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ACE-inhibitory activity and antioxidant properties of a low MW rice bran protein hydrolysate

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ABSTRACT

Rice bran (RB), a widely available protein-rich waste derived from the rice production chain, represents a potential source of bioactive peptides. In this study a two-step, easily scalable enzymatic hydrolysis protocol to obtain RB protein hydrolysates (RBPH) is described. Firstly, a pre-treatment with commercial carbohydrase mixtures was performed to selectively remove polysaccharides of RB cell wall. The resulting protein-enriched fraction was then hydrolyzed with a combination of two different proteases, *i.e.,* Alcalase® 2.4L and Flavourzyme®. RBPH was separated by membrane ultrafiltration and the fraction with low MW peptides (RBPH U < 1 kDa) was subjected to ACE-inhibitory and antioxidant assays, the latter both *in vitro* and at cellular level. RBPH U < 1 maintains the same ACE-inhibitory activity (68.7 %) before and after simulated gastrointestinal digestion. Moreover, RBPH U < 1 shows the ability to effectively scavenge ABTS and DPPH radicals, besides reducing ferric ions, and it can significantly mitigate oxidative stress-induced damage on human intestinal Caco-2 cells. Interestingly, its antioxidant capacity is maintained also after simulated gastrointestinal digestion. Results obtained suggest that peptides from RB proteins have the potential to be used in the development of functional foods that support intestinal health and alleviate illnesses linked to oxidative stress.

1. Introduction

Rice is one of the most important cereals for human nutrition, particularly in Asian countries. The huge production (526 million tonnes in 2022/23) (FAO Food and Agriculture Organization, 2024) results in a corresponding amount of rice by-products/waste. Rice grain contains 3 main parts: endosperm or white rice (~70%), hull/husk (~20%) and bran (~10%) (Fig. 1). Rice bran is obtained during the rice milling process (Fig. 1) and contains around 10–23 % lipids, 37–60 % carbohydrates, 9–14 % ash, and 13–19 % proteins (Scarabattoli et al., 2023). Specifically, rice bran proteins are mostly storage proteins, *i.e.*, albumin (37%, easily soluble in water), globulin (36%), prolamin (5%), and glutelin (22%) (Fabian & Ju, 2011).

Although rice bran proteins (RBP) have been reported to have high nutritional value, RBP concentrates and isolates are not commercially available due to the lack of suitable RBP processing technologies (Chinma et al., 2014). In particular, structural complexity, scarce solubility and the tendency to aggregate make separation from other components of cell walls cumbersome, thus limiting the use of RBP as food ingredients (Liu et al., 2019). During the past few years, different methods to facilitate RBP extractions, including physical, chemical and enzymatic treatments, have been developed. Chemical processes, *i.e.,* alkaline or acid hydrolysis, tend to be difficult to control and cause protein denaturation, giving products with modified amino acids. On the contrary, enzymatic methods based on the use of carbohydrases and proteases are the most promising ones, as they significantly enhance protein recovery (Fabian & Ju, 2011; Scarabattoli et al., 2023). Indeed, carbohydrases can attack the cell wall components, thus favouring the release of protein from the polysaccharide matrix of bran and increasing extraction yield (Fabian & Ju, 2011). Furthermore, proteolytic enzymes hydrolyze peptide bonds thereby converting proteins into mixtures of peptides and amino acids that are more soluble. Enzymatic hydrolysis

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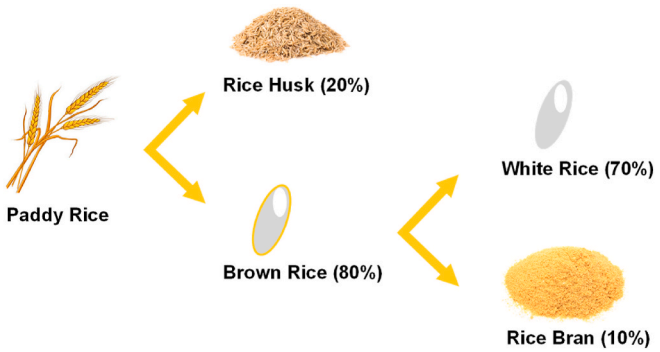

Fig. 1. Rice milling process.

can be performed under mild conditions, which avoid side reactions and preserve the nutritional value of the protein source (Liu et al., 2019). In addition, depending on enzyme specificity and the degree of hydrolysis (DH), it is possible to generate hydrolysates with functional, biological and nutritional properties distinct from those of the parent protein. Specifically, it is now widely accepted that biological properties of protein hydrolysates are strongly related to molecular weights (MW) of constituting peptides. Generally, peptides with low MW exhibit the highest bioactivities as they can easily cross the intestinal barrier to perform biological functions (Phongthai et al., 2018; Thamnarathip et al., 2016; Zhang et al., 2023). Ultrafiltration through decreasing MW cut-off membranes represents the most commonly used strategy for the fractionation of protein hydrolysates for several reasons, including high productivity, low cost, and maintenance of product purity under ambient conditions (Phongthai et al., 2018).

Short amino acid sequences, known as bioactive peptides, have frequently unique biological properties that go beyond their nutritional value (Zaky et al., 2022). Plenty of attention has been given to bioactive peptides that come from different food sources, such as rice, rice bran, soy *etc*., due to their potential impact on health (Chanput & Lawyer, 2020; Tadesse & Emire, 2020). Among the several health benefits, antihypertensive effect is one of the most frequently observed. Such an effect is generally attributed to low MW peptides and it is correlated to the capacity to inhibit the Angiotensin I Converting Enzyme (ACE), that catalyzes the conversion of angiotensin from an inactive form (angiotensin I) into an active one (angiotensin II, a potent vasoconstrictor) (Sangiorgio et al., 2022). Unlike synthetic ACE inhibitors, food-derived protein hydrolysates are more advantageous in terms of safety and side effects (Liu et al., 2019). Moreover, several low MW peptides (from 2 to 10 amino acid residues) have been reported to inhibit the oxidation of biomolecules by scavenging free radicals *in vivo* (Nam et al., 2006). Indeed, various diseases, such as diabetes, inflammatory disease and cardiovascular disorders are linked to the presence of reactive oxygen species (ROS) (Lin & Chang, 2005). In addition, oxidative processes caused by ROS play an important role in deterioration of food products with the consequent need of antioxidants (including synthetic ones) as food additives (Liu et al., 2019).

Due to the difficulty of protein extraction from the vegetable matrix, the preparation of bioactive hydrolysates from RB proteins results to be complex, and frequently involves numerous steps, such as pretreatments (*e.g.*, ultrasound-assisted extraction, alkaline hydrolysis and isoelectric precipitation), and/or further purification (*e.g.*, ion exchange chromatography) (Fathi et al., 2021; Piotrowicz et al., 2020; Thamnarathip et al., 2016; Wang et al., 2017; Zang et al., 2019). We report here an easily scalable protocol for the conversion of rice bran into a RB protein hydrolysate (RBPH) consisting of a two-step enzymatic hydrolysis that involves the sequential use of carbohydrases and proteases. RBPH obtained was fractionated by ultrafiltration and the fraction with low MW peptides (<1 kDa) was assayed for ACE-inhibitory and antioxidant properties, the latter both *in vitro* and at cellular level. Interestingly, both of activities resulted to be present also after simulated gastrointestinal digestion. Moreover, in order to elucidate the relationship between peptide structure and bioactivity, the peptidomic profile was evaluated thus demonstrating the potential of this important protein-rich waste for targeted applications in functional foods and nutraceuticals.

2. Experimental section

2.1. Materials and methods

All solvents and reagents were purchased from Merck, Fluorochem or Euroclone and were used without further purification. Rice Bran (RB) was kindly supplied by Ilsa S.p.A. (Arzignano, VI, Italy). % N content of RB and defatted RB (DRB) was 2.1% ± 0.3 and 2.7% ± 0.3, respectively, both evaluated by elemental analysis. Enzymes were kindly supplied by Novozymes® (Bagsværd, Denmark): *i*) Carbohydrases: cellulase Celluclast® 1.5L derived from *Trichoderma reesei* (activity: 11.67 μkat g^{-1} expressed as Endo-Glucanase Units (700 EGU g^{-1}) by the Manufacturer) and Ceremix® Plus MG, which is a mixture of enzymes (endo-1,4-xylanases, α-amylases, endo-1,3(4)-β-glucanase, neutral proteases; declared activities by the Manufacturer: 130 FXU g^{-1} (Farvet Xylan Units), 115 KNU-B g^{-1} (Kilo Novo Units) corresponding to 0.0167 μkat g^{-1}, 380 BGU g^{-1} (β-glucosidase Units) corresponding to 6.34 μkat g^{-1}, 0.3 AU-N g^{-1} (Anson Units) corresponding to 0.05 μkat g^{-1}, respectively), produced by submerged fermentation of several organisms: *Humicola insolens*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*; *ii*) Proteases: endopeptidase Alcalase® 2.4 L FG (declared activity: 2.4 AU g^{-1} (Anson Units) corresponding to 0.04 μkat g^{-1}) derived from *Bacillus licheniformis* and the mixture of endopeptidase and exopeptidase Flavourzyme® (declared activity: 1000 LAPU g^{-1} (Leucine AminoPeptidase Units) corresponding to 16.67 μkat g^{-1}) derived from *Aspergillus oryzae*. Pancreatin from porcine pancreas (8 x USP activity, considering that the 1 X minimum specification is 25 USP amylase units per mg, corresponding to 0.42 μkat mg^{-1}, 2 USP lypase units per mg, corresponding to 0.03 μkat mg^{-1}, and 25 USP protease units per mg, corresponding to 0.42 μkat mg^{-1}) and pepsin from porcine gastric mucosa (≥4.17 μkat/mg solid) were from Sigma-Aldrich. Hydrochloric acid (HCl), 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT), DPPH (1,1-diphenyl-2-picrylhydrazyl), TPTZ (2,4,6-Tripyridyl-*S*-triazine) and ROS assay kit were purchased from Sigma- Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), L-glutamine, phosphate-buffered saline (PBS), penicillin/streptomycin, 96- well plates were from Euroclone (Milan, Italy).

2.2. Pre-treatment of defatted RB with carbohydrases

Defatted Rice Bran (DRB, 10 g), prepared as previously reported (Bagnasco et al., 2013), was suspended in distilled H_2O (100 mL), the resulting mixture was heated to 50 °C and the pH was adjusted to 5.3 with 1 M HCl; then Celluclast® 1.5 L (250 mg) and Ceremix® Plus MG (250 mg) were added under mechanical stirring. Reaction conditions were set up according to an average of optimal pH and temperature for both enzymatic formulations (see Table 1). After 24 h, the enzymes were inactivated by heating at 85 °C for 15 min. Reaction mixture was then centrifuged at 9000 rpm for 15 min; the solid residue, namely defatted RB enriched in proteins (RBP), was recovered by filtration and it was dried in oven at 60 °C overnight; yield was calculated and expressed as dry weight with respect to the weight of the starting DRB, (% w_{RBP}/w_{DRB}).

2.3. Hydrolysis of RBP with proteases

Defatted rice bran enriched in proteins (RBP) derived from the previous step was suspended in distilled H_2O (at concentration of 10 g in

Table 1
List of optimal pH and temperature conditions for carbohydrases and proteases used.

Enzymes	pH	Temperature (°C)	References
Celluclast® 1.5L + Ceremix® Plus MG[a]	5.3	50	Gama et al. (2015) Peng et al. (2019)
Alcalase® 2.4 L	8.0	50	Ma et al. (2014)
Flavourzyme®	6.0	50	Rostammiry et al. (2017)

[a] Reaction conditions for the combination of two carbohydrase formulations were set as the average pH and temperature optimal values of the individual ones.

100 mL) and the resulting mixture was heated to 50 °C, then treated with proteolytic enzymes under mechanical stirring. Reaction conditions were set up according to optimal pH and temperature for each enzyme (see Table 1). Alcalase® 2.4L was added (100 mg) after adjusting pH to *ca.* 8.0 with 1M NaOH. After 2 h, the pH was adjusted to *ca.* 6.0 using 1M HCl and the mixture was further hydrolyzed by Flavourzyme® (200 mg) for 22 h. The enzymes were inactivated by heat treatment at 85 °C for 15 min. Reaction mixture was then centrifuged at 9000 rpm for 15 min, the supernatant (RB protein hydrolysate, RBPH) was collected, freeze-dried and stored at −20 °C. Yield was calculated and expressed as dry weight with respect to the weight of the starting RBP, (% w_{RBPH}/w_{RBP}).

2.4. Degree of hydrolysis

Degree of hydrolysis (DH) of the obtained RBPH was determined using the OPA-NAC method (Nielsen et al., 2001), with some modifications (**see Supplementary Data, section S1**). DH value was calculated using the following equation (Eq. (1)):

$$DH\ (\%) = \left(N_{\alpha}\ /\ N_{org}\right)*100 \qquad \text{(Eq. 1)}$$

where N_{α} is the α-amino nitrogen (mg g^{-1}) and N_{org} is the organic nitrogen (mg g^{-1}).

2.5. Elemental analysis

The protein content of RBP obtained from the carbohydrase pretreatment was assessed using the AOAC (Association of Official Analytical Chemists) method (Scarabattoli et al., 2023). A statistical factor of 6.25 was used to convert the results from elemental analysis (PerkinElmer, Series II CHNS/O analyzer, PerkinElmer, Massachusetts, USA) into the percentage of protein (%P, protein content).

2.6. Ultrafiltration

RBPH (*ca* 1.00 g) was dissolved in 100 mL HPLC grade H_2O and the resulting solution was filtered under stirring sequentially through 5 and 1 kDa cut-off membranes by using a 400 mL ultrafiltration cell system (Amicon® stirred cells, model 8400, 400 mL capacity, Merck Millipore Corporation). Nitrogen gas pressure was used for pressurizing the cell and was applied directly to the ultrafiltration cell. The pressure was kept constant at 60 psi. Three different fractions were recovered separately and freeze-dried: ultrafiltered hydrolysate with MW > 5 kDa, (U > 5); ultrafiltered hydrolysate with MW ranging from 5 to 1 kDa, (U < 5,1>); and ultrafiltered hydrolysate with MW < 1 kDa, (U < 1). Ultrafiltration yields (*w/w*,%, referring to the weight of the starting dry hydrolysate) are reported in Fig. 2. Fraction RBPH U < 1 was submitted to ACE-inhibitory activity and antioxidant activity assays.

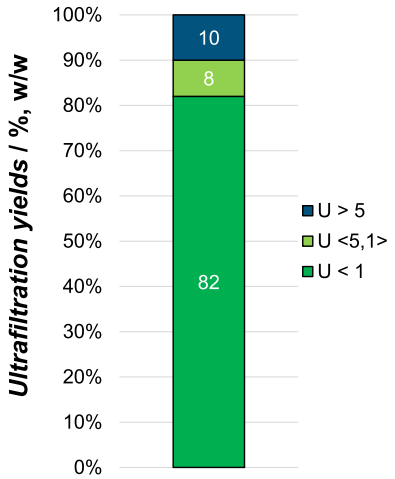

Fig. 2. Ultrafiltration yields (%w/w) obtained.

2.7. In vitro simulated digestion

The *in vitro* simulated digestion of RBPH U < 1 was carried out according to the method reported by (Xu et al., 2021), with slight modifications, as previously described (Sangiorgio et al., 2022). Briefly, for 103.6 mg of RBPH U < 1 dissolved in HPLC grade H_2O at a concentration of 10 mg mL^{-1}, 2 mg of pepsin at pH = 2 and 2 mg of pancreatin at pH = 7.5 were used. The resulting *in vitro*-digested RBPH (digested RBPH U < 1, 123.3 mg, 16% of salts) was submitted to ACE-inhibitory and antioxidant activity assays.

2.8. In vitro ACE-inhibitory activity assay

In vitro ACE inhibitory activity was evaluated by measuring the formation of hippuric acid (HA) from hippuryl-histidyl-leucine (HHL), a mimic substrate for ACE I, as previously reported (Boschin et al., 2014). Briefly, 100 μL of HHL (2.5 mmol L^{-1}) in tris-formic acid (100 mmol L^{-1}), NaCl (300 mmol L^{-1}) at pH 8.3 (buffer 1) was mixed with 30 μL of sample in the same buffer at five concentrations (186, 173, 345, 690, 1035 μg mL^{-1}). Samples were preincubated at 37 °C for 15 min, then 15 μL of 1.67 × 10^{-3} nkat μL^{-1} porcine kidney ACE (Sigma-Aldrich, Milan, Italy) solution in buffer 1 with 10 μM $ZnCl_2$, were added. Samples were incubated at 37 °C for 1 h, the reaction was then stopped with 0.1 M HCl (125 μL). The aqueous solution was extracted with ethyl acetate (2 × 600 μL); the solvent was evaporated, the residue was dissolved in 500 μL of buffer 1 and then analyzed by HPLC 1200 Series (Agilent Technologies, Santa Clara, US) in the following conditions: column, Lichrospher 100 C_{18} (4.6 × 250 mm, 5 μm; Grace, Italy); flow rate, 0.5 mL min^{-1}; detector, λ 228 nm; mobile phase, water and MeCN, gradient elution from 5 to 60% MeCN in 10 min and 60% MeCN for 2 min, then back to 5% MeCN in 3 min; injection volume, 10 μL. The determination of ACE inhibitory activity was based on the comparison between the concentrations of HA in the presence or absence of the inhibitor (Inhibitor Blank). The phenomenon of autolysis of HHL to give HA was evaluated by a Reaction Blank (sample with the higher inhibitor concentration but without enzyme). The percentage of ACE inhibition was computed considering the area of the HA peak with the following formula (Eq. (2)):

$$\text{ACE Inhibition}\ (\%) = \left(Area_{IB}\text{-}Area_{N}\right) / \left(Area_{IB}\text{-}Area_{RB}\right) * 100 \qquad \text{(Eq. 2)}$$

where $Area_{IB}$ is the area of HA in the Inhibitor Blank (IB) sample (with enzyme but without inhibitor), $Area_{N}$ is the area of HA in the samples containing different inhibitor amounts, and $Area_{RB}$ is the area of HA in the Reaction Blank (RB) sample (without enzyme and with inhibitor at the highest concentration). IC_{50} value is the concentration needed to observe a 50% inhibition of ACE activity (Boschin et al., 2014).

2.9. Evaluation of the direct antioxidant activity

2.9.1. Diphenyl-2-picrylhydrazyl radical (DPPH) assay

A slightly modified version of a typical technique was used to perform the DPPH assay (Liu et al., 2019). Briefly, 15 μL of RBPH U < 1 or digested RBPH U < 1 at concentrations of 0.5, 1.0 and 5.0 mg mL^{-1} in deionized H_2O was combined with 45 μL of DPPH solution in methanol (0.0125 mmol L^{-1}). The DPPH radical scavenging reaction was conducted at room temperature in the dark and, after 30 min of incubation, the absorbance was measured at 520 nm.

2.9.2. 2,2′-azino-Bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt assay

Trolox equivalent antioxidant capacity (TEAC) assay is based on the reduction of 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) radical by antioxidants. The ABTS radical cation (ABTS+•) was prepared by mixing potassium persulfate (2.45 mmol L^{-1}) (1:1) with a ABTS solution (7 mmol L^{-1}) (Sigma-Aldrich, Milan, Italy). It was then stored for 16 h at room temperature in the dark. To make the ABTS reagent, the ABTS+• was diluted in phosphate buffer (5 mmol L^{-1}) (pH 7.4) until a steady absorbance of 0.70 (±0.02) at 730 nm was obtained. In order to conduct the experiment, RBPH U < 1 or digested RBPH U < 1 (5 μL of water solutions) were combined with diluted ABTS+• (70 μL) at final concentrations of 0.005, 0.01, 0.05, and 0.1 mg mL^{-1}. After 5 min of incubation at 30 °C, the absorbance at 730 nm was measured using a microplate reader Synergy H1 (Biotek) (Bartolomei et al., 2021).

2.9.3. FRAP assay

The ability of a sample to convert ferrous ions (Fe^{2+}) from ferric ions (Fe^{3+}) is measured by the FRAP assay. A solution of TPTZ (10 mmol L^{-1}) (Sigma-Aldrich, Milan, Italy) in HCl (40 mmol L^{-1}), 1.3 mL of $FeCl_3 \times 6$ H_2O (20 mmol L^{-1}), and 1.3 mL of acetate buffer (300 mmol L^{-1}) (pH 3.6) were combined to create the FRAP reagent. Consequently, 70 μL of FRAP reagent was combined with 5 μL of RBPH U < 1 or digested RBPH U < 1 solutions at the final concentration of 0.5, 1.0, 2.5, and 5.0 mg mL^{-1} in deionized water. After the microplate was incubated for 30 min at 37 °C, the absorbance at 595 nm was measured. Absorbances were recorded using the Biotek Synergy H1 microplate reader (Phongthai et al., 2018).

2.10. Caco-2 cell culture

Caco-2 cells from ATCC (HB-8065, ATCC from LGC Standards, Milan, Italy) were routinely sub-cultured, according to a previously optimized protocol (Bartolomei et al., 2021). For more details, see **Supplementary Data, section S2.**

2.11. 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) assay

3×10^4 Caco-2 cells/well were cultured in 96-well plates and treated with 0.1–5 mg mL^{-1} of RBPH U < 1 or digested RBPH U < 1 in complete growth medium or vehicle (H_2O) for 48 h at 37 °C in a 5% CO_2 atmosphere, as previously reported (Fanzaga et al., 2023). For more details, see **Supplementary Data, section S2.**

2.12. Fluorometric intracellular ROS assay

Caco-2 cells were seeded in a 96-well plate and treated with Master Reaction Mix, RBPH U < 1, or digested RBPH U < 1 (1 mg mL^{-1}), followed by H_2O_2 exposure to assess ROS generation, with fluorescence detection (details in **Supplementary Data, section S2**).

2.13. High-resolution LC-MS/MS analysis and data elaboration

RBPH U < 1 sample have been analyzed using Dionex Ultimate 3000 nano-LC system (Sunnyvale CA, USA) connected to Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Scientific, Bremen, Germany) equipped with nano-electrospray ion source. Peptide mixture was pre-concentrated onto an Acclaim PepMap 100–100 μm × 2 cm C18 (Thermo Scientific) and separated on EASY-Spray column ES900, 25 cm × 75 μm ID packed with Thermo Scientific Acclaim PepMap RSLC C18, 3 μm, 100 Å. The temperature was set to 35 °C. The peptides were eluted using mobile phase A (0.1 % formic acid in water) and mobile phase B (0.1% formic acid in water/acetonitrile 20/80, v/v) at a flow rate of 300 μL/min according to the following gradient: 4%–28% of B for 40 min and then 28%–40% of B in 10 min, and to 95% within the following 3 min to rinse the column. The column was re-equilibrated for 20 min. The total run time was 90 min. The sample injection volume is 2 μL. One blank was run between samples to prevent sample carryover. MS spectra were collected over an *m/z* range of 375–1500 Da at 120 000 resolutions (*m/z* 200), operating in the data-dependent mode, using a cycle time of 3 s between masters scans. Higher-energy collisional dissociation (HCD) was performed with collision energy set at 35 eV and positive polarity. Spectra were processed using Mascot search engine (Matrix Science Mascot 2.2.04) against the *Oryza sativa* taxonomy Swiss-Prot database (2023, 569 213 sequences; 205 728 242 residues). The database search was performed with the following parameters: MS and MS/MS mass tolerance 10 ppm and 0.02 Da, respectively, semi trypsin was chosen as digestive enzyme, 1 missed cleavage was allowed. Proteins with at least one high-confidence peptide were validated. Peptide identifications from Mascot searches were filtered at 0.1% False Discovery Rate (FDR) confidence threshold, based on a concatenated decoy database search, using PD (Yang et al., 2006).

2.14. Statistical analysis

Statistical analyses were performed by One-Way ANOVA followed by Tukey's post-hoc tests using GraphPad Prism 9 (San Diego, CA, USA). Values were reported as means ± S.D, considering *p*-values <0.05 to be significant.

3. Results and discussion

3.1. Rice bran enzymatic hydrolysis

In order to enhance the protein recovery, defatted rice bran (DRB), obtained as previously reported (Bagnasco et al., 2013), was treated with commercially available carbohydrase formulations. Specifically, on the basis of a previous systematic study aimed at finding the most performing carbohydrases to promote the protein extraction of rice bran, we selected Ceremix® Plus MG and Celluclast® 1.5L as hydrolytic enzymes. Indeed, it has been shown that the combination of these two enzyme mixtures was the most efficient in degrading rice bran cell wall. Specifically, the presence of α-amylases in Ceremix® Plus MG and cellulases in Celluclast® 1.5L ensures the hydrolysis of RB polysaccharides, constituted mainly by starch (roughly 43%), and cellulose (21%), along with lignin-carbohydrate complexes (Scarabattoli et al., 2023). The protein-enriched fraction (RBP) was obtained in good yield (46%, calculated as % w/w) and with 25% of protein content. It was observed that the recovery of nitrogen (%N in RBP with respect to %N in DRB) is slightly lower than that obtained in our previous study (68% *vs* 73%) (Scarabattoli et al., 2023). This can be due to the different origin of the used rice bran; indeed, the variability of the starting material is a key point to be considered when dealing with protein hydrolysates obtained from different biomass sources.

The protein-enriched fraction (RBP) was then hydrolyzed by sequential treatment with Alcalase® 2.4 L and Flavourzyme®. It is noteworthy that Alcalase® has been reported to have highest capability for hydrolysis of rice bran protein concentrate compared to other enzymatic preparations (Ahmadifard et al., 2016) and its use in combination with Flavourzyme® was already proven to be effective in

producing bioactive peptides from soy protein isolate (Sangiorgio et al., 2022). To the best of our knowledge, a similar two-step hydrolytic protocol for the production of protein hydrolysates from rice bran has not been reported so far.

In order to evaluate the degree of hydrolysis (DH), OPA-NAC method, with some modifications, was used. In comparison to other protocols (Rutherfurd, 2010), such as TNBS method, the OPA method was found to be more accurate, simpler and faster to carry out, taking into account issues related to the poor solubility of the starting material (Adebiyi et al., 2008; Nielsen et al., 2001). A DH of 45 ± 4 % was obtained.

3.2. Ultrafiltration

The high value of DH suggested that RBPH obtained was largely constituted by low molecular weight peptides and amino acids. In order to fractionate this mixture, RBPH sample was ultrafiltered through decreasing molecular weight cut-off membranes (5 and 1 kDa). As a result, see Fig. 2, a fraction with the lowest MW (RBPH U < 1) was obtained, which accounted for 82% w/w of the entire RBPH. Such a peptide size distribution is due to the experimental conditions selected for enzymatic hydrolysis, *i.e.,* type of proteases and incubation time. Indeed, firstly endopeptidases of Alcalase® 2.4 L break down peptide bonds of rice bran protein, producing large peptides, then exopeptidases from Flavourzyme® remove amino acids or very small peptides from the N- or C-terminus position, thus resulting in an increased hydrolytic degradation, further favoured by prolonged incubation time (24 h). With the aim of assessing the potential use of rice bran as a functional food or nutraceutical, RBPH U < 1, which constitutes the largest portion of hydrolysate, was analyzed for peptidomic profile and investigated for its antioxidant and ACE-inhibitory properties, also after simulated gastrointestinal digestion.

3.3. ACE inhibition assay

ACE-inhibitory activity was thus evaluated both on RBPH U < 1 and on RBPH U < 1 subjected to a two-stage hydrolytic process simulating physiological digestion. To exert their health-promoting effects, ACE-inhibitory peptides must exhibit gastrointestinal stability and retain their bioactivity while reaching the cardiovascular system intact. It is well-documented that short-chain peptides, especially those with a proline residue in the C-terminus, demonstrate decreased susceptibility to proteolytic degradation (Daskaya-Dikmen et al., 2017). The importance of peptide size is linked to the absorption potential into the bloodstream, as well as to the ability to interact with targeted enzymes responsible for regulating blood pressure (Aluko, 2015).

RBPH U < 1 showed a 68.7% ACE-inhibitory activity at 1.0 mg mL^{-1}, with an IC_{50} value of 552.7 μg mL^{-1}. It is worth noting that fraction RBPH U < 5-1> kDa reached only 53.5% inhibitory activity at the same concentration, whereas the activity of RBPH U > 5 kDa fraction dropped to 49.4%. These data are in good agreement with those reported in literature for other food-derived peptides, such as soy and hempseed protein (Orio et al., 2017; Sangiorgio et al., 2022). After treatment with gastrointestinal proteases (pepsin and pancreatin), no significant differences in ACE-inhibitory activity were detected, suggesting that peptides present in RBPH U < 1, administered orally, could preserve their activity in the gastrointestinal tract (Zheng et al., 2019).

3.4. In vitro evaluation of the RBPH U < 1 and digested RBPH U < 1 direct antioxidant activity by DPPH, ABTS and FRAP assays

Numerous studies have demonstrated that protein hydrolysates derived from rice bran have the potential to be natural anti-oxidants (Rani et al., 2018). For instance, Wattanasiritham et al. identified antioxidant peptides in rice bran protein hydrolysates and demonstrated their ability to scavenge free radicals effectively (Wattanasiritham et al., 2016). Moreover, an anti-inflammatory activity of rice bran hydrolysates in LPS-THP-1 macrophage stimulated model was observed (Chanput & Lawyer, 2020).

With the aim of assessing the *in vitro* antioxidant properties of RBPH U < 1 and investigating whether the simulated digestion could cause a partial loss of its bioactivity, the most common bioassays to evaluate the free radical scavenging activity, *i.e.*, ABTS and DPPH, as well as the ferric ions reducing antioxidant power (FRAP) were performed (Benzie & Strain, 1996; Floegel et al., 2011; Shah & Modi, 2015).

As reported in Fig. 3, results suggest that RBPH U < 1 reduced the DPPH radical concentration by 25.14 ± 2.21%, 50.25 ± 2.09%, 72.51 ± 3.29% at 0.5, 1, and 5 mg mL^{-1} respectively, whilst *in vitro* digested RBPH U < 1 reduced the DPPH concentration by 6.41 ± 2.72%, 19.6 ± 1.05%, 22.12 ± 9.32% at 0.5, 1, and 5 mg mL^{-1}, indicating that, when subjected to simulated digestion and tested at the same concentrations, RBPH U < 1 lost part of its ability to scavenge DPPH free radical.

In addition, RBPH U < 1 was able to significantly reduce the ABTS radical by 34.75 ± 1.71%, 52.67 ± 1.53%, 63.90 ± 2.98%, 67.64 ± 1.64% at the concentrations of 0.005, 0.01, 0.05, 0.1 mg mL^{-1}, respectively (Fig. 3). *In vitro* digested RBPH U < 1 scavenged the ABTS radical by 29.75 ± 5.19%, 45.75 ± 5.68%, 64.73 ± 2.57%, 66.07 ± 1.54% at 0.005, 0.01, 0.05, 0.1 mg mL^{-1}, respectively, suggesting that the treatment with digestive enzymes did not cause any negative effect on the ABTS radical scavenging activity of RBPH U < 1.

Finally, RBPH U < 1 increased the FRAP by 326.30 ± 5.56%, 977.30 ± 3.19 %, 1590.00 ± 31.51%, 2028 ± 27.08% at the concentrations of 0.5, 1.0, 2.5, 5.0 mg mL^{-1}, respectively (Fig. 3). On the other hand, *in vitro* digested RBPH U < 1 augmented the FRAP by 270.30 ± 5.0 %, 783.80 ± 6.48%, 1383.00 ± 16.90 %, 2053.00 ± 53.92 % at the same tested concentrations, respectively, indicating that the simulated digestion led to a slight loss in the ability of the hydrolysate to reduce iron ions, except in the case of the highest concentration (5 mg mL^{-1}), where both hydrolysates show a comparable activity.

In a recent study, Song et al., starting from rice bran protein concentrate, prepared fractions with high antioxidant activity using proteolytic enzymes and experimental conditions different from those used in this work. Specifically, the fraction below 1 kDa showed higher activity in reducing the ABTS radical *in vitro*, while the 3 kDa fraction was more effective in the DPPH and FRAP assays (Song et al., 2023). These findings highlight that the antioxidant activity of food-derived hydrolysates is influenced not only by the type of peptides and the molecular weight distribution of the hydrolysate (*i.e.*, DH), but also by amino acid composition of peptides generated by enzymatic hydrolysis, since different amino acids composing the bioactive peptides can exert antioxidant activity with different mechanisms of action (see **Supplementary Data, section S3**). Indeed, Song et al. reported that peptides containing cysteine exhibit a more significant DPPH radical scavenging activity, while those containing aromatic amino acids (AAAs) are particularly effective in scavenging ABTS radicals (Song et al., 2023). Results from antioxidant assays at the cellular level, including the inhibition of ROS production in Caco-2 intestinal cells, as well as findings from previous biocompatibility studies on Caco-2 cells using the MTT assay, are provided in the **Supplementary Data, section S2.**

3.5. RBPH U < 1 peptides composition

Understanding the peptide profile is essential for elucidating the relationship between peptide structure and bioactivity. Thus, RBPH U < 1 was submitted to LC-MS analysis (see **Supplementary Data, section S4**) that led to the identification of several peptides derived primarily from glutelin, the major protein in rice grains, specifically Glutelin type-B 1, B 4, and A 1 (He et al., 2013). Despite the use of a 1 kDa cut-off ultrafiltration membrane, peptides with a molecular weight slightly above this threshold, such as those around 1.5 kDa, could be identified. Indeed, it is possible for peptides that slightly exceed the 1 kDa threshold to co-elute with the smallest ones or remain in the filtrate due to their

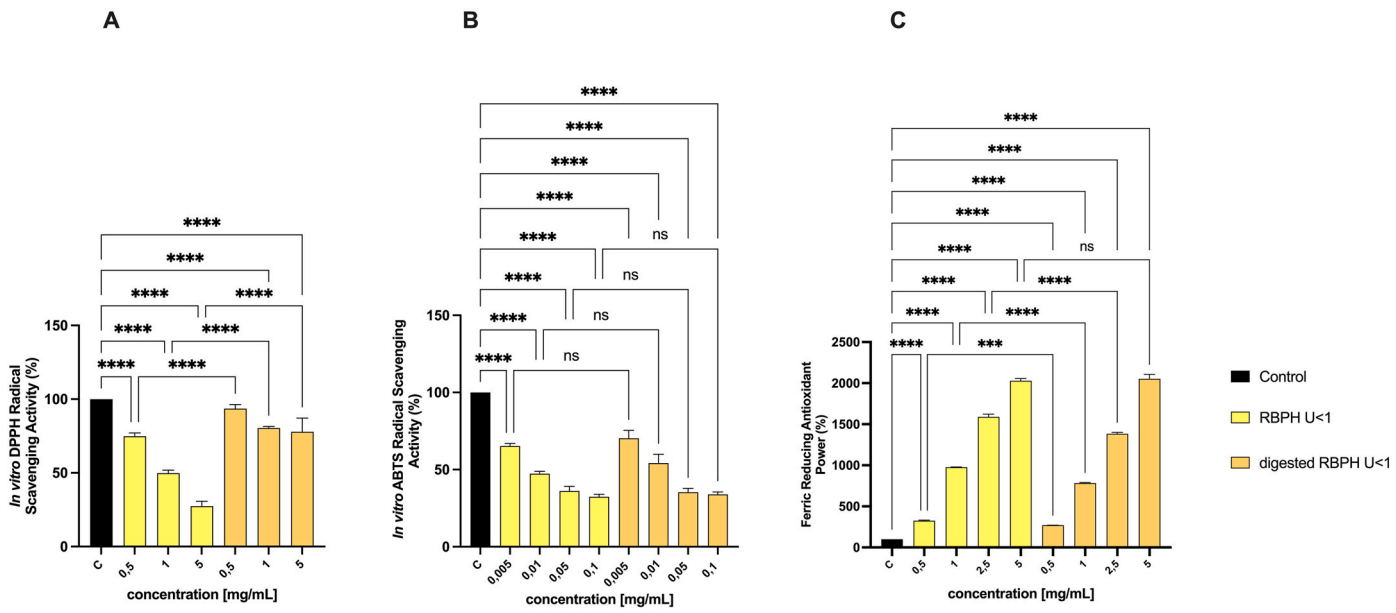

Fig. 3. *In vitro* direct antioxidant activity. Radical scavenging activity of RBPH U < 1 and digested RBPH U < 1 on DPPH (**A**) and ABTS (**B**) assays, respectively. Ferric Reducing Antioxidant Activity of RBPH U < 1 and digested RBPH U < 1 (**C**). Data represent the mean ± s.d. of two determinations performed in triplicate. All the data sets have been analyzed by One-way ANOVA followed by Tukey's post-hoc test. Ns: not significative, (***) $p < 0.001$, (****) $p < 0.0001$. C: control.

structural properties or interactions with other peptides. Moreover, membranes do not have 100% efficiency and it is possible that a small amount of peptides with size higher than the MWCO can pass through into the permeate (Aluko, 2015). At the same time, tri- and tetra-peptides, which produce a limited number of fragment ions due to their small size, were not detected since they were obscured by the presence of a greater amount of larger peptides. Thus, the results reported in **Supplementary Data, section S4** are referred to medium size peptides. Notable is the presence of peptides such as KTNANAFV, RVIQPQGLLVPRY, and RGQLLIVPQHYV from Glutelin type-B, which are of particular interest due to their potential bioactivities, including antioxidant and ACE inhibitory effects (Et, 2008).

Several studies have demonstrated that the biological activity of peptides is related to chain length, amino acid composition and sequence (Orio et al., 2017). For instance, the presence of hydrophobic amino acids, such as valine (V), leucine (L), and isoleucine (I), particularly near the C-terminus, as in RVIQPQGLLVPRY, has a strong influence on ACE-inhibitory activity (Orio et al., 2017; Sitanggang et al., 2021). This sequence also includes proline (P), highlighted as a key amino acid due to its cyclic structure which contributes to the stability and effectiveness of ACE inhibitory peptides (Xiang et al., 2023). Also RGQLLIVPQHYV contains multiple hydrophobic residues (L, I, V and P), thus aligning with characteristics of effective ACE inhibitory peptides.

Aromatic amino acids (AAAs) are known to enhance the ACE-inhibitory activity of peptides, thereby amplifying their antihypertensive effects (Aluko, 2015). Moreover, tyrosine (Y) and methionine (M), are reported to be part of numerous sequences of peptides with antioxidant activity (Di Bernardini et al., 2011). Both RGQLLIVPQHYV and RVIQPQGLLVPRY, which include tyrosine at the C-terminal, have the potential to be antioxidants as well as ACE inhibitors. Methionine, while less frequent, can be found in sequences like KTNANSMVSHLA, potentially contributing to antioxidant activity through its sulfur-containing side chain. Thus, these peptides could be responsible of the observed bioactivity of RBPH U < 1.

4. Conclusions

Considering the technological constraints which limit the use of rice bran, the valorisation of proteins of such an important agrifood by-product could significantly contribute to the sustainability of rice production. This study describes a green approach, based on a two-step, easily scalable hydrolytic protocol to prepare a rice bran protein hydrolysate (RBPH), which exploits cheap and commercially available cellulolytic (Ceremix® Plus MG and Celluclast® 1.5 L) and proteolytic (Alcalase® 2.4L and Flavourzyme®) enzyme formulations. The fraction with MW lower than 1 kDa (RBPH U < 1) was isolated by membrane ultrafiltration and subjected to a multidisciplinary investigation to assess its antihypertensive and antioxidant activities. RBPH U < 1 showed high ACE-inhibitory activity (68.7 %) at 1 mg mL^{-1}, as well as the ability to reduce ABTS and DPPH radicals, and to increase the ferric ions reducing antioxidant power (FRAP); moreover, with the aim to evaluate a possible protective effects on intestinal health, tests were performed on Caco-2 cells, showing that RBPH U < 1 sample reduced the H_2O_2-induced ROS overproduction. It is noteworthy that these biological activities are maintained also after simulated gastrointestinal digestion. Further studies are needed for deep understanding the mechanism underlying the antioxidant activities, such as the evaluation of the ability of peptides to modulate some key intracellular target like Nrf-2 and its related protein substrates. Furthermore, mass spectrometry analysis was conducted to characterize the peptidomic profile of the hydrolysate. This analysis has made it possible to identify in RBPH U < 1 certain peptide sequences, *i.e.*, RGQLLIVPQHYV, RVIQPQGLLVPRY and KTNANSMVSHLA, which are thought to contribute to the overall bioactivity. This study confirms the bioactive properties of enzymatically prepared rice bran protein hydrolysates, supporting their potential use in the development of nutraceuticals aimed at promoting intestinal health and mitigating oxidative stress-related conditions.

CRediT authorship contribution statement

Letizia Scarabattoli: Writing – original draft, Investigation, Formal analysis. **Melissa Fanzaga:** Writing – original draft, Investigation, Formal analysis. **Gilda Aiello:** Methodology, Investigation. **Giovanna Boschin:** Methodology, Investigation. **Lorenza d' Adduzio:** Investigation. **Carlo F. Morelli:** Writing – original draft, Validation, Formal analysis. **Marco Rabuffetti:** Validation, Formal analysis. **Carmen Lammi:** Writing – review & editing, Supervision, Conceptualization. **Giovanna Speranza:** Writing – review & editing, Supervision,

Resources, Project administration, Conceptualization.

Declaration of competing 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 paper.

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Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2025.117381.

Data availability

Data will be made available on request.

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