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A novel multi-biofunctional protein from brown rice hydrolysed by endo/endo-exoproteases

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Brown rice, which is a less allergenic food grain and contains essential amino acids, was hydrolysed by bromelain and PROTEASE FP51® to improve its functionalities and taste for food applications. The hydrolysate prepared by bromelain (eb-RPH) had high protein solubility, surface hydrophobicity, low molecular weight peptides, hydrophobic amino acids (leucine, valine and glycine) and flavor amino acids (glutamic acid and aspartic acid). The eb-RPH exhibited higher 1,1-diphenyl-2-picrylhydrazyl (DPPH[•]) and 2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic (ABTS^{•+}) radical-scavenging activities of 76.62% and 52.96%, respectively, and possessed a better foaming capacity (221.76%) and emulsifying capacity (32.34%) than the hydrolysate prepared by PROTEASE FP51® (ep-RPH) did. The eb-RPH gave the desired taste, which is attributed to volatile flavor compounds (benzaldehyde, benzeneacetaldehyde and 2-acetyl-1-pyrroline) and non-volatile flavor compounds, such as monosodium glutamate, 5'-guanosine monophosphate and 5'-inosine monophosphate (0.07, 0.03 and 0.05 mg mL⁻¹, respectively). Brown rice peptides generated by bromelain were novel bioactive peptides with multifunctional properties.

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Introduction

For centuries, enzymatic hydrolysis has been used to modify the nutritional and organoleptic qualities of proteins in traditional foods. During the last decade, the peptides generated from enzymatic hydrolysis under controlled proteolysis are reported to have biological activities and functional properties, as well as flavor characteristics.^{1,2} The biological antioxidant activities, functional properties and taste of the peptides are directly affected by various factors, such as the source of the plant protein, the operational conditions applied to hydrolyse the proteins, the degree of hydrolysis, the type of protease used, the amino acid composition, peptide sequences, molecular size, and surface hydrophobicity.^{3–5}

An increase in the biological properties might be the result of the cleavage of peptide bonds, whereas excessive hydrolysis primarily causes the loss of some functional properties. Several studies have reported that some low molecular weight peptides (2–20 amino acid residues and molecular masses of less than 6 kDa) containing tyrosine, tryptophan, methionine, histidine, cysteine, leucine, and lysine showed strong biological antioxidant activities.^{6,7} The functionality of the peptide, including protein solubility and its foaming and emulsifying

properties, could be improved by enzymatic hydrolysis under limited conditions.² Peptides ranging from 50 to 100 kDa had a higher emulsifying capacity than those of 20 kDa did.⁸ Apart from their functionalities, the 5–10 kDa fractions of enzymatic protein hydrolysates from different plant protein sources possess unique taste properties, including sweet, salty, bitter, and umami tastes. The taste resulted from the presence of components such as free amino acids, peptides and taste nucleotides.⁹ Most peptides with a high molar ratio of L-glutamic acid peptides and a hydrophilic C-terminal residue have also been reported to elicit an intense umami taste.⁵ However, peptides containing hydrophobic amino acids in their sequences or at the end of the peptide chain, which showed excellent biological properties, were known to give a distinct bitter taste.¹⁰

Numerous peptides from various cereal protein sources, namely mung beans, sesame, peanuts and soy, are hydrolysed by proteolytic enzymes and have been reported to exhibit biological antioxidant capacities, functional properties and flavor.^{2,5,9,11,12} In addition to these cereals, brown rice protein is a good plant protein source because rice proteins are a gluten-free and less allergenic food grain. Although the protein content of rice is not particularly high (7–9 g per 100 g), rice protein contains the complete essential amino acids, particularly lysine, which is a limited amino acid in other cereals¹³ and has been reported to be an antioxidative amino acid.¹⁴ Moreover, rice protein contains high proportions of hydrophobic amino acids (leucine, valine, isoleucine and

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histidine) and flavor amino acids (glutamic acid and aspartic acid), which are potential sources for the preparation of biological and functional peptides with a good taste. However, the application of rice protein as a nutritious and functional food is still limited because it consists mainly of alkali-soluble glutelin (~80%), which exhibits poor solubility and low digestibility at acidic and neutral pH. Various studies have reported that a number of endoproteinases, such as Alcalase, Chymotrypsin, Neutrase, Papain, and Flavorase could improve the desired functionalities of rice proteins.^{4,15} Among these enzymes, Alcalase, an alkaline proteinase, has good hydrolysis activity to improve peptide biological and functional properties, but the plant protein hydrolysates produced with Alcalase generate high salt in the neutralizing process, and have a bitter taste that greatly limits its application in food products.¹⁶

To overcome these problems, stem bromelain, which has an optimal activity over a wide pH range (pH 4.0–8.0), was used to improve the biological and functional properties of plant proteins, due to the remaining hydrophobic and non-polar amino acid residues at the C-terminus of the peptide after bromelain hydrolysis.¹⁷ Although the hydrolysates produced by bromelain give a slightly bitter taste, this taste had no negative impact on the food product.^{1,11} Nevertheless, endo-exoproteases, such as Flavourzyme® and PROTEASE FP51®, were applied to reduce the undesirable bitterness of protein hydrolysate. Flavourzyme® has been reported to cleave hydrophobic residues at the end of peptide chains to produce the free amino acid, which may reduce the antioxidant activity.^{15,18} In addition to Flavourzyme®, PROTEASE FP51® (Aspergillopepsin I), which cleaves hydrophobic residues at different sites compared with those of Flavourzyme®, could be used successfully to produce protein hydrolysates that had a less bitter taste, but still possessed biological and functional properties.¹⁹ There is no report on the application of bromelain and PROTEASE FP51® to generate biological and functional peptides with a good taste from brown rice protein. Therefore, the objective of this study was to evaluate the antioxidant activities, functional properties and taste characteristics of brown rice protein hydrolysates produced by bromelain and PROTEASE FP51®.

Results and discussion

Chemical properties of the brown rice protein hydrolysate

Degree of hydrolysis and protein solubility. A substantial number of reports have indicated that the hydrolysis must be performed under strictly controlled conditions to produce a specified size of peptides and degree of hydrolysis and obtain the desirable antioxidant activities, functional properties and taste of the protein hydrolysates.^{3,5} In our previous study, brown rice was hydrolysed by various concentrations of bromelain and PROTEASE FP51® (0–20% weight of enzyme/weight of brown rice powders) with different hydrolysis times (0.5–24 h) to find the optimal hydrolysis conditions. Statistical analysis shows that the DH value increased rapidly at 0–10% enzyme

concentration (w/w) and at 0–3 h of hydrolysis, thereafter reaching a plateau which could be due to the saturation of the enzyme/substrate or inhibitory effects of the end products.^{1,11} Therefore, the RPH produced by 10% enzyme (w/w) for 3 h was the optimal hydrolysis condition of both proteases because these conditions resulted in the highest % DH in the shortest hydrolysis time (data not shown). With the same protein substrate concentrations and same amount of enzymes, the RPH produced by PROTEASE FP51® (ep-RPH) showed higher DH values ($53.49 \pm 0.49\%$) than those of eb-RPH ($40.10 \pm 1.56\%$). The difference in the DH values might be attributed to the specificity of the enzyme because PROTEASE FP51® has both endo-proteinase and exo-peptidase activities, which hydrolysed the peptide bonds within the protein molecule and at the end of the protein structure. These results were confirmed by the protein solubility.

The solubilities of the ep-RPH ($76.54 \pm 2.93\%$) and eb-RPH ($69.41 \pm 1.02\%$) proteins were significantly greater than the solubility of the control ($17.21 \pm 1.04\%$). An increase in the nitrogen solubility of both RPHs over that of the native protein was the reason that enzymatic hydrolysis could lead to unfolded protein molecules and expose the charged residues and polar amino acid groups on the surface of the protein molecules, which could form hydrogen bonds with water.^{2,4} Similar to the degree of hydrolysis, the solubility of the ep-RPH protein was greater than that of eb-RPH. This could be due to the differences in the modes of action of the proteases used, which potentially influenced the molecular size, hydrophilic groups and polar groups of the protein hydrolysates. As a consequence, ep-RPH, which had a higher DH value than that of eb-RPH, also exhibited higher solubility in water.

Surface hydrophobicity. The surface hydrophobicity values (S_o), indicating the number of hydrophobic groups on the surface of a protein in contact with a polar aqueous envelopment, of eb-RPH and ep-RPH were measured, with ANS as the fluorescent probe. Measurement of S_o using ANS diluted in phosphate buffer (pH 7.0) is the most appropriate way to assess proteins and seems to be a useful fluorescent probe to monitor changes in the protein surface hydrophobicity of RPHs, which were dissolved in water at neutral pH but were aggregated in alcohol solution. The results showed that the native brown rice protein had a higher S_o value (1235) compared to eb-RPH (556) and ep-RPH (405). Basically, the proteolysis of protein into small peptides might cause an increase or decrease of the surface hydrophobicity, which mainly depends on the exposure of the hydrophobic groups that have been buried inside the core of the intact native protein.⁸ In the present study, the exposed hydrophobic surfaces of the RPHs were reduced as the DH increased. The possible reason is that peptides released from the native structure of the brown rice protein could have more hydrophilic groups on the surface, decreasing their surface hydrophobicity.⁴ This observation was consistent with the selectivity and specificity of bromelain to hydrolyse proteins at hydrophobic amino acid residues and non-polar amino acid residues within the molecules.¹⁷ However, the hydrolysis by PROTEASE FP51® could eliminate some hydro-

phobic amino acids, resulting in fewer hydrophobic binding sites than the peptides produced by bromelain.⁴

Molecular weight distributions. The molecular weight distributions of the hydrolysed proteins produced by the two proteases reflected the differences in the lengths of the peptide chains, which had a direct impact on their functionalities and flavor characteristics. Fig. 1 shows that brown rice exhibited several bands with molecular weights of approximately 10, 13, 18, 20, 25, 31, 37, 52, 57 and over 75 kDa. The bands at 13 kDa, approximately 18 and 25 kDa and over 200 kDa, approximately 18–20 kDa, and approximately 57 kDa might be prolamin, globulin, albumin and glutelin, respectively, as reported by Cao *et al.*²⁰ When the brown rice protein was hydrolysed by PROTEASE FP51®, the high MW peptides were hydrolysed to low MW peptides (approximately 57, 37 and less than 5 kDa). However, the eb-RPH was mainly composed of larger peptides (approximately 37 to less than 10 kDa), but the band representing rice glutelin (approximately 57 kDa) was completely absent due to a bromelain-specific cleavage at the arginine–alanine and alanine–glutamic acid bonds.^{17,19} Accordingly, glutelin, which contained higher amounts of arginine, glutamic acid, alanine and leucine compared to the other protein fractions, was completely hydrolysed by bromelain.¹³ The presence of the low molecular weight band of less than 5 kDa in ep-RPH revealed that PROTEASE FP51® was rather effective in degrading the brown rice protein into smaller peptides and free amino acids compared to bromelain. This result, which was consistent with the data for the DH values, suggested that the size of the peptides was influenced by the type of protease.

Amino acid compositions. The amino acid compositions of brown rice, eb-RPH and ep-RPH were determined using HPLC. Fifteen amino acids were found in brown rice treated with 6 N HCl at 110 °C for 24 h, resulting in complete hydrolysis to the

free amino acids. Table 1 shows that the proteins in brown rice contained a large amount of essential amino acids. As reported by García-Lara,¹³ glutamic acid was the most abundant amino acid in rice, followed by aspartic acid, arginine, leucine and alanine, respectively. After hydrolysis, the amino acid compositions of eb-RPH and ep-RPH had changed slightly compared to that of the brown rice protein, but the majority of the essential amino acids were also found in the two RPHs, with different amounts of the amino acids. It was also found that the amount of hydrophobic amino acids in eb-RPH and ep-RPH was higher than the hydrophilic amino acids. One of the possible explanations for these results was that bromelain and PROTEASE FP51® preferred to hydrolyse the proteins at hydrophobic amino acid residues, and non-polar amino acid residues remain at the C-terminus of the peptide, causing a larger amount of hydrophobic amino acids in the two RPHs.¹⁷ Based on the results of the amino acid compositions, the peptide content was evaluated by subtracting the free amino acids (without acid hydrolysis) from the total amino acids that were subjected to complete hydrolysis using 6 N HCl. The amount of free amino acids in eb-RPH and ep-RPH was lower than the total amino acids, indicating that most of the amino acids were present in the peptide form (Table 1). Moreover, ep-RPH had a higher content of free hydrophobic amino acids than that of eb-RPH, and some free amino acids, including aspartic acid, serine and glutamic acid, were not found in ep-RPH due to the site-specific hydrolysis of PROTEASE FP51®, which hydrolysed the hydrophobic amino acids at the end of the peptide chain.

The amino acid results of eb-RPH indicated that hydrophobic amino acids were present in peptides, but these amino acids were mostly liberated from the peptide to free amino acids in ep-RPH, which was in agreement with the results of the surface hydrophobicity. From the above results, the amino acid compositions and specific peptide sequences, as well as the chain length of the peptides from two hydrolysates, were different and might be involved in drastically different antioxidant activities, functional properties and taste characteristics of the protein hydrolysates.

Biological antioxidant activities of the brown rice protein hydrolysates

Fig. 2 shows that the DPPH and ABTS radical-scavenging activities of the eb-RPH (76.62% and 52.96%, respectively) and ep-RPH (54.08% and 37.29%, respectively) were significantly higher compared to those of the control (brown rice). The enhanced antioxidant activity of the RPHs is likely the consequence of some low molecular weight peptides or free amino acids that were cleaved by enzymes and were able to donate an electron to react with the free radicals to convert them to more stable molecules, resulting in the termination of the radical chain reaction.⁷ It was also found that eb-RPH had a greater scavenging activity against the DPPH and ABTS radicals than ep-RPH did. The differences in the radical scavenging ability could be due to the protease specificity, which influenced the peptide length, amino acid compositions and amino acid

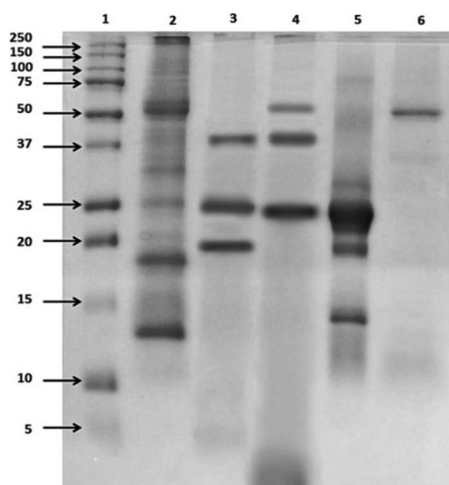


Fig. 1 Electrophoretic profile of the native brown rice protein, bromelain, PROTEASE FP51® and their hydrolysates. Lane 1 = standard MW marker; lane 2 = brown rice protein; lane 3 = eb-RPH; lane 4 = ep-RPH; lane 5 = bromelain; lane 6 = PROTEASE FP51®.

Table 1 Amino acid compositions of brown rice and its hydrolysates produced by bromelain and PROTEASE FP51®^g

Amino acids	Total amino acids of brown rice (g per 100 g of protein)	Amino acid content of eb-RPH (g per 100 g of protein)			Amino acid content of ep-RPH (g per 100 g of protein)		
		Total amino acids	Free amino acids	Bound-type amino acids ^a	Total amino acids	Free amino acids	Bound-type amino acids ^a
Aspartic acid	7.57 ± 0.12	6.41 ± 0.13	0.12 ± 0.00	6.29	7.19 ± 0.21	nd	7.19
Serine	4.58 ± 0.14	4.00 ± 0.20	0.52 ± 0.01	3.48	4.29 ± 0.11	nd	4.29
Glutamic acid	15.35 ± 0.32	8.58 ± 0.21	0.28 ± 0.00	8.30	10.25 ± 0.23	nd	10.25
Glycine	3.84 ± 0.09	3.54 ± 0.07	0.32 ± 0.01	3.22	3.02 ± 0.10	0.46 ± 0.00	2.56
Histidine	2.03 ± 0.09	1.38 ± 0.03	0.09 ± 0.00	1.29	nd	nd	—
Arginine	6.93 ± 0.07	4.30 ± 0.19	1.79 ± 0.02	2.51	3.89 ± 0.08	nd	3.89
Threonine	3.20 ± 0.04	2.89 ± 0.07	0.21 ± 0.01	2.69	3.79 ± 0.12	0.75 ± 0.01	3.04
Alanine	4.90 ± 0.10	3.83 ± 0.04	0.62 ± 0.02	3.21	3.56 ± 0.05	0.29 ± 0.01	3.27
Proline	3.73 ± 0.12	2.66 ± 0.16	nd	2.66	3.56 ± 0.07	0.43 ± 0.00	3.13
Cysteine	nd	nd	nd	nd	nd	nd	nd
Tyrosine	2.35 ± 0.03	1.46 ± 0.07	0.41 ± 0.01	3.05	2.89 ± 0.04	1.78 ± 0.01	1.11
Valine	4.80 ± 0.08	3.79 ± 0.18	0.28 ± 0.00	3.51	4.15 ± 0.06	1.49 ± 0.00	2.66
Methionine	nd	nd	nd	nd	nd	nd	—
Lysine	3.20 ± 0.09	2.94 ± 0.12	0.94 ± 0.02	3.00	3.69 ± 0.02	3.35 ± 0.01	0.34
Isoleucine	3.30 ± 0.11	3.04 ± 0.18	0.10 ± 0.00	2.93	2.41 ± 0.01	nd	2.41
Leucine	7.04 ± 0.17	4.15 ± 0.12	0.67 ± 0.02	3.48	4.27 ± 0.03	1.49 ± 0.01	2.78
Phenylalanine	4.26 ± 0.11	2.71 ± 0.05	0.29 ± 0.00	2.43	2.59 ± 0.01	2.03 ± 0.00	0.56
Total	77.08	55.69	6.64	52.05	59.56	2.40	47.49
Taste component							
Bitter ^b	29	21.26	1.75	19.51	20.01	5.91	14.11
Umami ^c	22.92	14.99	0.4	14.59	17.44	0.00	17.44
Sweet ^d	19.62	15.04	3.14	11.89	15.53	1.04	14.49
Salty ^e	—	—	—	—	—	—	—
Tasteless ^f	5.54	4.41	1.35	6.05	6.58	5.13	1.45
Total	77.08	55.69	6.64	52.05	59.56	12.07	47.49

^a Bound-type amino acids = total amino acids – free amino acids. ^b Bitter was calculated from the sum of glycine + histidine + proline + valine + methionine + isoleucine + leucine + phenylalanine. ^c Umami was calculated from the sum of aspartic acid + glutamic acid. ^d Sweet was calculated from the sum of serine + arginine + alanine + threonine. ^e Salty was calculated from cysteine. ^f Tasteless was calculated from the sum of tyrosine + lysine. ^g nd = not detected.

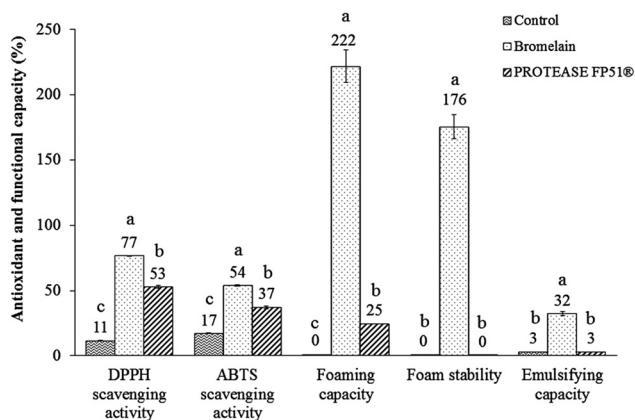


Fig. 2 DPPH scavenging activity, ABTS scavenging activity, foaming capacity, foam stability and emulsifying capacity of the brown rice protein hydrolysates produced by bromelain (eb-RPH) and PROTEASE FP51® (ep-RPH). ^{a,b,c} The values for the same parameter with different letters are significantly different ($p < 0.05$).

sequences of the peptides within protein hydrolysates.¹² Presumably, this may be because bromelain preferably hydrolyses peptide bonds by cleaving after the N-terminus of aromatic amino acids, which increased the number of small peptides containing aromatic amino acids at the N-terminus. In fact,

some aromatic amino acids, such as tyrosine, phenylalanine and histidine, were remarkably important to the antioxidant activity of peptides due to their special structures. Tyrosine and phenylalanine have phenolic groups that can specifically and easily donate protons to electron-deficient radicals. Meanwhile, the imidazole group in histidine could be a proton donor.¹² Interestingly, lysine, which was a limited amino acid in other cereals and is an antioxidative amino acid, was mostly present in the peptide form in eb-RPH, whereas lysine and hydrophobic amino acids were largely released into free amino acids in ep-RPH, causing the loss of their antioxidant activities. Therefore, the eb-RPH peptides contained a higher amount of hydrophobic and aromatic amino acids, had increased surface hydrophobicity, and exhibited higher antioxidant activities than ep-RPH did.

When the DPPH and ABTS radical-scavenging activities are expressed as the mmol equivalent of trolox, the scavenging activity of eb-RPH and ep-RPH against the DPPH radical (0.81 ± 0.002 and 0.60 ± 0.009 mM trolox, respectively) was lower than that for the ABTS radical (2.89 ± 0.03 and 2.02 ± 0.06 mM trolox, respectively). Although the DPPH assay was based on an electron transfer similar to the ABTS assay, ABTS^{•+} was reactive toward hydrophilic and hydrophobic antioxidant agents, whereas DPPH[•] was a stable radical that was applicable to hydrophobic systems.²¹ The water-soluble, low

molecular weight peptides of the two RPHs did not easily react with the ethanol-soluble DPPH[•] compared to the water-soluble ABTS^{•+}. Accordingly, the eb-RPH, which contains a higher content of low molecular weight hydrophobic peptides, showed a stronger ability to eliminate free radicals than that of ep-RPH. This finding showed that antioxidant peptides could be produced from brown rice.

Biofunctional properties of the brown rice protein hydrolysate

Various studies have reported that a controlled degree of hydrolysis usually improves peptide functional properties. The peptide sequence, surface hydrophobicity and a controlled size of the peptides were needed to obtain a desirable foaming and emulsifying capacity of the protein hydrolysates.^{3,20} The foaming capacity of the peptides in the eb-RPH and ep-RPH was determined by measuring their whippability. The eb-RPH had a greater foaming capacity ($221.76 \pm 12.32\%$) than that of ep-RPH ($24.64 \pm 0.00\%$), whereas the native brown rice protein did not have a foaming capacity (Fig. 2). Enzymatic modification significantly improved the foaming capacity of native brown rice protein by decreasing the peptide chain length and flexibility of the protein, which allowed the protein to encapsulate air particles. When comparing the endo-exoprotease with the endo-proteinase, eb-RPH had a higher exposure of the buried hydrophobic groups and was composed of larger molecular weight peptides compared to ep-RPH, leading to a better hydrophobic–hydrophilic balance, low surface tension and a strong tendency to form flexible films around the air bubbles. This is in accordance with other published studies on protein hydrolysates, which reported that a limited degree of hydrolysis usually improves the foaming capacities; however, excessive hydrolysis often causes loss of some of these functionalities.^{4,20} For foaming stability, only eb-RPH had foaming capabilities after 30 min ($175.56 \pm 9.24\%$), whereas ep-RPH did not have the ability to stabilise the air cells of the foam. The ep-RPH, which contained small peptides with low surface hydrophobicity, possessed poor foaming stability because the films were not strong enough to maintain their integrity.²⁰

The emulsifying activity of the RPHs produced with different types of proteases was determined by the turbidimetric method, as shown in Fig. 2. The results show that bromelain-mediated hydrolysis could significantly improve the emulsifying capacity of the brown rice protein ($32.34 \pm 1.54\%$), while there was no significant difference in the emulsifying capacity between the proteolytic hydrolysis by PROTEASE FP51® ($3.08 \pm 0.00\%$) and the control ($3.08 \pm 0.00\%$). The increase in the emulsion capacity of eb-RPH over than that of the ep-RPH peptides and the original proteins was due to the exposure of the buried hydrophobic groups during proteolysis, which increased the hydrophobic–hydrophilic balance and enhanced the peptides' ability to interact with both water and oil in food systems.^{4,20} The low molecular weight peptides are less efficient at reducing the interfacial tension and stabilising the emulsions because they cannot unfold and re-orient at the interface, as large peptides do.³ Accordingly, the eb-RPH, which contains larger molecular weight peptides, a higher

amount of hydrophobic amino acids in the peptides and higher surface hydrophobicity than that of ep-RPH, had the highest emulsifying capacity. Conversely, ep-RPH, with a higher solubility and lower surface hydrophobicity, might facilitate the diffusion and spread at oil–water interfaces and might not be amphiphilic enough to exhibit good emulsifying properties.^{4,20}

Volatile flavor, non-volatile flavor and taste of the brown rice protein hydrolysates

The volatile flavor compounds of all RPHs were detected using the DHS-SPME-GC-MS method and are summarized in Table 2. Compared with the native brown rice protein, the volatile flavor compounds of the RPHs were significantly increased. Benzaldehyde, benzeneacetaldehyde, 2,4-di-*tert*-butyl-phenol, 2-ethyl-hexanoic acid, linoleic acid ethyl ester and nonanal, which are described as having sweet, cereal-like, green, fruity, floral and fatty odor notes, were predominant in the two RPHs. The volatile flavor compounds found in the two RPHs were also reported as the volatile compounds of rice.²² The presence of amino acids in the RPHs might explain the odor of the two hydrolysates, such as aldehydes, which are formed by the oxidation of certain amino acids and fatty acids.¹ An increase in alcohols might be generated by lipid oxidation, while methyl ketones might occur by the beta-oxidation of fatty acids during heating.²³

It is generally recognized that 2-acetyl-1-pyrroline (2-AP, popcorn-like flavor compound) has been reported as an important flavor component of aromatic rice.²² It was assumed that the enhanced intensity of 2-AP might be due to hydrolysis by the enzyme, resulting in the release of volatile flavor compounds from brown rice. On the other hand, the lipid oxidation products (hexanal, nonanal and 1-dodecanol), which have been known to have a negative impact on the acceptability of rice, were derived from the degradation of polyunsaturated fatty acids either by auto-oxidation or by the action of enzymes, including lipoxygenase and fatty acid hydroperoxide lyase.²² The results showed that there were significant differences in the volatile flavor components and the intensity of the volatile compounds following hydrolysis by the different proteases (Table 2). The intensity of the volatile flavor components in the hydrolysate produced by PROTEASE FP51® was greater than that of the hydrolysate produced by bromelain. The intensity of the sweet, floral (2-acetyl-1-pyrroline, benzaldehyde and phenylethyl alcohol), which are considered as desirable aroma attributes, and fatty (octanoic acid, hexadecanoic acid methyl ester and linoleic acid ethyl ester) notes found in ep-RPH tended to be higher than those of eb-RPH. Meanwhile, the green and earthy notes (2-pentyl furan, octanal and 2-heptenal) of the two RPHs were not different. The possible explanations for the differences in the volatile flavor compounds could be the difference in the hydrolysis efficiency of the enzymes, resulting in the differences in the amino acid compositions, peptides and release of volatile compounds.

Non-volatile flavor compounds, such as glutamic acid and aspartic acid, have been known to be the major components in

Table 2 Volatile flavor compounds of the brown rice protein hydrolysates produced by bromelain and PROTEASE FP51®

Volatile compounds ^a	RI ^{a,b}	Odor description ^c	Brown rice	Correlative peak area (×10 ⁶)	
				eb-RPH	ep-RPH
5-Methyl-2-heptanone	1152	—	—	3.42	2.79
3-Methyl-1-butanol	1183	Ethereal, cognac, fruity, banana	—	0.94	—
Ethyl ester hexanoic acid	1207	Sweet, fruity, pineapple, waxy, fatty	3.42	—	—
1-Pentanol	1224	—	—	—	1.02
Octanal	1252	Aldehydic, waxy, citrus with green	2.14	1.72	—
2-Heptenal	1282	Green, vegetable, fatty	3.90	3.95	4.33
6-Methyl-5-hepten-2-one	1294	Fruity, creamy with slight cheesy, banana	2.89	1.76	1.35
1-Hexanol	1314	Fruity and alcoholic, sweet with a green top	3.01	3.07	3.03
Nonanal	1349	Aldehydic, waxy, citrus, orange	6.23	4.78	6.30
3-Octen-2-one	1364	Green, fatty, melon	—	—	1.53
2-Octenal	1387	Fatty, green, herbal	—	2.26	—
1-Octen-3-ol	1412	Earthy, green, oily, vegetative, fungal	4.70	4.07	5.39
1-Heptanol	1417	Musty, pungent, leafy green	2.02	—	—
2-Acetyl-1-pyrroline	1421	Sweet, woody, bread, caramellic	2.25	4.10	4.26
2-Ethyl-1-hexanol	1450	Citrus, fresh, floral, oily, sweet	8.51	4.57	7.86
Benzaldehyde	1477	Almond, fruity, nutty	13.62	25.23	17.06
Ethyl ester nonanoic acid	1490	Sweet and fruity	1.94	—	1.81
1-Octanol	1515	Waxy, green, citrus, aldehydic, floral with sweet	6.33	4.15	7.90
2-Octen-1-ol	1570	Green	5.91	3.86	6.08
Benzeneacetaldehyde	1590	Green, sweet, floral, honey, cocoa	—	13.30	16.12
2-Furanmethanol	1612	Sulfuraceous estery chemical, sweet, caramellic	—	—	4.82
Phenylethyl alcohol	1851	Sweet, floral, rose	—	0.71	2.46
Benzothiazole	1891	Sulfury, vegetable, cooked nutty, meat	2.87	2.29	3.53
1-Dodecanol	1906	Fresh, waxy, rose, soapy	4.16	2.29	—
2-Ethyl-hexanoic acid	1911	Sweet, fruity, pineapple, fatty	—	4.99	5.04
Dihydro-5-pentyl-2(3H)-furanone	1959	Sweet, creamy, coconut, fatty	2.19	1.99	3.43
Ethyl ester tetradecanoic acid	1981	Sweet, waxy	—	—	4.05
Octanoic acid	1950	Fatty, waxy, rancid, vegetable, cheesy	—	2.49	3.77
6,10,14-Trimethyl-2-pentadecanone	2110	Oily, herbal, jasmine, celery, woody	6.02	1.52	5.81
Eugenol	2141	Sweet, spicy, clove, woody	8.38	3.57	34.85
2-Methoxy-4-vinylphenol	2156	Spicy, clove, smoky, phenolic, peppery, woody	—	—	10.32
Nonanoic acid	2202	Waxy, dirty, cheesy	—	7.43	—
Hexadecanoic acid methyl ester	2223	Oily, waxy, fatty	1.94	1.01	5.92
Hexadecanoic acid ethyl ester	2270	Waxy, fruity, creamy, milky	6.21	1.01	5.29
2,4-Di-tert-butyl-phenol	2301	Phenolic	14.99	11.55	19.46
2-Methoxy-4-(1-propenyl)-phenol	2310	Spicy, clove, smoky, phenolic, peppery, woody	—	—	1.87
Methyl ester-9-octadecenoic acid	2472	Mild fatty	2.45	0.73	18.97
Ethyl oleate	2476	Fatty, oily, dairy and milky	5.48	0.73	32.40
Linoleic acid ethyl ester	2510	Mild fatty, fruity	5.51	5.59	35.80
Vanillin	2566	Sweet, vanilla, creamy, phenolic	—	0.73	—
Total			127.05	129.82	284.61

^a All compounds were identified by comparison with mass spectra and the retention index database. ^b RI (retention index) calculated with a DB-Wax stationary phase using a series of alkanes between C₈ and C₂₀ as reference standards. ^c The odor descriptions were cited from <http://www.flavornet.org> and a recent report.

the taste sensation of umami, the Japanese word for savory and delicious.²⁴ In addition to glutamic acid, its salt, monosodium glutamate (MSG), and the 5'-ribonucleotides, such as 5'-guanosine monophosphate (5'-GMP) and 5'-inosine monophosphate (5'-IMP), have also provided umami taste characteristics.²⁵ The results showed that the MSG, 5'-GMP and 5'-IMP components identified by HPLC were found in eb-RPH and ep-RPH (Fig. 3). The MSG, 5'-GMP and 5'-IMP found in the two RPHs could be naturally synthesized and are present in brown rice.²⁶ The proteolytic process could break down these nucleotides that are bound to the proteins, resulting in the release of non-volatile flavor compounds. Therefore, the MSG, 5'-GMP and 5'-IMP contents in ep-RPH were higher than those in eb-RPH because of the high hydrolysis efficiency of PROTEASE FP51®.

The results of the volatile and non-volatile compounds were confirmed by sensory evaluation. As shown in Fig. 4, the sensory profile of the control (without enzymatic hydrolysis) was rice odor, sweet odor, bitter taste, sweet taste and umami taste, which received scores of 4.0, 2.0, 1.2, 1.2 and 1.0, respectively. When the brown rice protein was hydrolysed, the intensity of the rice odor and sweet odor was increased because the rice and sweet volatile compounds were liberated by enzymatic hydrolysis. In accordance with the volatile profile identified using GC-MS (Table 2), the ep-RPH resulted in greater rice odor and sweet odor scores than those of eb-RPH. For the taste characteristics described by the panellists, eb-RPH and ep-RPH were predominated by a sweet taste, followed by a moderate umami and slight bitter taste (Fig. 4). Remark-

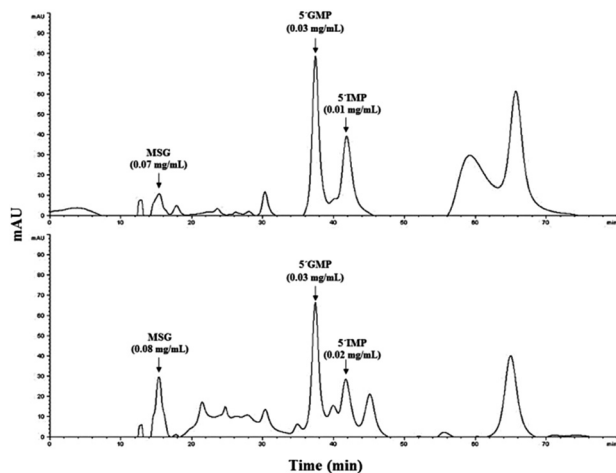


Fig. 3 Chromatogram of the non-volatile compounds, 5'-guanosine monophosphate (5'-GMP), 5'-inosine monophosphate (5'-IMP), and monosodium glutamate (MSG), that were present in the RPH hydrolysed by bromelain (top) and PROTEASE FP51® (bottom).

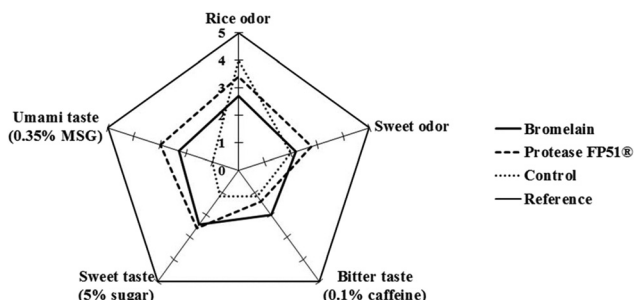


Fig. 4 Sensory profiles of the brown rice protein hydrolysates produced by bromelain and PROTEASE FP51® compared to the control (without enzymatic hydrolysis) and the taste reference solutions.

ably, ep-RPH had a higher intensity of the umami taste, but it had a lower bitter taste intensity than that of eb-RPH. Meanwhile, the sweet taste of ep-RPH showed a similar score to eb-RPH (Fig. 4). It was inconsistent with the calculated amino acids, which revealed that the umami and bitter amino acids of eb-RPH were lower than those of ep-RPH, while sweet amino acids of the two RPHs did not differ (Table 1). Although the aggregated free bitter amino acids in ep-RPH were higher than in eb-RPH, the ep-RPH had a lower bitter taste score. The explanation for these observations could be that PROTEASE FP51® liberated hydrophobic amino acids at the end of the peptide and decreased the peptide chain length of the hydrophobic bitter peptides, which frees the hydrophobic amino acids, and the short hydrophobic bitter peptides gave a lower bitterness than the large hydrophobic bitter peptides did.²⁴

The taste difference between two hydrolysates was likely caused by different factors, including the molecular size, composition of the free amino acids and the amino acid sequences of the peptides, which resulted from the specificity of the protease used.¹⁰ It has been reported that amino acids are highly

correlated with the taste characteristics in food. For example, leucine might impart a bitter taste, whereas alanine and arginine might impart a sweet taste.²⁴ Additionally, numerous peptides generated by enzymatic hydrolysis from plant proteins have also been reported to elicit unique tastes.⁵ Therefore, the distribution of the free amino acids and flavor peptides found in the RPHs might explain the sensory perception of the two hydrolysates. The bitter taste may be caused by the presence of hydrophobic amino acids (histidine, valine, and proline) and strongly hydrophobic bitter peptides.¹⁰ The sweet taste in the RPHs may be associated with the sweet amino acids, such as serine, arginine, alanine, and threonine.²⁴ The umami taste may be related to the presence of free glutamic acid and aspartic acid, which were present in high amounts in the RPHs. Moreover, MSG, 5'-GMP, 5'-IMP and peptides consisting of high molar ratios of L-glutamic acid could have contributed to the umami taste of the two hydrolysates.^{5,9} As expected, the use of endo-exoproteases, such as PROTEASE FP51®, could minimize the bitterness in the hydrolysable product because it produced fewer bitter peptides, with a lower amount of hydrophobic amino acids than bromelain.¹⁸ This result was in agreement with the surface hydrophobicity and amino acid composition results, which showed that eb-RPH had a higher surface hydrophobicity and contained a higher content of total hydrophobic amino acids than that of ep-RPH.

Experimental

Materials and chemicals

Brown rice (Khao Dawk Mali 105) was purchased from a local market in Thailand. PROTEASE FP51® (E.C. 3.4.23.18; 91 470 U g⁻¹) was kindly provided by Siam Victory Chemicals Corporation. Stem bromelain (E.C. 3.4.22.32; 97 044 U g⁻¹) was purchased from K-Much Industry Co., Ltd, Bangkok, Thailand. One unit was defined as the amount of enzyme that liberated 1 mg of tyrosine from a casein substrate in 10 min at a pH of 6.0 and a temperature of 50 °C. All reagents were purchased from Sigma-Aldrich (Milan, Italy) and were of analytical grade. The brown rice (containing 9.38% protein, 0.62% fat, 0.24% crude fiber, 1.88% ash, and 87.88% carbohydrate on a dry weight basis) was ground and sieved through an 80-mesh screen. The powder was packed in plastic bags and stored at -20 °C prior to proteolytic hydrolysis.

Enzymatic hydrolysis of brown rice protein

Firstly, the brown rice powder was pasteurised using an autoclave at 121 °C for 15 min for sterilization and inactivation of autolytic enzymes. 10 g of the sterilized brown rice powder was dispersed in 100 mL of sterile distilled water, and the pH of the samples was adjusted to 6.0 with 1 N HCl. The sample was then pre-incubated at 50 °C for 10 min for efficient enzymatic hydrolysis at the start of the reaction. The dispersions were incubated at 50 °C for 3 h with bromelain or PROTEASE FP51® at an enzyme to substrate ratio (w/w) of 10%. After hydrolysis, the reaction was stopped by heating at 75 °C for

15 min to inactivate the enzyme. The samples were centrifuged at 5000g for 15 min, and the obtained supernatant was then filtered through filter paper (Whatman no. 1). The enzymatic bromelain brown rice protein hydrolysates (eb-RPH) and the enzymatic PROTEASE FP51® brown rice protein hydrolysates (ep-RPH) were stored in brown glass bottles at -20°C prior to analysis. All tests were performed in triplicate.

Determination of the chemical properties

Degree of hydrolysis and protein solubility. The degree of hydrolysis (% DH) was performed by the method described by Laohakunjit *et al.*¹ The % DH indicated the efficiency of the enzyme for cleaving the peptide linkages, which is defined as the percentage of soluble protein in trichloroacetic acid (TCA). The brown rice hydrolysates (10 mL) were mixed with 10 mL of 20% TCA and then centrifuged at 5000g for 15 min at 25°C . The supernatants were analysed by the Kjeldahl method.²⁹ The degree of hydrolysis was calculated by the following formula:

$$\% \text{ degree of hydrolysis} = \frac{20\% \text{ TCA soluble N} - \text{blank}}{\text{Total N}} \times 100$$

where 20% TCA soluble N is the number of proteins that were solubilized in TCA, blank is the number of proteins of the RPH that were solubilized in TCA without enzymatic hydrolysis, and total N is the number of nitrogens in the hydrolysed protein solution.

The protein solubility of the RPHs was expressed as the percentage of the ratio between the total number of soluble proteins in TCA and the total number of proteins in each sample.²⁷ The protein solubility was calculated by the following formula:

$$\% \text{ protein solubility} = \frac{\text{protein content in supernatant}}{\text{total protein content in sample}} \times 100$$

Surface hydrophobicity. The surface hydrophobicity of each RPH was determined using 1,8-anilino naphthalene sulphate (ANS) as a fluorescent probe, according to the method of Wu *et al.*,⁸ with minor modifications. The samples were serially diluted in 0.01 M phosphate buffer (pH 7.0) to obtain protein concentrations ranging from 0.00125 to 0.03%. 20 μL of ANS (8×10^{-3} M ANS in 0.01 M phosphate buffer, pH 7.0) were added to 4 mL of the diluted protein solutions. The fluorescence intensity (FI) was measured at 390 (excitation) and 470 (emission) nm at $20 \pm 0.5^{\circ}\text{C}$, with a constant excitation and emission slit of 5 nm. The FI for each sample with a probe was then computed by subtracting the FI attributed to the protein in the buffer. The initial slope of the FI *versus* the protein concentration plot was calculated by linear regression analysis and used as the surface hydrophobicity (S_0).

Molecular weight distribution. The RPHs from each hydrolysis condition were analysed for their molecular size using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Gel electrophoresis was conducted using a 15% separating gel and a 4% stacking gel. The RPHs were diluted in a 1:1 ratio with reducing SDS sample buffer and heated at 95°C for 5 min. The treated samples (15 μL) were injected into

the gel wells and separated. Electrophoresis was performed at 40 mA until the tracker dye reached the bottom of each gel. After electrophoresis, the gels were stained with 0.2% (w/v) Coomassie brilliant blue R-250 in 10% (v/v) acetic acid:50% (v/v) methanol for 45 min and de-stained with a 10% acetic acid:40% methanol solution until the bands were clear. The molecular weight markers of 2, 5, 10, 15, 20, 25, 37, 50, 75, 100, 150 and 250 kDa (Precision Plus Protein™ Dual Xtra standards, Bio-Rad) were used as a reference.

Amino acid compositions. The amino acid compositions of the brown rice powder and its hydrolysates were analysed using high-performance liquid chromatography (HPLC). Prior to the determination of the total amino acid composition, the brown rice sample and all hydrolysates were treated with 6 N HCl at 110°C for 24 h and derivatised using the AccQ-Fluor reagent, followed by HPLC analysis (Waters Alliance 2695, USA). However, the free amino acid content of the two RPHs was determined by the same analysis without acid hydrolysis. All sample solutions were then analysed using HPLC coupled to a fluorescence detector (JASCO FP2020, Japan), with the excitation and emission wavelengths set at 250 and 395 nm, respectively. A Hypersil GOLD column (4.6 mm $\times$ 100 mm, 5 μm , Thermo Scientific, USA) was used and eluted at a flow rate of 1 mL min^{-1} in gradient mode using a mixture of eluent A (sodium acetate buffer, pH 4.90) and eluent B (60% acetonitrile). The amino acid composition was identified and quantified by comparison with a standard mixture of amino acids (Sigma-Aldrich, USA) and was reported as grams of amino acid per 100 g of protein in the sample solution.

Determination of the biological antioxidant activities

1,1-Diphenyl-2-picrylhydrazyl (DPPH') radical-scavenging activity. 1 mL of each RPH solution (100 μL of sample + 900 μL of distilled water) was added to 2 mL of 0.1 mM DPPH' dissolved in 95% ethanol. The mixture was shaken and left for 30 min in the dark at room temperature, and the absorbance was measured at 517 nm. The scavenging effect was expressed as the percentage of disappearance of the purple colour of DPPH' at 517 nm, and calculated by the following equation:

$$\text{DPPH radical scavenging effect (\%)} = \frac{A_{\text{Blank}} - (A_{\text{Sample}} + A_{\text{Control}})}{A_{\text{Blank}}} \times 100$$

where A_{Blank} is the absorbance at 517 nm of 1 mL distilled water + 2 mL of 0.1 mM of the ethanolic DPPH' solution, A_{Sample} is the absorbance at 517 nm of 1 mL aqueous sample + 2 mL of 0.1 mM of the ethanolic DPPH' solution, and A_{Control} is the absorbance at 517 nm of 1 mL of the aqueous sample + 2 mL of ethanol.

2,2'-Azino-bis 3-ethylbenzthiazoline-6-sulfonic (ABTS^{•+}) radical-scavenging activity. The scavenging activity of ABTS^{•+} was measured by dye decolorization. The radical cation was prepared by mixing 7 mM ABTS with 7 mM $\text{K}_2\text{S}_2\text{O}_8$ (ratio 2:1), and the mixture was incubated in the dark at room temperature for 12–16 h to complete the radical generation. The

ABTS^{•+} solution was diluted with distilled water to give an absorbance at 734 nm of 0.700 ± 0.030 before use. The photometric assay was conducted on 1 mL of the ABTS^{•+} solution and 10 μ L of sample and stored for 6 min in the dark. The absorbance was measured at 734 nm using distilled water as a blank. The antioxidant activity of the sample was calculated using the same equation as that for DPPH[•].

Determination of biofunctional properties

Foaming capacity. The brown rice protein hydrolysates (5.0 mL) were mixed with distilled water (5.0 mL) and then whipped by a homogenizer (Ultra-Turrax T25 basic, Germany) at a speed of 10 000 rpm for 1 min at room temperature to incorporate air. The volume of the whipped sample was then recorded. The volumes before and after whipping were recorded and the foam capacity was calculated according the following equation:

$$\text{Foaming capacity (\%)} = \frac{\text{total volume of foam}}{\text{total volume of solution}} \times 100$$

Emulsifying capacity. Refined peanut oil (2.5 mL) and 10 mL of the 50% brown rice protein hydrolysates were mixed, homogenized using a homogenizer (Ultra-Turrax T25 basic, Germany) at a speed of 10 000 rpm for 1 min, and then centrifuged at 5000g for 5 min at 25 °C. The emulsifying capacity was calculated as the volume of cream (emulsifying volume) to the volume of the solution multiplied by one hundred.

Determination of the bioflavor characteristics

Volatile flavor compounds. The composition of volatile compounds in all the RPHs was analysed on a dynamic headspace solid-phase microextraction gas chromatography-mass spectrometry (DHS-SPME-GC-MS) system (789DA MS, Agilent Technologies, Santa Clara, CA). Each sample (3 mL) was placed into a 20 mL vial and heated at 60 °C for 10 min in a GC-MS heating block for headspace analysis. The volatile compounds were absorbed onto an SPME fiber (50/30 μ m DVB/CarboxenTM/PDMS StableFlexTM, Supelco, USA) for 20 min. After equilibrium, the SPME fiber was desorbed into the injector port at 240 °C for 20 min, and the injector was operated in splitless mode. Helium was used as the carrier gas at a constant velocity of 1.0 mL min⁻¹. The volatile compounds were separated using a DB-WAX capillary column (30 m $\times$ 0.25 mm, 0.25 μ m film thickness; J&W Scientific Inc., Folsom, CA, USA). The oven temperature program was as follows: initial temperature of 55 °C; increased to 180 °C at 5 °C min⁻¹; increased to 200 °C at 8 °C min⁻¹; and held at 200 °C for 10 min. The volatile compounds were detected using MSD (scan range of 35–350 amu) at 230 °C. The identification of the compounds was based on the comparison of their retention times and mass spectra with the data in the Wiley 275 and NIST libraries at a quality match greater than 85%. The data were also compared to previously published literature values. A series of *n*-alkanes (C₈–C₂₀) was analysed by direct injection on the GC-MS to obtain the RI values.

Non-volatile flavor compounds. The 5'-nucleotides were analysed as described by Taylor *et al.*²⁸ The RPHs were filtered using a 0.45 μ m nylon syringe filter prior to analysis. The non-volatile flavor compounds were measured by high-performance liquid chromatography (Agilent 1200 Series Rapid Resolution LC system). The separation was accomplished at a column temperature of 25 °C using an Agilent Eclipse XDB-C18 reverse phase column (4.6 mm $\times$ 150 mm, 5 μ m). The mobile phase was 0.5 M KH₂PO₄/H₃PO₄ (pH 4.0). The flow rate was 0.10 mL min⁻¹ for 80 min and the injection volume was 20 μ L, which was analysed using a UV-visible diode array wavelength at 254 nm. Each 5'-nucleotide (5'-GMP, 5'-guanosine monophosphate; 5'-IMP, 5'-inosine monophosphate and monosodium glutamate) was quantified by the calibration curve of the authentic standard.

Sensory profile analysis. The sensory evaluation panellists consisted of 5 males and 10 females between the ages of 25 and 35 years old who had previously been trained to recognize basic tastes such as bitterness, saltiness, sourness, sweetness and umami taste. They were trained using a triangle test with deionised water, following an increasing order of concentration of reference solutions over at least three training sessions to assess their accurate sensory evaluation abilities. Fifteen panellists performed the quantitative descriptive analysis test according to the method of Rhyu and Kim with slight modification.⁹ Evaluation was done in a separated sensory booth at a temperature of 25 ± 2 °C. The liquid RPHs (5 mL) were served in opaque disposable plastic cups. For evaluation, the fully-trained panellists were asked to evaluate the sample taste with regards to intensity on a scale ranging from 0 to 15 (0: no taste; 15: very strong taste) for 5 attributes including two odors for rice odor and sweet odor, and three tastes of bitter, sweet, and umami. The cooked rice (10–50% w/v), caffeine (0.05–0.10% w/v), sugar (1–5% w/v) and monosodium glutamate (0.15–0.35% w/v) (Ajinomoto Co., Ltd, Thailand) were dissolved in hot distilled water and used as reference solutions for the rice odor and sweet odor, and bitter, sweet and umami tastes, respectively. Each reference solution was assigned 5 points on the 15-point intensity scale.

Statistical analysis. All tests were performed in triplicate. The statistical analysis was performed based on a randomized complete block design (RCBD). The significant differences between each sample were obtained by analysis of variance and Duncan's Multiple Range Test (DMRT) using version 9 of the SAS program (SAS Institute Inc., Cary, NC, USA).

Conclusions

When comparing the brown rice protein hydrolysates produced by bromelain with those produced by PROTEASE FP51®, the eb-RPH, which exhibited high surface hydrophobicity and was composed of low molecular weight hydrophobic peptides, has distinct antioxidant activities and more favorable functional properties than that of ep-RPH. However, ep-RPH had a quite good ability to scavenge DPPH[•] and ABTS^{•+} radi-

cals and gave desirable flavor characteristics. Although eb-RPH gave a slightly bitter after-taste, it is composed of MSG, 5'-GMP and 5'-IMP, which are flavor enhancers. Moreover, eb-RPH had a sweet odor from 2-acetyl-1-pyrroline and had a sweet taste, as accepted by the panellists. Accordingly, eb-RPH could be used as a foaming agent, emulsifier and flavor enhancer for various food products. Nevertheless, PROTEASE FP51® appeared to be an ideally suited protease for reducing the bitter taste in protein hydrolysates. The multifunctional properties of brown rice protein hydrolysed by bromelain may be a value-added utilization of brown rice. Further research on purification and identification of bioactive peptides from brown rice should be carried out.

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