin protein extract. The precipitate was re-extracted to obtain a second extract. The crude glutelin extracts were then subjected to alkali removal by dialysis against distilled water using a 10 kDa MWCO membrane before freeze-drying and storage at −20 ℃ for further analysis.

2.3. Protein determination

The protein content of the extracted samples was determined using the Lowry method as described by Lowry et al. [19]. Bovine serum albumin (BSA) was used to create a standard curve. The protein profiles of the extracted samples were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method by Laemmli [20]. This technique allows for the separation of proteins based on their molecular weight, providing a detailed protein pattern analysis for each rice storage protein fraction and its protein hydrolysate.

2.4. Antioxidant activity of rice protein

The scavenging activity against the free radical 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS•+) was assessed to determine the

antioxidant potential of the rice protein extracts. The method followed the one previously described by Khammuang and Sarnthima [21]. Initially, the ABTS•+ solution was prepared to achieve an initial absorbance of approximately 0.7 at 734 nm. Subsequently, 800 µL of the ABTS•+ solution was thoroughly mixed with 200 µL of the protein extract. The reaction mixture was then allowed to stand at room temperature for 30 min. After incubation, the absorbance of the reaction mixture was measured at 734 nm using a spectrophotometer. The percentage of free radical scavenging activity was calculated using the following equation:

$$\text{Scavenging activity (\%)} = [(\text{Absorbance of control} - \text{Absorbance of sample} / \text{Absorbance of control}) \times 100]$$

2.5. Preparation of protein hydrolysate from Thai red glutinous rice

Crude glutelin, the most abundant storage protein in Thai red glutinous rice, was extracted and hydrolyzed using pepsin at an enzyme-to-substrate ratio of 1:20 (w/w) under simulated gastric conditions to generate antioxidant peptides. This choice was based on the enzyme's relevance to physiological conditions and its established effectiveness in generating antioxidant peptides from plant proteins. The digestion reaction was carried out in 200 mM Glycine-HCl buffer (pH 2.0) and incubated at 37 ℃. To monitor the hydrolysis progression, samples were collected at 60, 120, and 240 min and terminated by heating the samples at 100 ℃ for 10 min. The resulting mixture was then centrifuged at 10,000× g for 10 min to obtain a clear supernatant, which was subsequently stored at −20 ℃ for further analysis. The protein profile of the hydrolysate was analyzed using SDS-PAGE to compare it with the undigested control reaction. Additionally, the antioxidant activity of the hydrolysate was evaluated.

2.6. Simulation of the digestion of rice protein glutelin in the human gastrointestinal tract and prediction of antioxidant activity

In the in silico simulation, glutelin digestion was modeled using PeptideCutter (https://web.expasy.org/peptide_cutter/), applying pepsin, trypsin, and chymotrypsin to represent a comprehensive gastrointestinal digestion process. This approach aims to generate a broader range of potential bioactive peptides, despite only pepsin being used in the experimental (in vitro) digestion. The amino acid sequence of rice protein glutelin was retrieved from the National Institutes of Health (NIH) protein database (https://www.ncbi.nlm.nih.gov/protein/AGT59178.1?report.=genpept). The sequence of glutelin [*Oryza sativa* Indica Group] GenBank: AGT59178.1 was in FASTA format. This sequence was utilized to gastrointestinally (GI) simulate the digestion of protein chains by three types of enzymes found in the human gastrointestinal tract: (1) chymotrypsin—with both high specificity and low specificity (C-terminal to [FYWML], not before P), (2) pepsin (pH 1.3), and (3) trypsin. Simulations were conducted using the PeptideCutter program available at https://web.expasy.org/peptide_cutter/ [22].

Subsequently, the peptide fragments obtained from the digestion simulations were subjected to prediction of antioxidant activity using the AnOxPePred-1.0 program, accessible at https://services.healthtech.dtu.dk/services/AnOxPePred-1.0/ [23]. This computational tool evaluates the potential antioxidant properties of peptides based on their amino acid sequences and structural

characteristics.

2.7. Molecular docking of peptides with ABTS and DPPH radicals

The molecular docking study was conducted using Autodock Vina, following the previously described method with some modifications [24–26]. The ABTS (PubChem CID: 90658258) and DPPH (PubChem CID: 15911) radical structures were sourced from the PubChem database. The peptide structure was generated using Discovery Studio 2021 (Accelrys, San Diego, CA, USA). Before docking, polar hydrogen atoms were added to all molecules, and Kollman charges were automatically assigned by AutoDock Vina. The docking simulation was executed with 100 independent runs, with only the pose exhibiting the lowest binding energy retained. The results of the molecular docking were expressed in terms of binding affinity.

2.8. Antioxidant activity of rice glutelin-derived synthetic peptides

The selected peptides, according to molecular docking results, were chemically synthesized by U2Bio (Thailand) with >90% purity. The peptides were dissolved in appropriate solvents and measured for ABTS radical scavenging activity according to the method described previously. DPPH radical scavenging activity was assessed as previously described [27]. The stock of 10 mM of DPPH• was diluted in ethanol ($A_{515} \approx 0.7$) before reacting with the peptides.

2.9. Cytotoxic assay of selected synthetic peptides

A549 human lung adenocarcinoma cells, SW480 human colorectal adenocarcinoma cells, MDA-MB-231 human triple-negative mammary adenocarcinoma cells, and HepG2 human hepatocellular carcinoma cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in RPMI 1640 (A549 and SW480) or DMEM (MDA-MB-231 and HepG2) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic. The cells were maintained at 37 ℃ in a humidified CO_2 incubator.

MTT assay was employed to evaluate the cytotoxicity of test samples against cancer cell lines as previously described [28]. The cells were plated into 96-well plates at 5×10^3 cells/well (A549 and SW480) or 1×10^4 cells/well (MDA-MB-231 and HepG2) and further incubated for 24 h. Subsequently, cells were treated with peptides at a concentration of 10 μM, while Doxorubicin (2 μM) was used as a positive control, for 48 h. After that, wells were replaced with media containing MTT (0.5 mg/mL) and incubated for another 2 h. Then, wells were replaced with DMSO to lyse the cells and dissolve MTT formazan crystals. The absorbance at 550 nm was measured using a microplate reader and subtracted from the absorbance at 650 nm. Cell viability was determined from the absorbance and expressed as a percentage compared to the control group.

2.10. Statistical analysis

All assays were performed in triplicate. Results were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) and Duncan test ($p < 0.05$) were used for antioxidant capacity using IBM SPSS Statistics (Version 29.0.0).

3. Results and discussion

3.1. Rice storage protein extraction

Five local varieties of Thai rice, including Thai red glutinous rice (Niew-Daeng), Red jasmine (Mali-Daeng), Hom-Nil, Tubtim-Chumphae, and Hom-Baitoey, were subjected to protein extraction using various solvents. The extraction process yielded four types of seed storage proteins: albumin, globulin, prolamin, and glutelin, each extracted in two rounds as detailed in Table 1.

Table 1 illustrates the protein content and distribution across the rice varieties. Overall, most rice varieties showed higher crude glutelin content than albumin, apart from Hom-Nil, where albumin content was slightly higher than glutelin. Notably, Hom-Baitoey exhibited equally high levels of albumin and glutelin. Among the varieties, Niew-Daeng, Hom-Nil, and Tubtim-Chumphae showed higher albumin concentrations than globulin, while Mali-Daeng had a slightly higher percentage of globulin. Prolamin content was minimal across all varieties, with negligible differences observed in Hom-Baitoey.

Analyzing the protein concentrations revealed distinct patterns. In Hom-Baitoey, the first extraction yielded the highest concentration of glutelin (6.055 mg/mL), while in other rice varieties, concentrations ranged from 3.376 to 5.115 mg/mL (Table 1). Globulin concentrations were generally higher in the first extraction, whereas prolamin concentrations were consistently lower in the first extraction in all rice samples. In contrast, the lower prolamin concentrations may indicate challenges in specific solvent extraction (70% ethanol), potentially extracting non-proteinaceous compounds, especially evident in pigmented rice varieties such as Niew-Daeng and Hom-Nil.

The proportions of seed storage proteins observed in this study align broadly with previous reports, emphasizing glutelin as the dominant protein (60.08%–84.94%), followed by albumin (7.60%–26.35%) and globulin (4.07%–12.73%), with prolamin representing the smallest fraction (0.84%–3.39%) (Table 1). Among rice storage proteins, the content of glutelin is reported to be the largest, which accounts for more than 80% of the total endosperm protein; on the other hand, albumin accounts for about 10%, globulin 5%–10%, and prolamin 5% [4,5]. Even though the other three types of rice seed storage proteins have slightly different amounts, the most abundant is always glutelin. According to a capillary electrophoresis study (CE) in Australian rice protein isolates, the major fraction (about 75% of total) was glutelin. The globulin fraction was around 15%, followed by albumin at 6% and prolamin at 2.7% [29]. Additionally, Wattanasiritham et al. [30] reported similar proportions in white jasmine rice bran protein 105 (RBP), highlighting variations due to rice cultivar and growth conditions. Among the five Thai rice varieties analyzed, prolamin consistently exhibited the lowest abundance, while glutelin was the most dominant protein fraction, highlighting a common distribution pattern of seed storage proteins in rice. In contrast, the levels of globulin and albumin varied noticeably among the varieties, suggesting genotype-specific differences in their protein profiles. These differences highlight the impact of both genetic and environmental factors on the composition of rice protein. The methodology employed in protein extraction, including solvent ratio, duration, and number of extractions, significantly impacts protein yield and composition. While performing more than two extraction rounds can increase protein content, it requires careful consideration to prevent compromising specificity. Future studies could explore optimizing extraction protocols to enhance prolamin recovery and refine protein proportions in rice varieties.

Table 1. Storage protein amounts in each crude protein extract of various Thai rice varieties analyzed by the Lowry method using bovine serum albumin (BSA) as a standard.

Rice sample	Protein name	Extraction order	Dissolved volume (mL)	Protein content (mg/mL)	Total Protein (mg)	Protein (%) d.wt.	Total protein (twice extraction)	Total protein (%) d.wt.	Protein proportion
Niew-Daeng (8 g)	Albumin	1	33.00	0.279	9.207	0.12	12.606	0.16	8.58
		2	11.00	0.309	3.399	0.04			
	Globulin	1	9.00	0.901	8.109	0.10	11.160	0.14	7.60
		2	9.00	0.339	3.051	0.04			
	Prolamin	1	19.00	0.053	1.007	0.01	2.092	0.03	1.42
		2	7.00	0.155	1.085	0.01			
	Glutelin	1	25.00	4.017	100.425	**1.26**	121.050	1.51	**82.40**
		2	25.00	0.825	20.625	0.26			
Mali-Daeng (8 g)	Albumin	1	31.00	0.348	10.788	0.13	20.563	0.26	10.73
		2	17.00	0.575	9.775	0.12			
	Globulin	1	8.00	0.815	6.520	0.08	22.580	0.28	11.78
		2	22.00	0.730	16.060	0.20			
	Prolamin	1	21.00	0.105	2.205	0.03	6.469	0.08	3.38
		2	8.00	0.533	4.264	0.05			
	Glutelin	1	34.00	3.455	117.470	**1.47**	142.050	1.78	**74.11**
		2	20.00	1.229	24.580	0.31			
Hom-Nil (8 g)	Albumin	1	45.00	0.851	38.295	0.48	51.335	0.64	26.35
		2	16.00	0.815	13.040	0.16			
	Globulin	1	10.00	1.788	17.880	0.22	24.801	0.31	12.73
		2	9.00	0.769	6.921	0.09			
	Prolamin	1	20.00	0.013	0.260	0.00	1.628	0.02	0.84
		2	8.00	0.171	1.368	0.02			
	Glutelin	1	25.00	3.376	84.400	**1.06**	117.020	1.46	**60.08**
		2	20.00	1.631	32.620	0.41			

Continued on next page

Rice sample	Protein name	Extraction order	Dissolved volume (mL)	Protein content (mg/mL)	Total protein (mg)	Protein (%) d.wt.	Total protein (twice extraction)	Total protein (%) d.wt.	Protein proportion
Tubtim-Chumphae (8 g)	Albumin	1	25.00	0.473	11.825	0.15	15.275	0.19	7.60
		2	15.00	0.230	3.450	0.04			
	Globulin	1	11.00	0.404	4.444	0.06	8.194	0.10	4.07
		2	10.00	0.375	3.750	0.05			
	Prolamin	1	22.00	0.224	4.928	0.06	6.824	0.09	3.39
		2	6.00	0.316	1.896	0.02			
	Glutelin	1	30.00	5.115	153.450	**1.92**	170.805	2.14	**84.94**
		2	15.00	1.157	17.355	0.22			
Hom-Baitoey (8 g)	Albumin	1	30.00	0.812	24.360	0.30	37.308	0.47	14.94
		2	13.00	0.996	12.948	0.16			
	Globulin	1	15.00	1.200	18.000	0.23	27.270	0.34	10.92
		2	10.00	0.927	9.270	0.12			
	Prolamin	1	18.00	0.253	4.554	0.06	7.434	0.09	2.98
		2	6.00	0.480	2.880	0.04			
	Glutelin	1	25.00	6.055	151.375	**1.89**	177.763	2.22	**71.17**
		2	18.00	1.466	26.388	0.33			

3.2. Antioxidant activity of crude protein extracts

The antioxidant activity of crude protein extracts from five indigenous rice varieties was evaluated by their ability to scavenge ABTS•+ free radicals. Since it is relatively simple and fast and requires only a few steps, it can be used in both aqueous and lipid systems. ABTS assay relies on color change upon radical scavenging, providing a clear and immediate visual or spectrophotometric readout. This makes it excellent for initial or comparative screening of antioxidant activity across multiple protein samples for rice varieties. Among the four types of crude protein extracts (albumin, globulin, glutelin, and prolamin), glutelin consistently exhibited the highest ABTS•+ free radical scavenging activity across all rice varieties in both the first and second extractions. Specifically, in the first extraction, glutelin demonstrated ABTS•+ scavenging activities of 66.43%, 78.00%, 64.25%, 92.79%, and 56.43% for Niew-Daeng, Mali-Daeng, Hom-Nil, Tubtim-Chumphae, and Hom-Baitoey varieties, respectively (Figure 1a–e).

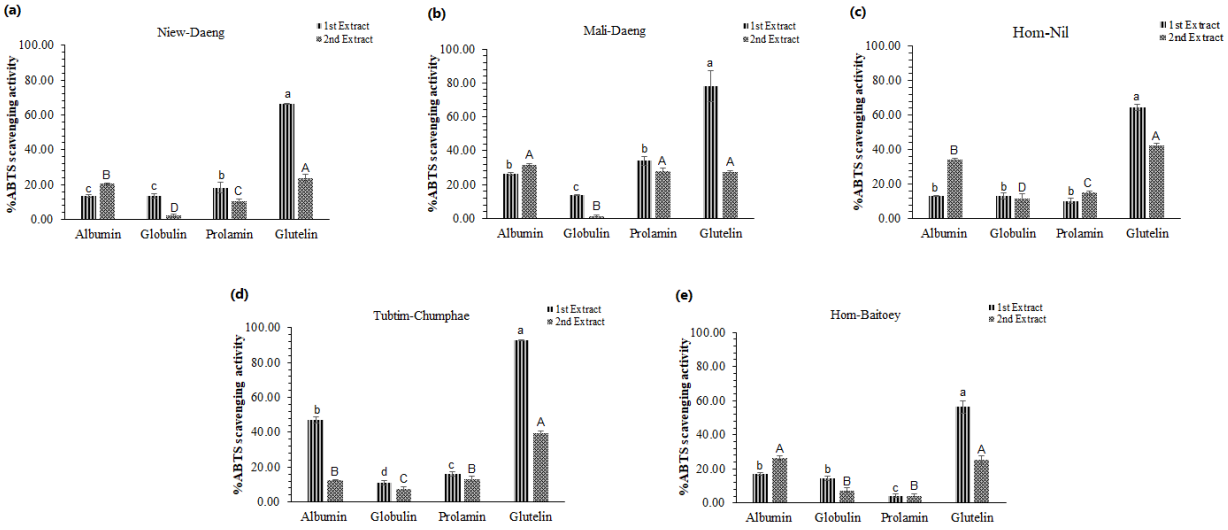

Figure 1. ABTS•+ free radical scavenging percentages of the crude storage protein extract from certain Thai rice varieties (protein concentration 1 mg/mL).

Note: All data are presented as the mean ± SD (n = 3). Values with the same letters indicate no significant difference in the ABTS radical scavenging rate ($p > 0.05$).

Across all rice types, albumin also demonstrated notable ABTS•+ scavenging activity, but generally lower than glutelin. The scavenging activity of prolamin was slightly higher than that of globulin in the first extraction, except for Hom-Nil and Hom-Baitoey varieties. In the second extraction, most rice varieties (Niew-Daeng, Mali-Daeng, and Hom-Nil) continued to show stronger ABTS•+ scavenging activity in glutelin compared to albumin, prolamin, and globulin. This difference in activity between the first and second extractions may be attributed to the presence of interfering molecules. The observed superior antioxidant activity of glutelin aligns with previous findings indicating that rice glutelin possesses stronger ABTS•+ scavenging capabilities compared to prolamin [13].

Moreover, the observed variation in antioxidant activity between the first and second extractions of each rice sample underscores the influence of extraction methods and potential interference molecules on protein functionality. Notably, the second extraction generally yielded lower antioxidant activity across all protein types, suggesting differential extraction efficiencies. Comparatively, similar trends in antioxidant activity have been reported in studies involving barley hordein and rice bran protein fractions, which also highlighted glutelin's significant antioxidative potential [31]. Additionally, research on peptides derived from brown rice hydrolysates has demonstrated their efficacy in combating oxidative stress, further emphasizing the diverse antioxidant capabilities of rice-derived proteins [8,32]. Overall, these findings emphasize the variability in antioxidant activity among different rice protein extracts, influenced by both intrinsic protein characteristics and extraction methodologies. Future research could explore optimizing extraction protocols to enhance the antioxidant potential of specific rice protein fractions, thereby maximizing their beneficial health effects.

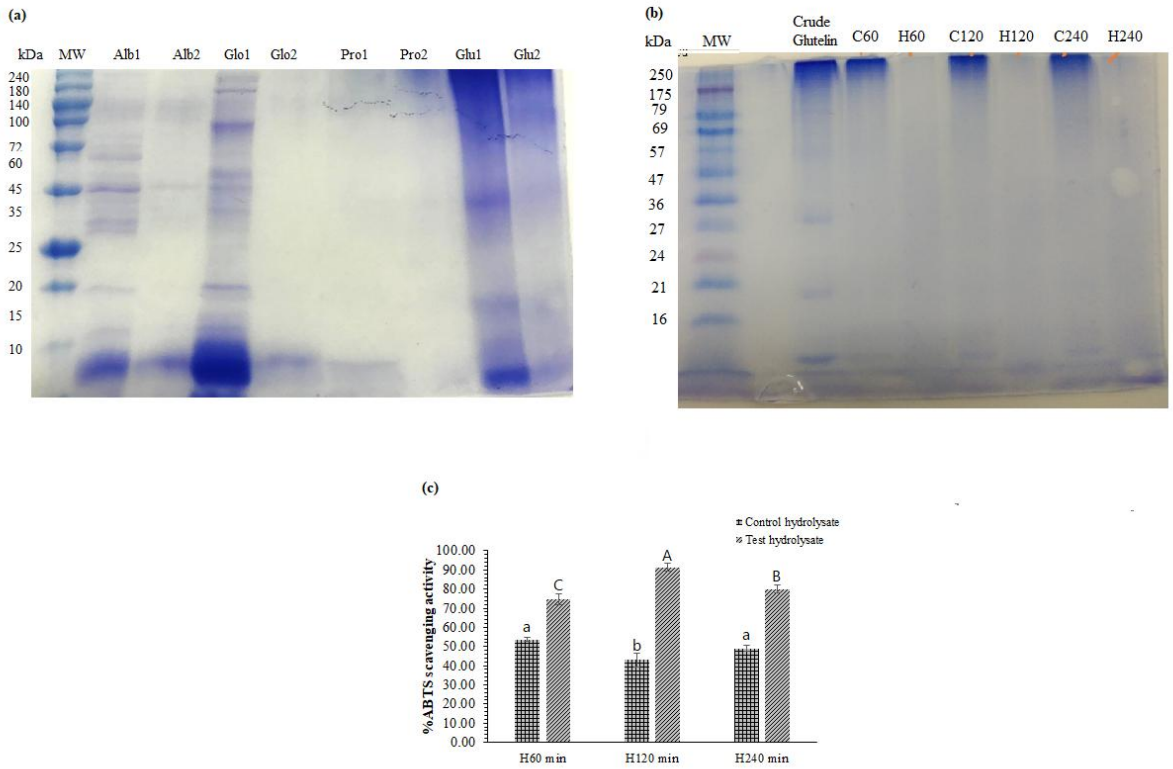

Figure 2. 12% SDS-PAGE of four seed storage proteins from Thai red glutinous rice (a), glutelin hydrolysate prepared by digestion with pepsin at various times (b), and ABTS radical scavenging activity of its glutelin hydrolysate at 60, 120, and 240 min.

Note: MW: molecular weight markers; Alb1 and Alb2: albumin first and second extract; Glo1 and Glo2: globulin first and second extract; Pro1 and Pro2: prolamin first and second extract; Glu1 and Glu2: glutelin first and second extract. C60, C120, C240: control reaction at 60, 120, and 240 minutes; H60, H120, H240: glutelin hydrolysate at 60, 120, and 240 minutes. All data are presented as the mean ± SD (n = 3). Values with the same letters indicate no significant difference in the ABTS radical scavenging rate ($p > 0.05$). Protein loaded in each lane, 20 μg.

3.3. Crude seed storage protein patterns

We valued the people in northeastern Thailand who prefer to eat glutinous rice over conventional rice; therefore, we chose the red glutinous rice variety for additional research and digestion. Despite not having the greatest glutelin content among the five strains examined, only red glutinous rice is sticky. To be consistent with the local context, we have chosen it as the model in this report. The SDS-PAGE analysis of crude storage proteins extracted from red glutinous rice seeds revealed distinct protein patterns for each fraction (Figure 2a). The crude albumin fraction exhibited protein bands spanning a molecular weight (MW) range from approximately 140 kDa down to less than 10 kDa. In contrast, crude globulin showed bands with MW ranging from approximately 180 kDa to below 10 kDa. The predominant seed storage fraction, crude glutelin, displayed protein bands distributed between 240 kDa and less than 10 kDa, indicative of its high molecular weight profile. Conversely, crude prolamin in red glutinous rice exhibited bands primarily around less than 10 kDa. These findings are consistent with previous reports highlighting the diverse molecular weight distributions of rice protein fractions [33–35].

3.4. Protein hydrolysate preparation and antioxidant activity

Given the superior antioxidant activity observed in crude glutelin, it was selected for further study to prepare protein hydrolysates using pepsin digestion over varying durations. Pepsin was chosen as the sole proteolytic enzyme for in vitro digestion due to its physiological relevance and efficiency in mimicking gastric conditions. SDS-PAGE analysis following enzymatic digestion (Figure 2b) revealed a reduction in protein band intensity over time at 60, 120, and 240-min intervals, suggesting effective breakdown of high molecular weight proteins into smaller peptides. This is consistent with previous studies reporting that pepsin generates bioactive peptides from cereal proteins under simulated gastric conditions [33]. The disappearance of protein bands suggests extensive hydrolysis, which aligns with the aim of producing antioxidant peptides for further screening. Although the degree of hydrolysis was not quantitatively measured, SDS-PAGE provided qualitative evidence of successful protein breakdown.

The antioxidant activity of the glutelin hydrolysates, evaluated by ABTS•+ radical scavenging assay, demonstrated a time-dependent increase in free radical scavenging ability (Figure 3c). Specifically, the ABTS•+ radical scavenging activity of glutelin hydrolysates after 60, 120, and 240 minutes of digestion was 47.59%, 91.33%, and 80.07%, respectively. This trend aligns with findings by Wang et al. [13], who reported enhanced ABTS•+ scavenging activity with prolonged pepsin digestion of rice glutelin. The improved antioxidative properties of protein hydrolysates post-digestion can be attributed to the release of amino acid residues capable of donating electrons, which are typically sequestered within the folded protein structure.

Comparable enhancements in antioxidative and reducing activities have been reported in protein hydrolysates from barley proteins following digestion with pepsin and trypsin [31]. These studies underscore the potential of enzymatic digestion to enhance the bioactive properties of rice protein fractions, thereby broadening their applications in functional foods and nutraceuticals.

3.5. Simulation results of the digestion of rice protein glutelin and prediction of antioxidant activity

Currently, full-length glutelin sequences specific to our local varieties are not available in public databases. The amino acid sequence of rice protein glutelin (*Oryza sativa* Indica Group; GenBank: AGT59178.1) was selected because it is a well-characterized and publicly available glutelin sequence from *Oryza sativa*, which shares high sequence homology with the glutelin proteins found among rice cultivars.

Using the amino acid sequence of rice protein glutelin, we simulated the digestion of protein chains by human digestive enzymes, including (1) chymotrypsin with high specificity (C-term to [FYW], not before P) and low specificity (C-term to [FYWML], not before P), (2) pepsin (pH 1.3), and (3) trypsin, using the computational chemistry program PeptideCutter (https://web.expasy.org/peptide_cutter/). This GI simulation resulted in the identification of 99 peptides (with four duplicates) ranging in length from 2 to 13 constituent amino acid residues, and molecular masses between 162.145 and 1565.573 Da. Subsequently, 95 of these peptides were assessed for their antioxidant activity using the AnOxPePred-1.0 program [23] (https://services.healthtech.dtu.dk/services/AnOxPePred-1.0/), and the top 5 peptides with the highest predicted scores are presented in Table 2.

Table 2. Peptides obtained from a simulated digestion of rice glutelin protein. Predicted free radical scavenger activity (predicted free radical scavenger, FRS score) and metal ion binding properties (predicted chelation, CHEL score), sorted by the top 5 highest scores.

FRS score	**Sequence**	**Name**
0.51274	CH	AGT59178.1 glutelin_Oryza sativa Indica Group_
0.50343	GEEH	AGT59178.1 glutelin_Oryza sativa Indica Group_
0.48115	IH	AGT59178.1 glutelin_Oryza sativa Indica Group_
0.46026	EP	AGT59178.1 glutelin_Oryza sativa Indica Group_
0.45710	TNTPGVVY	AGT59178.1 glutelin_Oryza sativa Indica Group_
CHEL score	**Sequence**	**Name**
0.30898	SP	AGT59178.1 glutelin_Oryza sativa Indica Group_
0.30177	EP	AGT59178.1 glutelin_Oryza sativa Indica Group_
0.30088	IH	AGT59178.1 glutelin_Oryza sativa Indica Group_
0.29592	TQQQEQAQAQDQY	AGT59178.1 glutelin_Oryza sativa Indica Group_
0.29559	CH	AGT59178.1 glutelin_Oryza sativa Indica Group_

3.6. Molecular docking

Molecular docking simulations were conducted to determine the binding affinities of various peptides with ABTS and DPPH radicals. Glutathione was employed as a positive control. A more negative binding affinity value signifies a stronger interaction between the peptide and the radical, suggesting potential for enhanced radical scavenging activity (Table 3). The molecular docking analysis revealed that the peptide TQQQEQAQAQDQY displayed the most favorable binding interaction with the ABTS radical, as evidenced by the lowest calculated binding energy of −3.70 kcal/mol. Similarly, TNTPGVVY demonstrated the strongest binding affinity toward the DPPH radical with a binding energy of −4.26 kcal/mol. In comparison, the positive control, glutathione, exhibited relatively weaker binding affinities of −2.25 and −2.77 kcal/mol for ABTS•+ and DPPH•,

respectively. These findings suggest that these peptides may have promising antioxidant properties.

Table 3. The binding affinity of rice glutelin-derived peptides against ABTS and DPPH radicals in comparison to glutathione.

Peptides	**Binding affinity (kcal/mol)**	
	ABTS•+	**DPPH•**
CH	−2.30	−2.50
EP	−2.55	−2.55
IH	−2.53	−3.06
SP	−2.10	−2.22
GEEH	−2.84	−3.35
TNTPGVVY	−3.49	**−4.26**
TQQQEQAQAQDQY	**−3.70**	−4.21
Glutathione	−2.25	−2.77

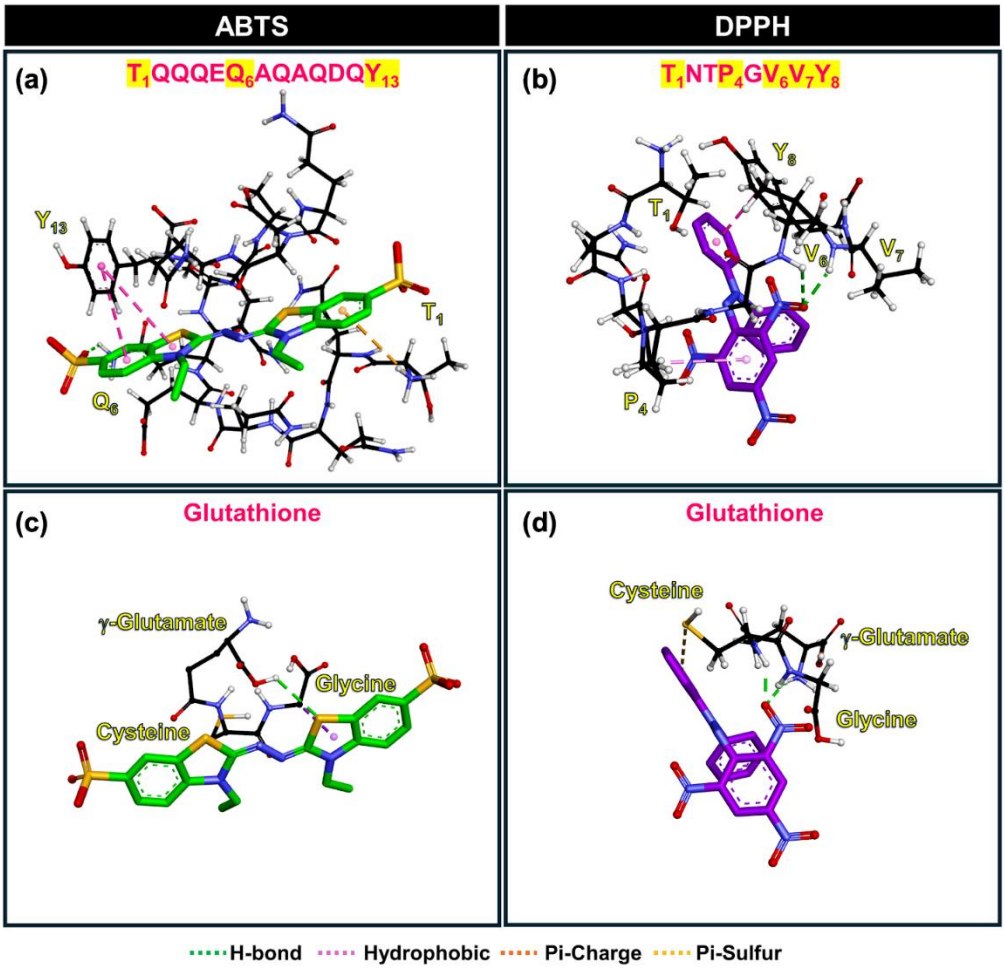

Figure 3. Binding interactions between peptides from rice glutelin protein and glutathione with free radicals. Peptide TQQQEQAQAQDQY with free radicals ABTS•+ (a), peptide TNTPGVVY with the free radicals DPPH• (b), binding interactions of glutathione with ABTS•+ (c), and glutathione with DPPH• (d).

Analysis of the interaction patterns revealed distinct binding modes between the peptides and the radicals (Figure 3). The peptide TQQQEQAQAQDQY formed four primary interactions with the ABTS radical (Figure 3a). These interactions included hydrogen bonds with the glutamine (Q) residue at position 6, as well as pi-charge and two hydrophobic interactions involving the threonine (T) residue at the N-terminus and the tyrosine (Y) residue at the C-terminus. Conversely, the peptide TNTPGVVY interacted with the DPPH radical (Figure 3b) primarily through four interactions: two backbone hydrogen bonds involving valine (V) residues at positions 6 and 7 and two hydrophobic interactions, namely a pi-alkyl interaction with proline (P) at position 4 and a pi-pi stacking interaction with tyrosine (Y) at position 8. The binding patterns of glutathione with both ABTS radical (Figure 3c) and DPPH radical (Figure 3d) were less complex. For the glutathione/ABTS•+ interaction, a single hydrogen bond and a pi-alkyl hydrophobic interaction were observed. In contrast, the glutathione/DPPH• interaction involved three interactions: two hydrogen bonds and a pi-sulfur interaction. Molecular docking studies highlighted the potential of these peptides as potent antioxidants. The observed electrostatic, hydrogen bonding, and hydrophobic interactions with ABTS•+ and DPPH• molecules suggest a mechanism for their free radical scavenging activity.

These findings align with previous studies on antioxidant peptides derived from food sources, highlighting the importance of hydrogen bonds and hydrophobic interactions in enhancing radical scavenging activities [36,37,38]. Moreover, the peptides studied in this research were derived from simulated gastrointestinal digestion of rice glutelin, indicating their resistance to gastrointestinal enzymes. However, the biological activities of these peptides may vary under different processing conditions, such as NaCl concentration, temperature, pH, and enzymatic digestion, as shown in studies on bioactive peptides from various sources [39,40]. The dipeptide Ile-His (IH) is reported as a dipeptidyl peptidase IV inhibitor (DPP IV inhibitor) and DPP-III inhibitor. The dipeptides Glu-Pro (EP) and Ser-Pro (SP) also have bioactivity as DPP IV inhibitors according to the BIOPEP-UWM: Bioactive peptides database [41] (https://biochemia.uwm.edu.pl/biopep/start_biopep.php). Notably, the 13-amino acid peptide TQQQEQAQAQDQY has also been predicted to possess antihypertensive properties through the AHTpin database [42], highlighting its potential therapeutic applications beyond antioxidative effects (AHTpin database). It is noted that among 31 peptides from GI simulated digestion of glutelin (composed of 5-13 amino acid residues), 15 peptides were predicted as potential anticancer peptides (ACPs) by AntiCP 2.0 [43] (https://webs.iiitd.edu.in/raghava/anticp2/index.html). Meanwhile, 11 out of 15 peptides were also predicted to have non-toxin properties by ToxinPred 3.0 [44] (https://webs.iiitd.edu.in/raghava/toxinpred3/).

3.7. Antioxidant activity of synthetic peptides

These computational results were supported by in vitro antioxidant assays. The radical scavenging activity of the synthetic peptides, tested at 425 μM, revealed that TQQQEQAQAQDQY exhibited stronger radical neutralization, achieving 8.26% ABTS•$^{+}$ and 31.3% DPPH• scavenging activity (Figure 4a). In contrast, TNTPGVVY demonstrated lower activities (4.47% and 5.38%, respectively), aligning with the predicted docking energy trends. These findings confirm that both structural binding affinity and physicochemical properties of the peptide, such as amino acid composition and hydrophobicity, influence antioxidant activity [45]. Despite having a slightly lower docking score than TNTPGVVY, TQQQEQAQAQDQY has a stronger DPPH• scavenging activity.

This could be because of its longer sequence and higher glutamine (Q) and acidic residue (E, D) content, both of which are known to aid in radical stabilization [46]. Furthermore, because DPPH and ABTS have different polarities and redox potentials, the limited activity of TNTPGVVY in the ABTS•+ assay indicates selectivity in radical type and mode of action [47].

3.8. Cytotoxicity of peptides in cancer cell lines

The synthetic peptides TNTPGVVY and TQQQEQAQAQDQY were tested at 10 µM against four human cancer cell lines (A549, SW480, MDA-MB-231, HepG2) for 48 h. Both peptides showed low cytotoxicity, with cell viabilities remaining above 94% across all lines. The most notable reduction was seen in A549 cells treated with TQQQEQAQAQDQY (94.3%) and TNTPGVVY (95.4%), compared to 14.0% viability in the doxorubicin-treated group. No significant cytotoxic effects were observed in HepG2 cells. These findings suggest that both peptides are non-toxic at the tested concentration (Figure 4b). Cell viabilities remained above 94%, in contrast to the strong cytotoxic effect of the positive control (doxorubicin), affirming the safety profile of these peptides.

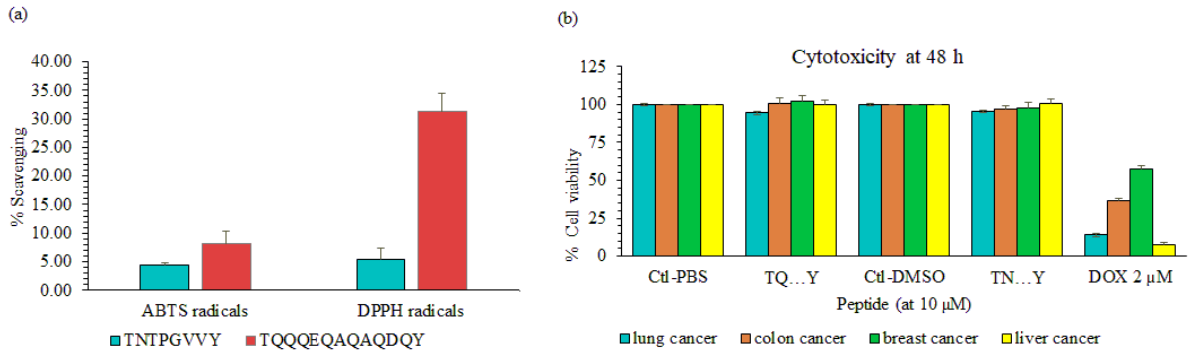

Figure 4. ABTS•+ and DPPH• scavenging activities of the synthetic peptides TQQQEQAQAQDQY and TNTPGVVY (a), and cytotoxicity against certain cancer cell lines (b).

Note: Ctl-DMSO and Ctl-PBS: control solvents for the peptide TQ…Y and TN…Y, respectively; TQ…Y: TQQQEQAQAQDQY; TN…Y: TNTPGVVY; DOX: Doxorubicin.

4. Conclusions

This study explored the potential of rice protein glutelin from diverse Thai rice varieties as a source of bioactive peptides with antioxidant properties. The analysis of crude protein extracts revealed glutelin as the predominant fraction across all varieties, exhibiting significant ABTS•+ radical scavenging activity. Simulated gastrointestinal digestion of glutelin identified peptides with strong binding affinities to ABTS•+ and DPPH• radicals, surpassing glutathione in some instances. Molecular docking elucidated critical interactions such as hydrogen bonds and hydrophobic interactions crucial for radical scavenging. The glutelin-derived synthetic peptides TNTPGVVY and TQQQEQAQAQDQY showed the most promising antioxidant binding affinity, exhibited in vitro radical scavenging, and did not exert significant cytotoxic effects in vitro. Notably, peptides derived

from rice glutelin showed promise in antioxidant activity, highlighting their potential for application in functional foods and nutraceuticals. Further optimization of extraction methods, peptide purification, and identification, as well as exploration of in vivo efficacy, are warranted to fully exploit these bioactive peptides.

Author contributions

Saranyu Khammuang: Molecular docking design, Analysis, Writing – review & editing; Kamonpan Sanachai: Molecular docking experiment, Writing – review & editing; Methus Kittika: Sample preparation, Antioxidant investigation; Kriengsak Lirdprapamongkol: Cytotoxicity experiments, Analysis, Writing – review & editing; Jisnuson Svasti: Cytotoxicity analysis, Supervision; Rakrudee Sarnthima: Conceptualization, Funding acquisition, Project administration, Validation, Writing – original draft, review & editing. All authors have read and agreed to the published version of the manuscript.

Use of Generative-AI tools declaration

The authors declare that they have not used artificial intelligence tools in the creation of this article.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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