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Isolation and characterisation of antioxidative peptides from bromelain-hydrolysed brown rice protein by proteomic technique

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Abstract

In this study, proteins from Thai brown rice (Khao Dawk Mali 105) were separated into albumin (2.18 %), globulin (3.98 %), glutelin (84.23 %), and prolamin (9.61 %) fractions, and were hydrolysed with various bromelain concentrations and hydrolysis times. Liquid chromatography-electrospray ionization/mass spectrometry (LC-ESI-MS/MS) was conducted to assess the composition, molecular weight (MW) distribution, and sequence of the resulting peptides, and showed that most peptides have a MW below 2000 Da (60–70 %). Glutelin fraction hydrolysates exhibited the highest 2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic (ABTS^{•+}) radical-scavenging (0.69 ± 0.04 μ M trolox) and copper chelating (4.12 ± 0.01 mg ethylenediaminetetraacetic acid; EDTA) activities, which was further fractionated into six fractions using reversed-phase high-performance liquid chromatography. The fourth fraction showed the highest ABTS^{•+} scavenging (1.08 ± 0.03 mM trolox) and copper chelating (5.00 ± 0.02 mg EDTA) activity. LC-MS/MS analysis revealed that the peptides with MW less than 1500 Da and hydrophobic or aromatic N-terminal residues, such as SPFWNINAHS, MPVDVIANAYR, VVYFDQTQAQA, and VEVGGGARAP, possibly contributed to the highest antioxidant activity in fourth fraction.

Keywords

Brown rice; bromelain; LC-ESI-MS/MS; glutelin hydrolysate; antioxidant peptides

1. Introduction

In recent years, food-derived protein hydrolysates have gained considerable attention from nutraceutical and cosmetic industries because of their diverse functions, especially biological activities beyond their nutritional effects [1–3]. Rice (*Oryza sativa* L.) proteins have become one of the best sources of bioactive peptides for utilization as ingredients in functional foods, drugs, or cosmetics owing to their excellent nutritional and hypoallergenic properties compared to other cereal and legume proteins [4]. Rice proteins can be grouped

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into albumins, globulins, prolamins, and glutelins according to their solubility [5]. The major storage protein of rice is the alkali-soluble glutelin, which exhibits poor solubility and low digestibility under strong acidic and neutral pH conditions [6]. Accordingly, numerous endoproteinases are used to improve the nutritional and functional qualities of rice proteins, including alcalase, chymotrypsin, neutrase, trypsin, papain, flavorase, proteases M, N, S, and P, and pepsin. Furthermore, enzymatic hydrolysates of rice protein show radical scavenging activity and lipid peroxidation inhibition in a linoleic acid emulsion system with angiotensin converting enzyme [7–9].

The exact mechanisms underlying the biological activity of rice peptides have not been fully characterized, although the molecular weight (MW), hydrophobicity, and amino acid composition of these peptides show a strong correlation with antioxidant activity. Several rice derived peptides such as RPNYTDA, TSQLLSDQ, TRTGDPFF and NFHPQ from rice residue [10], FRDEHKK and KHNRGDEF from rice endosperm [7], and YSK from rice bran [11] have been reported to exert strong antioxidant activities. Generally, the antioxidant peptides in rice contain 3–30 amino acids with MW from 500 Da to 3611 Da and aromatic or hydrophobic residues, such as tyrosine, tryptophan, methionine, histidine, cysteine, leucine, and lysine [7,8,10,12,13]. Interestingly, peptides obtained from digestion of different rice protein fractions with different proteolytic enzymes had different amino acid sequences, resulting in different biological and functional properties [13,14].

Two major proteomics techniques, the Edman degradation reaction and liquid chromatography mass spectrometry (LC–MS/MS), have been applied to explore the relationship between the biological activities of rice protein hydrolysates and their primary sequences [15]. Compared with the Edman degradation method, LC–MS/MS is a rapid and highresolution method to identify bioactive peptides in protein hydrolysates, which contains a mixture of peptides with different molecular masses, amino acid sequences, and concentrations [16]. Mass spectrometry has been successfully applied to isolate and identify bioactive peptides from rice bran [12,13] and endosperm proteins [7,8,10].

Rice is the main staple food and export product of Thailand. Khao Dawk Mali 105 (KDML 105), a low amylose rice, has gained much attention because of its good aroma, nutrition, and high cooking quality [13]. Especially, brown rice consists of the bran, the germ and the endosperm, and as a result has a higher protein content than endosperm protein or bran itself. The biological and functional properties of brown rice hydrolysate have been published in our previous work [9], however, peptides with different amino acid composition often show unique functions [12,13] and the sequence of bioactive peptides from bromelain-hydrolysed brown rice proteins has not been reported to date. Therefore, the aims of the present study were to identify bioactive peptides in brown rice protein hydrolysates from four protein fractions (albumin, globulin, glutelin, and prolamins) and further characterize structural features associated with their biological activities using LC–ESI–MS/MS.

2. Materials and methods

2.1. Materials

Brown rice (Khao Dawk Mali 105) used in this study was harvested in November 2014 from Surin province, northeast of Thailand. The nutritional composition of brown rice corresponded to $9.38 \pm 0.24\%$ protein, $0.62 \pm 0.09\%$ fat, $0.24 \pm 0.03\%$ crude fibre, $1.88 \pm 0.04\%$ ash, and $87.88 \pm 1.25\%$ carbohydrate on a dry weight basis. The samples were ground and sieved through an 80- mesh screen. The powder was packed in aluminium foil bags and stored at $-20\text{ }^{\circ}\text{C}$ until use. Stem bromelain (E.C. 3.4.22.32; 80,518 U/g) was obtained from KMuch, Bangkok, Thailand. One unit is defined as the amount of enzyme that liberates 1 mg of tyrosine from a casein substrate in 10 min at pH 6.0 and $50\text{ }^{\circ}\text{C}$. Sequencing grade modified trypsin was obtained from Promega (Madison, WI, USA). All chemicals and reagents used in this work were of analytical grade.

2.2. Preparation of brown rice protein fractions (BRPF)

Firstly, the powder was defatted by extracting twice with three volumes of hexane. The defatted sample was air-dried under a fume hood overnight. Free phenolic compounds in the defatted sample were then extracted by shaking twice (30 min each) with 80% chilled ethanol (1:5, defatted brown rice: chilled ethanol) at $4\text{ }^{\circ}\text{C}$. Dispersions were centrifuged at $5000\times g$ for 15 min at $4\text{ }^{\circ}\text{C}$, and the residue was air-dried overnight in a fume hood, ground, and sieved using an 80-mesh screen. For protein extraction, the defatted and phenolic extracted brown rice powder (10 g) was fractionated according to Ju et al. [17] with slight modifications. The albumin fraction was first extracted by shaking with distilled water (1:6, sample:water) at $20\text{ }^{\circ}\text{C}$ for 4 h and centrifuged at $5000\times g$ for 15 min at $4\text{ }^{\circ}\text{C}$. After water extraction, the residue was extracted with 60 mL of 2% NaCl at $20\text{ }^{\circ}\text{C}$ for 4 h to obtain the globulin extract. Glutelin was then extracted from the residue with 60 mL of 0.1 N NaOH at $20\text{ }^{\circ}\text{C}$ for 30 h. The residue after glutelin extraction was extracted with 40 mL of 70% ethanol to yield the prolamin fraction. The albumin, globulin, glutelin and prolamin fractions were obtained by adjusting the pH of supernatants with 0.1 N HCl to their isoelectric points (pI) of 4.1, 4.3, 4.8 and 4.5, respectively. Next, the precipitated proteins (albumin, globulin, glutelin, and prolamin) were centrifuged at $5000\times g$ for 20 min at $4\text{ }^{\circ}\text{C}$ and washed twice with distilled water. All protein fractions were dialyzed using ultra filtration cartridges with a MWCO of 3500 Da (Slide-A-Lyzer Dialysis Cassettes, Thermo Scientific, USA) in distilled water for desalting. The concentrated protein fractions were freeze-dried and kept at $-20\text{ }^{\circ}\text{C}$. The MW profile and amino acid composition of all BRPFs were analysed. The yield (%) of each fraction was calculated as a percentage of the amount of freeze-dried protein in each fraction to the total protein of defatted rice flour. All protein fractions were hydrolysed by trypsin following the method described by Wattanasiritham et al. [13], trypsin digested protein in each fractions were analysed by LC–ESI–MS/MS to confirm proteins samples were well purified.

2.2.1. Molecular weight distribution—The purity and molecular size of all BRPFs was analysed using a 4–20% precast polyacrylamide gel (Mini-PROTEAN TGX Gels, Bio-Rad, USA) according to the method of Selamassakul et al. [9]. Samples were diluted at a 1:1 ratio with sample buffer (0.5M Tris–HCl pH 6.8, 30% glycerol, 10% SDS, 0.5%

bromophenol blue, v/w/w) and heated at 95 °C for 5 min. Next, 10 mL of treated samples (20 mg protein) was loaded onto the gels. 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 the stained gel was de-stained using a 10% acetic acid:40% methanol solution until the bands were clear. MW markers of 2, 5, 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa (Precision Plus Protein Kaleidoscope prestained standards, Bio-Rad) were used to construct a standard curve for MW estimation.

2.2.2. Amino acid composition—The amino acid compositions of four BRPFs were identified using high-performance liquid chromatography (HPLC), following the AOAC method [18]. Samples were hydrolysed with 6 N HCl at 110 °C for 24 h and derivatised using the AccQ-Fluor reagent. All sample solutions were then analysed using HPLC (Waters Alliance 2695, USA) coupled to a fluorescence detector (JASCO FP2020 Plus, Japan), with the excitation and emission wavelengths set at 250 and 395 nm, respectively. A AccQ Tag column (3.9mm×150 mm, 4 mm, Waters, USA) was used and eluted at a flow rate of 1 mL/min in gradient mode using a mixture of eluent A (sodium acetate buffer pH 4.90) and eluent B (60% acetonitrile). Amino acid compositions were identified and quantified by comparison with a standard mixture of 17 amino acids (Sigma-Aldrich, USA) and were shown as g of amino acid per 100 g of protein. Under strong acid hydrolysis condition, the recovery of each amino acid located between 80% and 110% regardless of whether they were partially destroyed. The determination of cysteine and methionine content, the samples were oxidized with performic acid before hydrolysis with 6N HCl [19] and tryptophan was determined by alkaline hydrolysis at 105 °C for 24 h with 4M NaOH [20].

2.3. Preparation of brown rice protein hydrolysates

Firstly, the protein concentration of four freeze-dried protein fractions was adjusted to 50 mg protein/mL with LC–MS grade water (Pierce Water, Thermo Fisher Scientific, USA). Sample solutions were heated to 50 °C for 10 min to reach efficient enzymatic hydrolysis at the start of the reaction, and then submitted to enzymatic hydrolysis using bromelain at concentration of 202, 404, 606 and 808 CDU/g of brown rice protein. Hydrolysis was conducted at 50 °C for 0, 3, and 6 h and inactivated by heating at 75 °C for 15 min. The hydrolysate was separated by centrifugation at 5000×g for 15 min, and the resulting supernatants were freeze-dried and stored at –20 °C prior to analysis. All tests were performed in triplicate. Protein concentration (mg/mL) of four freeze-dried fractions and of eb-RPHs was determined using a standard curve of bovine serum albumin and the Bradford assay [21]. Freeze-dried eb-RPHs from all hydrolysis conditions were analysed for peptide composition and antioxidant activity. The MW profiles of eb- RPH with high antioxidant activity from each BRPFs were also identified.

2.4. Peptide identification

Freeze-dried eb-RPHs were dissolved in 0.1% aqueous formic acid. Peptides were analysed using an Orbitrap Fusion Lumos mass spectrometer with a Nano ESI source (Thermo Scientific, Waltham, MA) coupled to a Waters nanoAcquity UPLC system (Waters, Milford, MA) according to the method of Troyer et al. [22] with slight modifications. One mL of each sample was loaded on a C18 column (nanoAcquity UPLC Trap Column, 180mm×20

mm, 5 mm) at 5 mL/min flow rate with 3% acetonitrile containing 0.1% formic acid. Peptide mixtures were separated using an Acquity UPLC Peptide BEH C18 column (100mm×100 mm, 1.7 mm) following a 120-min gradient at a flow rate of 500 nL/min. The nanoLC mobile phase consisted of 0.1% formic acid in H₂O (mobile phase A) and 0.1% formic acid in ACN (mobile phase B). The concentration of formic acid in B was increased from 3% to 10% in 3 min, 10% to 30% in 102 min, 30% to 90% in 3 min, held at 90% for 4 min, decreased from 90% to 3% in 1 min, and held at 3% for 7 min. Mass spectrometry data were acquired in positive ionization mode with a 2400 V spray voltage and the ion transfer tube was held at 300 °C. The mass spectrometer was operated in the data dependent acquisition mode. Full scan MS data were acquired in the orbitrap analyser with a resolution at 120 K at m/z 200. Low resolution MS/MS data were acquired using the linear ion trap. Precursor isolation window (m/z) on the quadrupole was 1.6. Automatic gain control (AGC) of full and MS/MS scans were set to 4.0×10⁵ and 104, respectively. The most intense precursor ions with charge 2–7 were selected for fragmentation under top speed data-dependent mode using collisioninduced dissociation (CID) with a 35% normalized collision energy and a dynamic exclusion of 60 s. Thermo Scientific Proteome Discoverer 2.1 software was used to analyse raw data files, and the Sequest HT search engine was applied to search against the Uniprot O. sativa protein database including the bromelain enzyme sequence. Mass tolerances for precursor and fragment ions were set at ±10 ppm and 0.6 Da, respectively. A maximum of two missed cleavage sites was allowed. Carbamidomethylation of cysteine was specified as a static modification and oxidation of methionine was specified as a dynamic modification. The overall false discovery rate (FDR) at the protein level was less than 1%.

2.5. Determination of radical scavenging activity

To facilitate comparison, the eb-RPH from each hydrolysis condition was adjusted to the same protein concentration (0.5 mg protein/mL) with purified water (Milli-Q) before analysis.

2.5.1. ABTS radical scavenging activity—The ABTS%+ scavenging activity of eb-RPHs was measured using dye decolourisation according to Selamassakul et al. [9]. The radical cation was prepared by mixing 7mM ABTS with 7mMK₂S₂O₈ (ratio 2:1), and the mixture was incubated in the dark for 12–16 h to complete the radical generation. The ABTS%+ solution was diluted with purified water (Milli-Q) to give an absorbance at 734 nm of 0.700 ± 0.030 prior to use. Briefly, 20 mL of samples were transferred in triplicate to a 96-well, flat bottom, polystyrene microplate, followed by addition of 200 mL of the ABTS %+ solution. The mixture was shaken and incubated for 6 min in the dark. Absorbance was measured at 734 nm using a microplate reader (TECAN model Infinite M200). Trolox, a strong ABTS radical-scavenger, and water were used as positive control and blank, respectively. The scavenging effect is expressed as the percentage of disappearance of the blue colour of ABTS%+ at 734 nm, and calculated using the following equation:

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

2.5.2. Copper chelating activity—Cu²⁺ chelating ability was determined using pyrocatechol violet as the metal chelating indicator according to Carrasco-Castilla et al. [23]. Briefly, 280 mL of 50mM phosphate buffer pH 6, 6 mL of 4mM pyrocatechol violet prepared in the same buffer, and CuSO₄·5H₂O (10 mL) were added to samples (10 mL). Absorbance at 632 nm was measured using a microplate reader. Copper chelating activity was calculated as:

$$\text{Chelating activity(\%)} = \frac{(A_{\text{control}} - A_{\text{Sample}})}{A_{\text{Control}}} \times 100$$

2.6. Fractionation of protein hydrolysates

The eb-RPHs showing strong radical scavenging activity were selected and further fractionated by a reversed-phase high-performance liquid chromatography (RP-HPLC) system equipped with a controller (Waters 600, USA) and a photodiode array detector (Waters 2996, USA). Purification of the antioxidant peptides from the glutelin hydrolysate was performed as described by Yan et al. [10], Freeze-dried eb-RPH (100 mg protein/mL) was dissolved in LC–MS grade water and filtrated through a syringe filter (polyvinylidene difluoride membrane, 0.45 mm), and then the supernatant was fractionated using RP-HPLC on a Discovery BIO Wide Pore C18 HPLC column (5mm particle size, 250mm×2.1 mm). A 36 min LC gradient consisting of 0.1% trifluoroacetic acid (TFA) in both water (mobile phase A) and acetonitrile (mobile phase B) was performed to fractionate protein hydrolysates at a flow rate of 4 mL/min. Specifically, mobile phase B kept 0% at first 2.5 min, then increased to 30% in 20 min and to 100% in 7.5 min, and finally stay at 100% for 6 min. The wavelength of UV detector was set up at 215 nm. Six fractions (F1–F6) were collected at 6 min intervals, then concentrated by a rotary evaporator following by freeze-dried. Each RP-HPLC fraction was dissolved in LC–MS grade water and used for antioxidant capacity (ABTS radical scavenging activity and Cu²⁺ chelating activity) determination and for peptide identification with Orbitrap-based mass spectrometers (LC–ESI–MS/MS). Furthermore, the antioxidant activity of identified peptides was predicted by the database BIOPEP.

2.7. Statistical analysis

Experiments were performed in triplicate and the data are expressed as mean ± standard deviation (SD). Statistical analyses of the data were carried out using the statistical analysis system (SAS) for windows version 9.0 (SAS Institute, USA). Least significant difference (LSD) test was used to make comparisons among mean values. Means at 95% confidence interval ($P < 0.05$) were accepted as significantly different.

3. Results and discussion

3.1. Protein yield, molecular weight profile, and amino acid composition of BRPF

According to Osborne [24], four fractions with different protein components, albumin, globulin, glutelin, and prolamin, can be extracted from brown rice flour under water, salt, alkali, and alcohol conditions, respectively. The protein percentage in each fraction is

glutelin ($84.23 \pm 1.52\%$), albumin ($2.18 \pm 0.09\%$), globulin ($3.98 \pm 0.12\%$), and prolamin ($9.61 \pm 0.77\%$), which is consistent with the protein content of KDML 105 [13]. Brown rice has low amounts of prolamin, a type of gluten protein, as well as albumin and globulin, which are the major allergenic proteins in patients with rice allergy [25,26]. This suggests that brown rice (KDML 105) proteins are less allergenic, which could be a competitive gluten-free protein ingredient in the food ingredient market.

The MW distribution of each extracted fraction was estimated by SDS-PAGE (Fig. 1). Each BRPFs exhibited different MW pattern in kDa showing as following: albumin with 5–10, 14, 22 a, and >35 kDa; globulin with 8–12, 13, 18, 25, 30, 37, 53, 63 kDa; glutelin with 13, 22, 35 kDa; and prolamin with 10, 13, 16, 22, 28, 32 kDa. Our results were well consistent with others [5,14] and slight differences were also observed possibly due to differences in extraction methods and cultivars used. Proteins in each fractions were digested by trypsin following by a LC–MS analysis, which identified peptides of albumin, globulin, glutelin and prolamin were of 503, 219, 242 and 115 matching against Uniprot *Oryza sativa* protein database search. The majority of the identified peptides were rice albumin, globulin, glutelin and prolamin peptides which involved in *Oryza sativa* L. ssp. indica. This data confirmed that the extracted protein was indeed extracting the target proteins.

The amino acid composition of four BRPFs is given in Table 1, and clear differences can be observed among the four fractions. Glutamic acid, aspartic acid, arginine, valine, lysine, leucine, phenylalanine, and histidine were the most predominant residues in the BRPFs, indicating that these fractions are a good source of essential, flavour (glutamic and aspartic acids), and bioactive amino acids (hydrophobic amino acids). Among the four fractions, glutelin had the highest content of essential amino acids (50.48% of the total amino acids) and hydrophobic amino acids (36.05% of total amino acids). Theoretically, biological antioxidant activity of protein hydrolysates is strongly dependent on the amount of antioxidant amino acids such as tyrosine, methionine, histidine, and lysine [27,28]. In our experiments, glutelin had the highest content of these antioxidant amino acids compared with globulin, prolamin, and albumin. Moreover, albumin served as an excellent source of branched-chain amino acids like leucine (9.92 ± 0.05 g/100 g protein), isoleucine (4.84 ± 0.08 g/100 g protein), and valine (8.61 ± 0.06 g/100 g protein), which may promote muscle protein synthesis [29]. These results show that all BRPFs had well-balanced amino acid compositions, and can be used as nutritional supplements, functional ingredients, and flavour enhancers in foods.

3.2. Effects of protein type and hydrolysis condition on peptide composition of eb-RPHs

Four BRPFs were subjected to hydrolysis with bromelain at various enzyme concentrations and hydrolysis time. The peptide profiles and bromelain proteolytic efficiencies on hydrolysates from BRPFs were determined using LC–ESI–MS/MS. Determination of peptide sequences was performed using the Proteome Discoverer software and the Sequest HT search engine with a probability of certainty greater than 90%. The number of identified peptides is summarised in Table 2. It can be seen that the number of identified peptides was different among the four BRPFs and hydrolysis conditions. Hydrolysates from the albumin fraction had the highest number of identified peptides, followed in decreasing order by those

of glutelin, prolamin, and globulin. The differences may result from the fact that each BRPF had different amino acid compositions and sequences, which affected the bromelain-specific cleavage sites and defined the peptide bond cleavages. Theoretically, bromelain cleave the peptide chain at arginine-alanine and alanine glutamic acid bonds, and it shows preference for glutamic acid, aspartic acid, lysine, or arginine in the P1 site [30]. Therefore, the higher number of identified peptides in the albumin and glutelin hydrolysates could result from the high amounts of those preferred amino acids, especially arginine, allowing bromelain to cleave a greater number of peptide bonds (Table 1). The increase in enzyme concentration and hydrolysis time might cause an increase or decrease in the number of identified peptides owing to the availability of cleavage sites in the substrate under saturation of enzyme/substrate [31]. Moreover, some bromelain-derived peptides were detected in the eb-RPH produced with bromelain at concentration of 808 CDU/g of brown rice protein, which could result from an excess of bromelain. This result was in agreement with our previous study, which reported that the degree of hydrolysis value was not different between hydrolysate produced by bromelain at concentration of 606 and 808 CDU due to the enzyme/substrate saturation [9].

After bromelain digestion, proteins in each fraction were completely hydrolysed to low MW bands with MW less than 15 kDa while peptides with MW below less than 5 kDa were not observed due to the technical limitations of SDS-PAGE (Fig. 1). All hydrolysates were subsequently analysed by LC-ESI-MS/MS to identify the peptide sequences of the antioxidant peptides with MW below 5 kDa as noted by other researchers [7,8,10,12,13]. Results showed that they contained a mixture of peptides with MW ranging between 900 and 5000 Da. Bromelain hydrolysates of BRPFs were mainly composed of oligopeptides (500–2000 Da, 60–70%), followed by polypeptides (> 2000 Da, 20–30%). These results showed that bromelain being an effective protease for hydrolysing brown rice proteins into low-MW peptides.

3.3. Effects of protein type and hydrolysis condition on antioxidant activity of eb-RPHs

ABTS radical scavenging and copper-chelating assays were used to determine the antioxidant activity of eb-RPHs. The results show that all eb-RPHs had the capacity to scavenge ABTS radicals and to chelate copper ions (Table 2). At the same protein concentration, glutelin hydrolysates showed higher antioxidant activity than those derived from other protein fractions. Glutelin hydrolysates produced by incubation with bromelain at concentration of 606 CDU/g of brown rice protein for 3 h had both the highest ABTS radical scavenging (0.69 ± 0.04 mM trolox) and copper-chelating (4.12 ± 0.01 mg EDTA) activities. Size and type of amino acid residues present are known to contribute to the antioxidant ability of peptides [27].

Antioxidant peptides contain hydrophobic and aromatic amino acid residues [1,2], whereas histidine and arginine are effective for copper-chelating activity [32]. It can be seen in Table 1 that over 30% of total amino acid residues found in BRPFs were hydrophobic, and the glutelin fraction contained the highest amount. LC-ESI-MS/MS analysis also suggest that glutelin hydrolysates are rich in proton-donor (glutamic acid, phenylalanine, tyrosine, threonine, glycine, and aspartic acid) and hydrophobic (phenylalanine, tyrosine, serine,

leucine, alanine, valine, isoleucine, and proline) amino acids (data not shown). Presumably, these amino groups in the side chain can act as hydrogen donor and metal ion chelator. The higher antioxidant and copper-chelating activities of peptides found in rice glutelin hydrolysates compared to other protein fractions could be attributed to the higher amount of histidine, which exhibits strong radical scavenging activity owing to the decomposition of its imidazole ring, and aromatic amino acids such as tyrosine and phenylalanine, which can easily donate protons to electron-deficient radicals while maintaining their stability via resonance structures [1,23]. Furthermore, antioxidant activity has been previously reported for rice proteins in short peptides in the 300–1,000 Da range (2–8 amino acid residues in length), owing to easy absorption in the body and strong radical scavenging activity [7,8,11], and in longer peptides, ranging from 10 to 21 amino acids and with a MW of 1000–3611 Da [12,13]. In this study, results obtained from mass spectrometry demonstrated that a large portion (> 60%) of peptides found in all hydrolysates have MW below 2000 Da (data not shown). As shown in Fig. 2, the hydrolysates with the highest antioxidant activity from each protein fraction had a different abundance of low-MW peptides (1000–2000 Da). As a consequence, the low-MW peptides containing histidine, arginine, and hydrophobic residues found in hydrolysates of each BRPFs are thought to play a crucial role in the corresponding antioxidant ability. Rice bran albumin hydrolysate exhibited the best antioxidant activity than other fractions [13,14], however, our work, for the first time, proved brown rice glutelin hydrolysates showed better antioxidant activities than albumin fraction. Considering the brown rice glutelin hydrolysate for food applications, this knowledge can help to optimise hydrolytic conditions to be as quick and effective as possible.

3.4. Identification of antioxidative peptides from glutelin hydrolysates

In addition to the presence of the relevant amino acid residues, their correct positioning in the peptide sequence, its molecular size, and higher hydrophobicity are known to be related to high antioxidant properties [1,28]. Accordingly, glutelin hydrolysates produced by incubation with bromelain at concentration of 606 CDU/g of brown rice protein for 3 h, which had the highest antioxidant activity, were fractionated using RP-HPLC in a Discovery BIO Wide Pore C18 HPLC Column, which not only separated peptides based on their difference of hydrophobicity. The 100 mg peptide samples were separated by LC-MS and fractionated in every 6 min interval. Generally, peptides with low hydrophobicity and/or small MW eluted first while peptides with high hydrophobicity and/or large MW eluted later. Each fraction was evaporated, freeze-dried, and assayed for antioxidant activity using the ABTS radical scavenging and copper-chelating assays. The corresponding antioxidant activities are shown in Fig. 3. At a concentration of 0.4 mg protein/mL, fraction 4 (F4) had the highest ABTS radical scavenging and copper-chelating activity with values of 14.03% and 33.65%, respectively (1.08 ± 0.03 mM trolox and 5.00 ± 0.02 mg EDTA), followed by fraction 5 (F5) with values of 11.66% and 22.77%, respectively (0.91 ± 0.04 mM trolox and 3.30 ± 0.03 mg EDTA). As shown in Table 3, LC-ESI-MS/MS revealed that the identified peptides in the glutelin fraction hydrolysate contained one or more of proton donor amino acids, such as tyrosine (Y), phenylalanine (F), glycine (G), glutamic acid (E), threonine (T) and aspartic acid (D), and hydrophobic amino acids, including tyrosine (Y), phenylalanine (F), valine (V), leucine (L), isoleucine (I), proline (P), alanine (A), and glycine (G). Clearly, the bioactive peptides in each fraction exhibited different antioxidant activities owing to their

different amino acid sequences. The peptides SPFWNINAHS (MW=1172.55 Da), MPVDVIANAYR (MW=1248.64 Da), and VVYFDQTQAQA (MW=1269.61 Da) in F4 and VEVGGGARAP (MW=912.49 Da) in F5 contain >50% of antioxidant amino acids. The large number of antioxidant amino acids in these peptides may play a role in the high levels of antioxidant activity compared to other fractions. In addition to the presence of the relevant amino acid residues, the electronic and hydrogen-bonding properties, the correct positioning of the imidazole group, and the steric properties of the amino acid residues at the C- and N-termini are also closely related to the antioxidant activity of peptides [1,33]. The presence of antioxidant and hydrophobic amino acids such as F, Y, H, V, L, I, or A at the N-terminus of the peptides HIAGKSSIFRA (MW=1186.67 Da), VVYFDQTQAQA (MW=1269.61 Da), FDTADLPSGKGYL (MW=1383.68 Da), AVYVYDVNNANQ (MW=1271.62 Da), VEVGGGARAP (MW=1483.68 Da), YNILSGFDTEL (MW=1271.62 Da), and VVSNFGKTVFDGVL (MW=1481.80 Da) seem to positively contribute to the high ABTS % + scavenging and copper-chelating activity of F4 and F5 via their ability to donate a proton from the benzene ring and imidazole group [34]. Calculation of the frequency of bioactive fragments occurrence in protein sequence (A) using the BIOPEP database suggested that peptides in F4 and F5, including SPFWNINAHS (A=0.1000), MPVDVIANAYR (A=0.0909), VVYFDQTQAQA (A=0.0909), AVYVYDVNNANQ (A=0.2308), YNILSGFDTEL (A=0.0909), EFFDVSNELFQ (A=0.0909) and QNIFSGFSTELLS (A=0.0769) could be potential candidate function as natural antioxidants. Besides bioactive peptides reported in the literature [7,10,11], our study is the new finding demonstrated that peptide consisting of low hydrophobic value such as valine, alanine and methionine at N-terminal also showed great antioxidant activity. The sequence identification of bioactive peptides may aid us to better understand the amino acids attribution in antioxidant activity and to discovery/develop natural antioxidants for industrial applications.

4. Conclusions

The results obtained from LC–ESI–MS/MS show that the hydrolysis of brown rice protein with bromelain produces low-MW peptides with hydrophobic or aromatic N-terminal residues, which can donate electrons to electron-deficient radicals and copper ions. F4, with the peptides SPFWNINAHS, MPVDVIANAYR, HIAGKSSIFRA, VVYFDQTQAQA, FDTADLPSGKGYL, and AVYVYDVNNANQ, and F5, with the peptides VEVGGGARAP, YNILSGFDTEL, and VVSNFGKTVFDGVL were recognized as enhancing the antioxidant activity of the glutelin hydrolysates. Results suggested that brown rice (Khao Dawk Mali 105) glutelin fractions hydrolysed with bromelain at concentration of 606 CDU/g of brown rice protein and its purified peptides could be used as nutraceutical ingredients in foods to boost antioxidant activity. Peptides reported here should be further synthesized and their antioxidant activities need to be evaluated in other in vitro or in vivo assays.

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Fig. 1.

Electrophoretic profile of isolated rice proteins and the bromelain brown rice protein hydrolysates with high antioxidant activity from each brown rice protein fraction. 10 mL of a 20 mg protein concentration of denatured protein sample was loaded per well. Lane 1 and 6, standard molecular weight marker; lane 2, albumin fraction; lane 3, globulin fraction; lane 4, glutelin fraction; lane 5, prolamin fraction; lane 7, bromelain hydrolysate of albumin fraction; lane 8, bromelain hydrolysate of globulin fraction; lane 9, bromelain hydrolysate of glutelin fraction; and lane 10, bromelain hydrolysate of prolamin fraction.

Fig. 2.

Molecular weight range of peptides identified by LC-MS/MS in hydrolysates with high antioxidant activity from albumin, globulin, glutelin, and prolamins hydrolysed using bromelain.

Fig. 3. Purification of the glutelin hydrolysate by reversed-phase high-performance liquid chromatogram on a C18 column. (A) Chromatographic representation of peptide fractions (F1-F6) and their ABTS scavenging activity and Cu^{2+} chelating activity (B).

Table 1

Amino acid composition of brown rice protein fractions.

Amino acids	Amino acid content (g/100 g protein)			
	Albumin	Globulin	Glutelin	Prolamin
Aspartic acid	13.03 ± 0.11	8.75 ± 0.07	2.47 ± 0.01	3.58 ± 0.05
Serine	0.84 ± 0.05	7.68 ± 0.05	5.31 ± 0.08	7.79 ± 0.11
Glutamic acid	23.44 ± 0.12	21.01 ± 0.14	6.65 ± 0.09	7.19 ± 0.10
Glycine	4.85 ± 0.08	4.84 ± 0.04	1.83 ± 0.02	2.92 ± 0.03
Histidine	5.52 ± 0.06	6.62 ± 0.07	11.30 ± 0.16	10.06 ± 0.08
Arginine	8.25 ± 0.07	5.65 ± 0.11	11.96 ± 0.17	17.04 ± 0.28
Threonine	2.22 ± 0.10	4.39 ± 0.09	5.40 ± 0.07	8.83 ± 0.13
Alanine	2.70 ± 0.10	0.10 ± 0.08	4.33 ± 0.01	7.35 ± 0.08
Proline	0.93 ± 0.09	7.29 ± 0.06	1.44 ± 0.06	0.33 ± 0.07
Cysteine	1.60 ± 0.09	0.92 ± 0.02	2.67 ± 0.07	2.66 ± 0.10
Tyrosine	0.20 ± 0.01	2.72 ± 0.07	12.87 ± 0.16	5.35 ± 0.08
Valine	8.61 ± 0.06	5.05 ± 0.09	6.14 ± 0.05	3.50 ± 0.09
Methionine	0.11 ± 0.01	3.62 ± 0.05	1.57 ± 0.10	0.53 ± 0.08
Lysine	9.11 ± 0.08	6.84 ± 0.10	5.33 ± 0.04	3.69 ± 0.05
Isoleucine	4.84 ± 0.08	2.38 ± 0.09	3.20 ± 0.09	1.70 ± 0.09
Leucine	9.92 ± 0.05	6.87 ± 0.07	4.98 ± 0.09	6.75 ± 0.07
Phenylalanine	3.81 ± 0.02	5.27 ± 0.02	12.57 ± 0.14	10.73 ± 0.11
Total essential amino acids ^a	44.15	41.04	50.48	45.79
Total hydrophobic amino acids ^b	35.78	35.42	36.05	33.80

^aTotal essential amino acid values were calculated from the sum of histidine + threonine + tyrosine + valine + lysine + isoleucine + leucine + phenylalanine contents.

^bTotal hydrophobic amino acid values were calculated from the sum of alanine, isoleucine, leucine, methionine, phenylalanine, valine, proline, and glycine contents.

Values are expressed as the mean ± standard deviation (n = 3).

Table 2

Number of identified peptides and antioxidant activity of brown rice protein fraction hydrolysates.

Hydrolysis condition			Albumin						Globulin						Glutelin						Prolamin					
Hydrolysis time (h)	Enzyme concentration (CDU/g of protein)	Identified peptide No.	Antioxidant activity *			Identified peptide No.			Antioxidant activity *			Identified peptide No.			Antioxidant activity *			Identified peptide No.			Antioxidant activity *					
			%ABTS scavenging activity	%Copper chelating activity	Rice (5–2 kDa)	Enz.	Rice (<2 kDa)	Enz.	%ABTS scavenging activity	%Copper chelating activity	Rice (5–2 kDa)	Enz.	%ABTS scavenging activity	%Copper chelating activity	Rice (<2 kDa)	Enz.	%ABTS scavenging activity	%Copper chelating activity	Rice (5–2 kDa)	Enz.	%ABTS scavenging activity	%Copper chelating activity				
0h	0	-	0	0.01±0.00 ^b	0.23±0.00 ^g	-	-	0	0.01±0.00 ^b	0.07±0.00 ^h	-	-	0	0.04±0.00 ^b	0.34±0.02 ^g	-	-	0	0.05±0.00 ^h	0.00±0.00 ^g	-	-	0	0.05±0.00 ^h	0.00±0.00 ^g	
3h	202	27	41	4.90±0.10 ^a	9.69±0.45 ^a	14	8	0	14.09±0.09 ^a	7.15±0.47 ^a	13	75	0	11.51±0.42 ^c	3.56±0.02 ^e	14	31	0	5.11±0.20 ^d	0.88±0.24 ^{d,e}	-	-	-	-	-	
	404	44	63	2.80±0.16 ^c	9.46±0.25 ^a	16	12	0	4.05±0.32 ^f	6.58±0.32 ^b	43	104	0	15.42±0.19 ^a	27.69±1.89 ^a	27	69	0	6.17±0.44 ^c	1.28±0.13 ^{c,d}	-	-	-	-	-	
	606	61	136	2.53±0.25 ^c	8.01±0.43 ^c	21	16	0	4.63±0.29 ^e	6.03±0.07 ^c	51	138	0	15.68±0.17 ^a	28.00±0.81 ^a	33	86	0	6.30±0.36 ^c	1.47±0.51 ^c	-	-	-	-	-	
	808	68	137	2.28±0.12 ^c	6.82±0.31 ^d	44	15	0	7.71±0.25 ^d	6.35±0.10 ^{b,c}	46	139	0	13.15±0.53 ^b	23.70±0.18 ^b	33	82	0	3.42±0.42 ^f	0.47±0.11 ^e	-	-	-	-	-	
6h	202	32	20	3.23±0.14 ^b	8.99±0.14 ^b	7	12	0	10.40±0.56 ^b	6.53±0.41 ^b	40	79	0	13.30±0.20 ^e	3.32±0.15 ^{e,f}	16	31	0	4.18±0.10 ^e	0.52±0.18 ^e	-	-	-	-	-	
	404	30	26	3.58±0.12 ^b	9.55±0.18 ^a	9	15	0	8.52±0.22 ^c	4.70±0.10 ^d	59	83	0	15.27±0.13 ^b	4.61±0.39 ^d	63	66	0	8.58±0.40 ^b	4.27±0.04 ^b	-	-	-	-	-	
	606	33	87	1.88±0.03 ^d	8.90±0.14 ^b	3	57	0	3.23±0.29 ^{d,g}	3.99±0.21 ^e	48	92	0	14.94±0.61 ^b	18.06±0.84 ^b	44	71	0	8.61±0.40 ^b	5.62±0.24 ^a	-	-	-	-	-	
	808	53	94	0	0.13±0.03 ^e	6.32±0.19 ^e	5	61	3	2.74±0.10 ^g	3.36±0.33 ^f	42	103	19	15.48±0.83 ^a	16.09±0.18 ^c	40	97	10	9.48±0.21 ^a	4.05±0.68 ^b	-	-	-	-	-

^{a,b,c} Samples were tested in triplicate and values are expressed as mean ± standard deviation (n = 3).

^{*} ABTS scavenging and copper chelating activities of hydrolysates were tested at 0.5 mg/mL protein.

Table 3

Amino acid sequence, ABTS^{•+} scavenging activity and Cu²⁺ chelating activity of peptides from brown rice glutelin hydrolysed using bromelain and determined by LC-MS/MS.

Fraction no.	Sequence ^a	Theo. MH+ [Da]	ABTS ^{•+} scavenging activity	Cu ²⁺ chelating activity
F1	VVALALI	698.48	1.70±0.12 ^f	2.37±0.17 ^f
F2	AQQQEQAQQQEQ	1443.65	2.17±0.12 ^d	4.66±0.36 ^d
F3	AAAAAGGGELEG	917.40	1.84±0.06 ^e	2.21±0.11 ^e
	YQETSSSSSQ	1103.45		
	RVQVVNNNGK	1127.63		
	HNAVDSQIAGKA	1210.62		
	YSEEQQPSTR	1224.55		
	QVVRSDQGSVR	1230.65		
	YQETSSSSSQE	1232.49		
	LPENAKVDQVK	1240.69		
	TMAEYHHQDQ	1259.51		
	GEQQQPGMTR	1259.58		
	AGTGAGAGGGAGTKTSS	1307.62		
	SEALGVSSQVARQ	1331.69		
	YQETSSSSSQEQ	1360.55		
	GGRLDGKQPPRQ	1395.75		
	QAGTGAGAGGGAGTKTSS	1435.68		
	KKSKADLTEVTHK	1484.84		
	QQFQQSGQAQLTE	1492.70		
	AAVKKMYDIQAKK	1493.85		
F4	GKGEGGGGLA	802.41	14.03±0.14 ^a	33.65±0.44 ^a
	EFFDVSNEQ	1114.47		
	SPFWNINAHS	1172.55		
	HIAGKSSIFRA	1186.67		
	MPVDVIANAYR	1248.64		
	VVYFDQTQAQA	1269.61		
	TAGTGGGQFQPMR	1307.62		
	KEFLLAGNNRA	1346.72		
	FDTADLPSGKGYL	1383.68		
	AVYVYDVNNANQ	1483.68		
F5	VEVGGARAP	912.49	11.66±0.26 ^b	22.77±0.82 ^b
	YNILSGFDTEL	1271.62		
	EFFDVSNELFQ	1374.62		
	QNIFSGFSTELLS	1442.72		
	SPFLQSAAFQLRN	1478.78		

Fraction no.	Sequence ^a	Theo. MH+ [Da]	ABTS ⁺⁺ scavenging activity	Cu ²⁺ chelating activity
	VVSNFGKTVFDGVL	1481.80		
F6	SSAPGGGRP	785.39	2.52±0.05 ^c	4.74±0.39 ^c

^aSingle letter abbreviations of amino acids were used: alanine (A), arginine (R), asparagine (N), aspartic acid (D), cysteine (C), glutamic acid (E), lutamine (Q), glycine (G), histidine (H), isoleucine (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V).