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Potential Anti-Sarcopenia Effect and Physicochemical and Functional Properties of Rice Protein Hydrolysate Prepared through High-Pressure Processing

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Abstract: Rice protein is a suitable alternative protein source for dairy protein in infant formulas on account of its unique nutrition and hypoallergenicity. Rice protein was isolated through enzymatic hydrolysis (Alcalase, papain, bromelain, Flavourzyme®) in combination with high-pressure processing (HPP) (400 MPa for 15 min at 25 °C) to enhance its functional properties and broaden its food processing applications. The effect of the HPP-treated rice protein hydrolysate on dexamethasone (DEX)-induced atrophy in C2C12 myotubes was also studied. The length of myotubes was observed under a light microscope, and periodic acid Schiff staining. The results showed that even though enzymatic hydrolysis and HPP treatment affected the color of the resulting rice protein, the protein content (3120.31 ± 42.15), branched chain amino acid (BCAA) content (15.12 ± 1.03), peptide content (31.25 ± 0.55), and amino acid composition of the rice protein were significantly increased. Moreover, the combined enzymatic and HPP treatment effectively overcame the problem of limited solubility and water-holding capacity. Rice protein produced through enzymatic and HPP treatment exhibited a higher free radical scavenging activity and oxygen radical absorbance capacity. It also alleviated DEX-induced muscle atrophy in C2C12 myotubes as indicated by the increase in myotube length. In short, the enzymatic and HPP treatment of rice protein not only overcame limitations, but also produced rice protein with high functionality in terms of antioxidant and therapeutic effects on muscle atrophy. The rice protein hydrolysate produced through enzymatic hydrolysis and HPP treatment showed the potential for use as an ingredient for functional foods in the nutraceutical industry.

Keywords: anti-sarcopenia; rice protein; high-pressure processing; C2C12 cell atrophy model; periodic acid Schiff staining

1. Introduction

Sarcopenia is a condition that is characterized by the loss of muscle mass, strength, and function through adverse muscle changes that accrue over a lifetime. Muscle mass

decreases by 1% to 2% over the ages of 20–30 years and by 2% to 3% every year after the age of 60 [1]. The loss of muscle mass because of aging and disease-related sarcopenia, such as malnutrition, diabetes, renal insufficiency, and cancer affects the ability to perform daily physical activities due to the reduction in muscle strength, walking speed, and balance, and may lead to geriatric diseases, such as sarcopenia and frailty [1,2]. Older adults with sarcopenia experience higher risks of frailty, disability, hospitalizations, and mortality, and a poor quality of life [2]. Therefore, the development of nutritional supplements and therapeutic agents is necessary to prevent or overcome the progression of muscle atrophy.

A database analysis by using the bibliometric method revealed that agricultural waste has been studied worldwide continuously for more than 60 years [3]. Related studies mainly focused on sustainable development to guarantee resource efficiency, sustainable production, and consumption, and reduce the negative environmental impact [3]. Recently, agricultural byproducts have been turned into a popular and potential source to produce plant-based proteins. Protein based on plant byproducts is not only applied to produce biofilms [4–6], but also as ingredients in food [7] and pharmaceutical [8] products.

Rice (*Oryza sativa* L.) represents one of the leading food crops in the world. Its global annual production is approximately 503.7 million metric tons (milled rice basis) [9]. It grows best in areas where annual daytime temperatures are within the range 20–30 °C; however, it can tolerate 10–36 °C. It is suitable to plant in light (sandy), medium (loamy), and heavy (clay) soils [9,10]. Furthermore, rice is a staple food and a crucial source of proteins in many regions. Rice proteins are mainly in the form of storage organelles called protein bodies and categorized in accordance with the solubility-based classification as albumin (water-soluble), globulin (salt-soluble), glutelin (alkali/acid-soluble), and prolamin (alcohol-soluble) [10]. The rice protein isolate (RPI) is considered as a hypoallergenic protein and is commonly used in infant formulas and diets for allergy diagnosis in children and adults [10]. In addition to casein, whey, and soy proteins, rice protein serves as an alternative food ingredient in sports nutrition [11]. Previous studies also demonstrated that RPI comprising specific peptide fractions has antioxidant [12], antiobesity [13], anticancer [14], and antihypertensive activities [15]. RPI, which has a high nutritional value, is suitable for human consumption and has potential for the development of nutrient supplies.

However, rice protein has limited industrial applications because of its poor solubility [10,16]. Typical rice bran, which contains high levels of fat (15–20% by weight), causes rancidity and off-flavors [10]. Meanwhile, the commonly used defatting processes, such as hexane extraction, mechanical pressing, and protein extraction procedures, for example, alkaline extraction, physical extraction, and enzymatic extraction may induce protein denaturation and aggregation, reduce protein purity, and lead to low protein solubility [10,17]. Therefore, studies must solve the problem of rice protein solubility while retaining the nutritional and functional attributes.

In food applications, rice protein can be extracted by using enzymatic methods, and the method is divided into two steps. First, the proteins are separated from the rice flour using enzymes that solubilize starch, including α-amylase, glucoamylase, and pullulanase. Then, the protein hydrolysates are generated from food-grade commercial proteases, such as papain, trypsin, neutrase, alcalase, Flavourzyme®, and bromelain [18]. Even though the rice protein hydrolysate (RPH) produced through enzymatic methods exhibits high digestibility and bioavailability, it still shows some drawbacks, such as high pH and dark color due to the increase in the Maillard reaction [19].

In consideration of the high costs and low protein contents of milled rice and rice flour, rice proteins can be favorably extracted from a low-cost source, such as rice dregs, a byproduct of the processing of rice syrups. The protein content of rice dregs is higher than 50% [20]. An RPH prepared from rice dregs using trypsin (2.4 h at pH 7.6, 52.8 °C) had a relatively high recovered protein content (75.81%) and a low degree of hydrolysis (6.95) [21].

The novel nonthermal minimal processing technique, and high-pressure processing (HPP) treatment induced protein denaturation and aggregation or gelation with improved

textural properties [6]. Previous studies demonstrated that the HPP treatment of plant proteins reveals a modification in the structure and confrontation of protein, and it can be potentially used to modulate the digestibility and rheological properties of plant-based proteins [6,22,23]. The gel formation of pea protein occurred with 16 g protein/100 g water concentration and 250 MPa HPP treatments; a greater extent of protein denaturation, aggregation, and network formation happened by raising the pressure level, due to the fact the protein tertiary and quaternary conformation changed [23]. This HPP treatment has the potential to better preserve the organoleptic and nutritional properties of the final products as compared to traditional, thermal-based processing methods.

Hence, this study focused on evaluating the effects of different rice protein extraction and treatment (enzymatic hydrolysis and high-pressure processing, (HPP)) methods on the physicochemical properties, including the amino acid composition, solubility, and antioxidant capacity of the RPI. Meanwhile, the effect of the RPI on dexamethasone (DEX)-induced myotube atrophy in C2C12 mouse skeletal muscle cells was determined.

2. Materials and Methods

2.1. Sample Preparation

Figure 1 presented the experimental design and sample preparation methods of the rice protein samples. The RPI (sample A) was prepared through the alkaline extraction method with different degrees of alkali treatment (0.2 M of NaOH), followed by precipitation with an acidic solution [12]. Rice flour was gelatinized with 1000 mL of distilled water under stirring for 1 h at boiling temperature. The mixture was desugarized by using 0.25% α- and β-amylase at 55 °C for 1 h. The enzyme was deactivated at 80 °C for 10 min. After centrifugation at 3000× *g* for 15 min to obtain the precipitate, the residue was oven-dried at 45 °C for 24 h. The dried residue was subjected to alkali treatment with 0.2 M NaOH for 3 h at 50 °C. After centrifugation at 3000× *g* for 15 min to obtain the supernatant, the protein was then purified through isoelectric precipitation at pH 4.3. Next, the mixture was centrifuged at 3000× *g* for 15 min to obtain the precipitate. All precipitates were collected and freeze-dried by using an FDM-2 Uniss Manifold Freeze Dryer (Bioman Scientific Co., Ltd. Taipei City, Taiwan) to obtain the RPI (sample A).

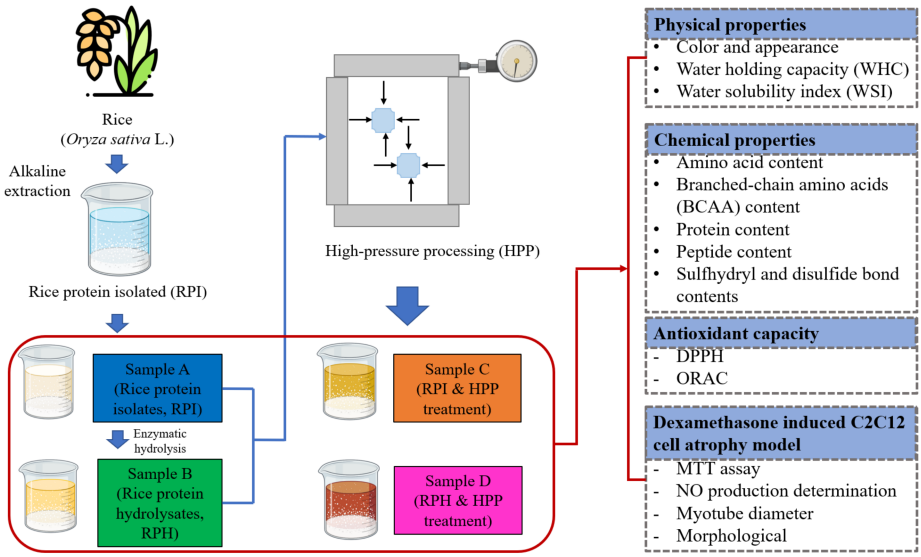


Figure 1. Experimental design and sample preparation methods of rice protein samples.

For the preparation of sample B (RPH), the pH of sample A was adjusted to pH 7.6. Then, 1.25% Alcalase and 1.25% papain and bromelain were added. Hydrolysis was carried out at 55 °C for 2 h. After hydrolysis, Flavourzyme® was added at pH 8 and 55 °C for 10 h

and deactivated at 90 °C for 10 min. The sample solution was freeze-dried by using the freeze dryer to obtain the RPH (sample B) [12,21].

Sample C, which is the HPP treatment sample, was prepared by subjecting sample A to HPP treatment (UHP/5 L/800 MPa, Bao Tou Kefa High-Pressure Technology Co. Ltd., Baotou, China) at 400 MPa for 15 min [12,21,24]. The HPP-treated sample solution was freeze-dried by using a freeze dryer to obtain sample C.

Sample D was prepared by using sample B. After enzymatic deactivation at 90 °C for 10 min, the sample solution was directly subjected to HPP treatment at 400 MPa for 15 min [12,21,24]. The sample solution was freeze-dried by using the freeze dryer to obtain sample D.

2.2. Amino Acid Composition

The amino acid composition of the rice protein samples was analyzed in accordance with a previously reported method with modifications [25]. Samples were hydrolyzed in 8 mL of 5 M hydrochloric acid (HCl) at 110 °C for 24 h. Next, the amino acid composition and composition of the hydrolysate were determined by utilizing an amino acid autoanalyzer (Shimadzu LC-30 AD) equipped with a C18 column (150 × 4.6 mm, 5 µm; Intact Co., Kyoto, Japan). Then, the column was equilibrated with 25 mM/L phosphate buffer as mobile phase A and acetonitrile/methanol/water at a ratio of 45:40:15 (*v*/*v*/*v*) as mobile phase B. The oven temperature was maintained at 40 °C. The instrument was operated by following the instructions of the manufacturer, and analytical results were generated by using LAB Solutions software (5.54SP v5, Shimadzu, Kyoto, Japan).

2.3. Protein, Branched Chain Amino Acid, and Peptide Contents

The protein content was measured by using a BCA protein assay kit (Thermo Scientific™, no. 23225, Waltham, MI, USA), and the procedure was carried out by following the BCA protein assay kit protocol. First, the copper was chelated with protein in an alkaline environment to form a light blue complex (Biuret reaction). In this reaction, peptides that contain three or more amino acid residues formed a colored chelate complex with cupric ions in an alkaline environment containing sodium potassium tartrate. The BCA reacted with the reduced (cuprous) cation. The intensely purple-colored reaction product resulted from the chelation of two molecules of the BCA with one cuprous ion. The BCA/copper complex exhibited a strong linear absorbance at 562 nm that could be measured by using a spectrophotometer (SH-U830 Vis-Spectrophotometer, Shishin Technology Co., Ltd., Taipei, Taiwan). The results were expressed as mg/mL.

A branched chain amino acid (BCAA) assay kit (BCAA assay kit, Abcam, no. 83374) was used to measure the BCAA content of the rice protein samples. BCAAs are amino acids with nonlinear aliphatic sidechains, namely, leucine (Leu), isoleucine (Ile), and valine (Val). The BCAA assay kit is based on an enzymatic assay in which the oxidative deamination of the BCAA produces NADH that reduces the probe, generating colored compounds with an absorbance that is measurable at the wavelength of 450 nm.

The rice protein sample was also assessed by using the OPA-assay, which enabled the measurement of the peptide content on the basis of the reaction with alpha amino groups. A sample (2.5 µL) was mixed with 100 µL of the OPA mixture (2.5 mL of sodium tetraborate, 1 mL of 5% [*w*/*v*] SDS, 100 µL of 40 mg/mL OPA in methanol, 10 µL of 2-mercaptoethanol, and 1.39 mL of water). Then, the mixture was left for 10 min at room temperature, and the resulting signal was measured at the wavelength of 340 nm.

2.4. Color and Appearance

The color of the rice protein samples was analyzed by using a Color Meter ZE-2000 (Nippon Denshku Industries, Tokyo, Japan). L^*, which represents the lightness measurement; a^*, which represents the redness-greenness value; and b^*, which represents the yellowness-blueness value, were evaluated to verify the changes in quality. The instrument

calibration involved the use of a standard black-and-white ceramic tile before measurement. Color measurements were carried out at room temperature with independent triplicates.

The hue angle (H, Equation (1)), total color difference (ΔE, Equation (2)), chroma, and browning index (BI, Equations (3) and (4)) were calculated using L^*, a^*, and b^* values as follows and used to determine the color changes as compared with the control.

$$\text{Hue angle, H} = \arctan b^*/a^* \quad (1)$$

$$\Delta E = \sqrt{(L_0 - L^*)^2 + (a_0 - a^*)^2 + (b_0 - b^*)^2} \quad (2)$$

where subscript "0" refers to the color reading of the control sample used as the reference, and a larger ΔE indicates the greater color change of the sample.

$$\text{Chroma} = \sqrt{(a^{*2} + b^{*2})} \quad (3)$$

$$\text{Browning index, BI} = \frac{100\ (x - 0.31)}{0.17} \quad (4)$$

where in Equation (4), x;

$$x = \frac{(a* + 1.75L)}{(5.645L* + a* - 0.012b*)} \quad (5)$$

2.5. Water Solubility Index and Water-Holding Capacity

The water-holding capacity (WHC) was measured by dispersing 1 g of the rice protein sample in 25 mL of distilled water in a centrifuge tube. The dispersions were stirred and left at 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, and 90 °C for 1 h, then centrifugated for 30 min at 3000× *g*. The supernatant was collected. The excess of water was removed by draining for 25 min at 50 °C, and the sample was reweighed.

The water solubility index (WSI) was determined by using AACC method No. 44-19 [26]. The sample powder (S_1, g) was dispersed in water in a centrifuge tube at a powder/water ratio of 0.02/1 (*w*/*w*) and ambient temperature. The dispersion was then incubated in a water bath at 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, and 90 °C for 30 min, then centrifuged at 1100× *g* for 20 min. The supernatant was carefully collected in a pre-weighed evaporating dish (S_2, g) and dried at 105 °C ± 2 °C. The evaporating dish with the residue was weighed again (S_3, g). The WSI was calculated by using the following formula:

$$\text{WSI (\%)} = (S_3 - S_2)/S_1 \times 100\% \quad (6)$$

2.6. Sulfhydryl and Disulfide Bond Contents

The free sulfhydryl group (FSH) and disulfide bond (S-S) contents of the rice protein samples A, B, C, and D were evaluated in accordance with the method described by Wang et al. (2016) with some modifications [16]. A total of 15 g of the protein samples was suspended in 10 mL of the Tris-Gly buffer (pH 8.0) containing 0.086 mol/L Tris, 0.09 mol/L glycine, 0.004 mol/L EDTA, and 8 mol/L urea, then centrifuged at 10,000× *g* for 10 min. For the determination of the FSH content, 50 µL of Ellman's reagent (DTNB in Tris-Gly buffer, 4 mg/mL) was added to 1 mL of the protein supernatant, and the solution was mixed thoroughly. After 5 min of binding, the absorbance at 412 nm was monitored. The contents of the FSH were determined as follows:

$$\text{mmol SH/g} = 73.53 \times A_{412}/C \quad (7)$$

where A_{412} is the absorbance at 412 nm; C is the protein concentration (mg/mL); and 73.53 is derived from $10^6/(1.36 \times 10^4)$, where 1.36×10^4 is the molar absorptivity of Ellman's reagent.

2.7. In Vitro Antioxidant Capacity

2.7.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The free radical scavenging activity of the rice protein samples was quantified in accordance with the method reported by Li et al. (2020) [27]. The antioxidant activity of the rice protein samples and the standard was assessed on the basis of the radical scavenging effect of the stable free radical activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH). The diluted working solutions of the test samples were prepared in methanol. Next, DPPH (1 mM) was prepared in methanol, and 2 mL of this solution was mixed with 1 mL of the sample and standard solutions separately. The mixture solution was kept in the dark for 30 min, and the optical density was measured at 518 nm by using a UV spectrophotometer (SH-U830 Vis-Spectrophotometer, Shishin Technology Co., Ltd., Taipei, Taiwan). The optical density was recorded, and the percentage of inhibition (% of inhibition) was calculated by using the formula given below:

$$\text{DPPH free radical scavenging activity (\%)} = (A_0 - A_1)/A_0 \times 100, \quad (8)$$

where A_0 = is the absorbance of the control, and A_1 = is the absorbance of the sample.

2.7.2. Oxygen Radical Absorbance Capacity (ORAC)

In this assay, 96-well microplates were used, and absorbance was read at the wavelengths of 485 nm (excitation) and 530 nm (emission) by using a BioTek's Epoch™ Micro-Volume Spectrophotometer (Bioman Scientific Co., Ltd., New Taipei City, Taiwan) [28]. The reaction was carried out at 37 °C and was started by the thermal decomposition of 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH) in 75 mM phosphate buffer (pH 7.0). A total of 50 µL of 78 nM FL and 50 µL of the sample were placed in each 96-well plate. The blank was PBS, and 20 µM Trolox was used as the standard. Next, 25 µL of 221 mM AAPH was added. The plates were heated to 37 °C for 15 min before the addition of AAPH to avoid measurement variations among wells due to the low conductivity of the 96-well plates. Fluorescence was measured as the relative fluorescence intensity (FI%), and measurements were carried out every 5 min until the value was less than 5% of the value of the initial reading. Each analysis and measurement were performed in triplicate.

2.8. DEX-Induced C2C12 Cell Atrophy Model

2.8.1. C2C12 Cell Culture and Differentiation

The mouse muscle myoblast cell line C2C12 (BCRC no. 60083) was seeded in cell culture dishes and cultured in proliferation media (90% Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose + 10% fetal bovine serum + 1% penicillin/streptomycin [P/S]) at the incubation temperature of 37 °C and 5% CO_2. When the cell reached 70% confluence, the medium was replaced with differentiation media (DMEM + 2% horse serum + 1% P/S) to induce differentiation. The medium was changed at least every 2 days.

2.8.2. Dosage Regimen

Fully differentiated myotubes were treated with 5 mM DEX; 50, 100, 200, 500, and 1000 mg/mL rice protein samples; and 20 µM EX-527, a SIRT1 selective inhibitor (Beyotime Biotechnology, Shanghai, China) for 24 h.

2.8.3. Cell Viability Analysis

The C2C12 cells (0.5×10^5 cells/well) were seeded in 24-well cell culture dishes and cultured in proliferation media for 24 h, then treated with 5 µM DEX with or without different concentrations of rice protein samples (100, 200, 500, and 1000 mg/mL) for 24 h. Subsequently, the culture medium was replaced immediately with a fresh medium. Then, a proportional amount of the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) reagent was added to the medium in proportion. After 40 min of incubation, the OD value of the

medium was detected at 450 nm by the microplate reader. Each experiment was repeated six times with three replicate wells each time.

2.8.4. Measurement of Myotube Diameter

The C2C12 myotubes were photographed by using an optical microscope. The diameters of the myotubes were measured by using IS Capture 4.1 software (Informer Technologies, Inc., Roseau, Dominica), and the average diameter of the myotubes was calculated by measuring the maximum diameter of each myotube. Ten random culture fields for each sample were selected, and at least 100 myotubes were counted.

2.8.5. Periodic Acid Schiff Staining

The C2C12 cells were rinsed with cold PBS and fixed for 5 min in 3.7% formaldehyde buffered with PBS. After three more cycles of washing with PBS, the cells were incubated in 1% periodic acid for 15 min and washed three times for 5 min with distilled water. The samples were subsequently incubated for 30 min in Schiff's reagent, then incubated three times with 0.5% potassium bisulfite-0.05 M HCl for 5 min each time. Next, the stained myotubes were photographed with a microscope.

2.9. Statistical Analysis

All experiments were performed with independent triplicates. All data are presented as means ± SD. The differences between groups were examined for statistical significance by a one-way analysis of variance using SPSS 20.0 Statistical Software Program (SPSS Inc., Chicago, IL, USA), and later determined with the least significant difference test. The level of statistical significance was defined as $p < 0.05$.

3. Results and Discussion

3.1. Color and Appearance of RPI

The color development of the rice protein samples subjected to different treatments was determined to measure the extent of browning. The results are presented in Figure 2. Sample A had the highest L^* value (lightness) among the samples (Figure 2a). No significant difference was found between the L^* value of sample B (32.29 ± 6.13) and C (33.43 ± 5.25). Sample D had the lowest L^* value, which was 29.13 ± 2.46. In contrast, sample D had higher a^* (17.25 ± 0.42) and b^* (29.12 ± 2.01) values than sample A (8.52 ± 0.19 and 17.25 ± 0.42) (Figure 2b,c). Meanwhile, sample D illustrated the highest total color difference (ΔE), which was 8.96 ± 0.26, as compared to sample A (4.32 ± 0.19) (Figure 2d). The total color difference (ΔE), which was a combination of the L*, a*, and b* values, was a colorimetric parameter extensively used to characterize the variation in colors depending on processing conditions. The hue angle values of the samples in all treatment groups followed the same pattern as the a* values (Figure 2e). Browning indexes (BI) in all treatment groups increased marginally from sample A to D (Figure 2f). A similar trend was also observed for ΔE and chroma values (Figure 2g) in all treatment groups. The chroma value of sample A was dramatically different from 12.29 ± 0.32, 13.31 ± 0.58, and 16.52 ± 0.37 to 18.87 ± 0.26. The primary criterion that the consumers consider about a product is its appearance; color has been considered to have a key role in the choice of food, food preference and acceptability, and may even influence taste thresholds, sweetness, perception, and pleasantness [25].

A previous study concluded that color change had the highest correlation with peptides, followed by free amino acids [29]. This correlation revealed that peptides and free amino acids were more associated with the Maillard reaction [29]. The reaction of aldose sugars and sugar degradation products with available amino groups leads to the formation of brown compounds [30]. The Maillard reaction, which is the reaction of carbohydrates with amino acids or proteins, is a consequence in many heated, dried, and stored foods. It leads to the development of flavoring compounds and browning products, as well as the formation of antioxidants that delay or inhibit food lipid oxidation and protein cross-linking [30,31].

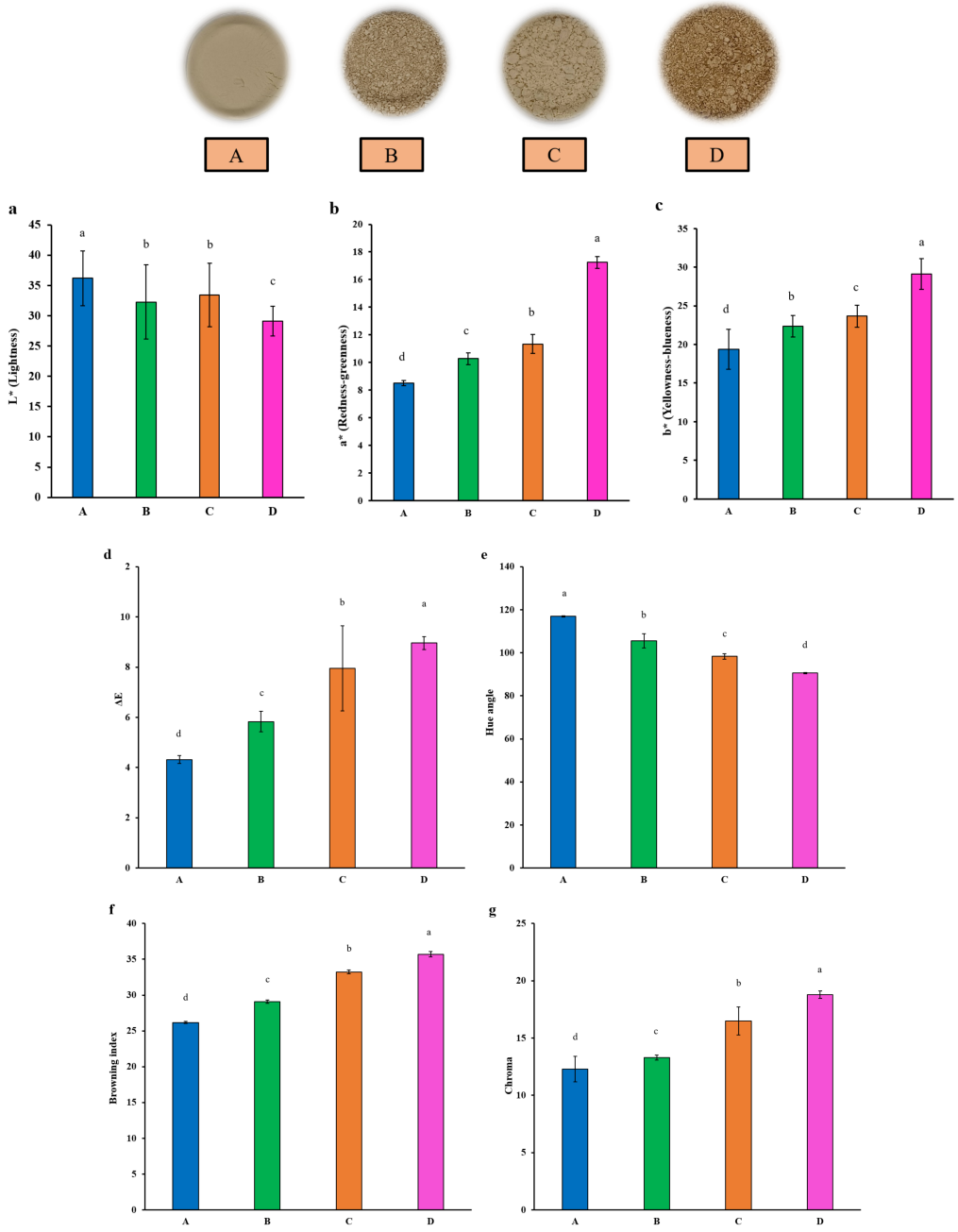


Figure 2. Appearance of the rice protein, and the (**a**) color in terms of L^*, (**b**) a^*, (**c**) b^* value; (**d**) total color difference (ΔE); (**e**) hue angles; (**f**) browning index; and (**g**) chroma. Lowercase letters in the same column indicate significant differences according to Duncan's multiple range tests ($p < 0.05$).

3.2. Amino Acid Composition of RPI

During the processing reaction, amino acids undergo thermal degradation, grafting with reducing sugars to form volatile compounds, and peptide cross-linking [10]. Given that the Maillard reaction is a process that uses up amino acids, the composition of the amino acid in the solution determines the balance between peptide and amino acid degradation and peptide cross-linking. Hence, the extent of the Maillard reaction and the degradation of amino acids can be better comprehended by quantifying the composition of amino acids. Table 1 presents the results of the amino acid composition analysis of the rice protein samples. The results revealed that rice protein contained eighteen amino acids, including eight essential amino acids, eight nonessential amino acids, and two partially essential amino acids. The total amino acid composition of samples A, B, C, and D were 66.41, 81.77,

86.5, and 90.73 g/100 g, respectively. Leucine (Leu) (6.10 ± 0.18 g/100 g), aspartic acid (Asp) (8.37 ± 0.21 g/100 g), proline (Pro) (11.16 ± 0.15 g/100 g), and glutamic acid (Glu) (16.92 ± 0.24 g/100 g) were relatively abundant for sample D. These results corresponded to the finding of a previous study [22].

Table 1. Amino acid composition of rice protein.

Amino Acid (g/100 g Protein)	A	B	C	D
		Essential amino acids		
Leucine	4.62 ± 0.08 ^{c}	5.65 ± 0.14 ^{b}	5.84 ± 0.27 ^{ab}	6.10 ± 0.18 ^{a}
Lysine	1.96 ± 0.11 ^{b}	2.36 ± 0.10 ^{ab}	2.43 ± 0.21 ^{a}	2.51 ± 0.14 ^{a}
Phenylalanine	3.37 ± 0.17 ^{c}	4.23 ± 0.23 ^{ab}	4.49 ± 0.18 ^{ab}	4.76 ± 0.09 ^{a}
Threonine	3.18 ± 0.12 ^{b}	3.84 ± 0.21 ^{ab}	3.95 ± 0.22 ^{a}	4.08 ± 0.10 ^{a}
Valine	3.07 ± 0.11 ^{c}	4.01 ± 0.14 ^{b}	4.37 ± 0.31 ^{b}	4.75 ± 0.18 ^{a}
Methionine	1.67 ± 0.25 ^{ab}	1.77 ± 0.17 ^{ab}	1.81 ± 0.25 ^{a}	0.94 ± 0.13 ^{c}
Tryptophan	0.64 ± 0.23 ^{c}	0.85 ± 0.21 ^{b}	0.95 ± 0.15 ^{a}	1.02 ± 0.08 ^{a}
Arginine	3.69 ± 0.18 ^{d}	4.23 ± 0.38 ^{c}	5.14 ± 0.19 ^{b}	5.98 ± 0.08 ^{a}
		Nonessential amino acids		
Tyrosine	2.73 ± 0.33 ^{c}	3.52 ± 0.12 ^{b}	3.81 ± 0.13 ^{ab}	4.11 ± 0.25 ^{a}
Cysteine	1.12 ± 0.25 ^{a}	1.21 ± 0.11 ^{a}	1.13 ± 0.26 ^{a}	1.07 ± 0.27 ^{ab}
Aspartic acid	3.74 ± 0.25 ^{d}	5.88 ± 0.11 ^{c}	7.16 ± 0.34 ^{b}	8.37 ± 0.21 ^{a}
Serine	3.36 ± 0.18 ^{c}	4.03 ± 0.27 ^{ab}	4.12 ± 0.38 ^{a}	4.24 ± 0.40 ^{a}
Glutamic acid	17.29 ± 0.31 ^{b}	18.82 ± 0.16 ^{a}	17.68 ± 0.21 ^{b}	16.92 ± 0.24 ^{c}
Proline	6.87 ± 0.21 ^{d}	9.19 ± 0.12 ^{c}	10.18 ± 0.29 ^{b}	11.16 ± 0.15 ^{a}
Glycine	2.60 ± 0.26 ^{d}	3.67 ± 0.14 ^{c}	4.18 ± 0.27 ^{b}	4.69 ± 0.17 ^{a}
Alanine	2.27 ± 0.11 ^{d}	3.16 ± 0.16 ^{bc}	3.59 ± 0.17 ^{b}	4.00 ± 0.15 ^{a}
		Partially essential amino acids		
Histidine	1.25 ± 0.26 ^{d}	1.67 ± 0.21 ^{c}	1.85 ± 0.14 ^{ab}	2.03 ± 0.18 ^{a}
Isoleucine	2.98 ± 0.15 ^{d}	3.68 ± 0.33 ^{c}	3.82 ± 0.33 ^{b}	4.00 ± 0.24 ^{a}
Total amino acids	66.41 ± 0.13 ^{d}	81.77 ± 0.24 ^{c}	86.5 ± 0.05 ^{b}	90.73 ± 0.10 ^{a}

Lowercase letters in the same column indicate significant differences according to Duncan's multiple range tests ($p < 0.05$).

Sample A, which is the RPI, exhibited the lowest amino acid composition compared with samples B, C, and D. After the enzymatic and HPP treatment, the content of amino acids, except for those of methionine, cysteine, and Glu, significantly increased.

Supplements of the BCAAs isoleucine (Ile), valine (Val), and Leu may be advantageous for counteracting muscle atrophy. The enzymatic and HPP treatment effectively increased the content of Ile from 2.98 ± 0.16 g/100 g (sample A) to 4.00 ± 0.24 g/100 g (sample D), Val from 3.07 ± 0.11 g/100 g (sample A) to 4.75 ± 0.18 g/100 g (sample D) g/100 g, and that of Leu from 4.62 ± 0.08 g/100 g (sample A) to 6.10 ± 0.18 g/100 g (sample D) g/100 g. The enzymatic hydrolysis of proteins is an effective tool for the modification of the functional properties of proteins for use in specific food applications. Furthermore, the enzymatic hydrolysis of proteins can enhance solubility, as well as other physicochemical properties, and may lead to the generation of bioactive peptides [32]. A previous study also concluded that HPP essentially boosted the digestibility of proteins via the proteolysis of peptide bonds and yielded low-molecular weight peptides with enhanced therapeutic properties for metabolic- and oxidative-stress-related diseases [33].

3.3. Protein and Peptide Content of RPI

Figure 3 illustrates the protein, BCAA, and peptide contents of the rice protein samples subjected to different processing methods. The protein contents of samples A, B, C, and D significantly differed. The protein contents of samples A, B, C, and D were 2214.13 ± 72.53, 2503.07 ± 69.54, 2912.05 ± 73.15, and 3120.31 ± 42.15 mg/mL, respectively. Meanwhile, the BCAA contents of samples A (10.25 ± 0.72 nmol/mg), B (13.51 ± 0.86 nmol/mg),

C (14.25 ± 0.65 nmol/mg), and D (15.12 ± 1.03 nmol/mg) significantly differed. As measured by the OPA method, sample D (31.25 ± 0.55 mM) had the highest peptide content, followed by sample B (26.29 ± 0.31 mM), sample C (21.23 ± 0.46 mM), and sample A (14.32 ± 0.36 mM). In short, the enzyme hydrolysis and HPP treatment successfully increased the protein, BCAA, and peptide contents of rice protein. Nonthermal technology (HPP treatment) affects the denaturation and aggregation of protein and peptides by altering the conformation and coagulation of their native structures through the disruption of interactive forces, mainly hydrophobic and electrostatic bonds [33]. A previous study on legume protein samples concluded that HPP treatment promotes the unfolding of peptide chains and the suppression of hydrophilic groups. The effects are not only helpful in increasing the emulsifying activity index values, but also the protein content of samples [23].

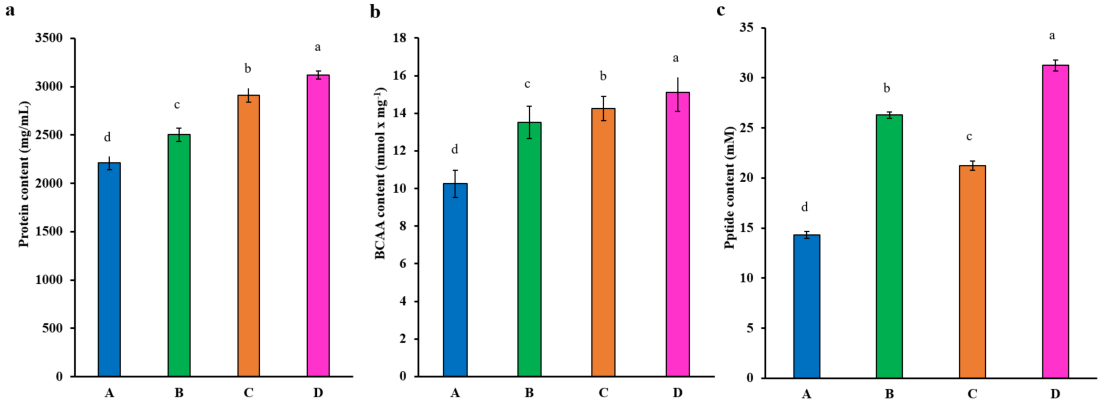


Figure 3. (**a**) Protein, (**b**) BCAA, and (**c**) peptide contents of rice protein. Lowercase letters in the same column indicate significant differences according to Duncan's mul-tiple range tests ($p < 0.05$).

3.4. WSI and WHC

The WSI and WHC of rice protein samples treated at different temperatures are shown in Figure 4. The results indicated that as the treatment temperature was increased, the solubility of the rice protein samples also increased. Meanwhile, the WHC of the rice samples also increased gradually with the treatment temperature. Sample D, which was subjected to enzymatic and HPP treatment, showed the highest WSI and WHC when compared with samples A, B, and C. Thermal processing based on structural destruction is commonly used to enhance digestibility and functionality and enhance the microbiological stability of proteins. Nevertheless, the high temperatures and long treatment times employed in traditional heating systems may lead to a notable reduction in quality sensory attributes as reflected by off-flavors and color degradation and in nutritional values [33]. Rice proteins are plant proteins with a high nutritional value but limited industrial applications due to their poor water solubility. The present study identified a valuable rice protein isolation method that can increase the solubility of rice protein. The HPP treatment at pressures up to 400 MPa increased the surface hydrophobicity of legume protein. Meanwhile, further pressure treatment reduced hydrophobicity [23]. Hence, the appropriate treatment pressure will affect the WSI and WHC of the samples effectively.

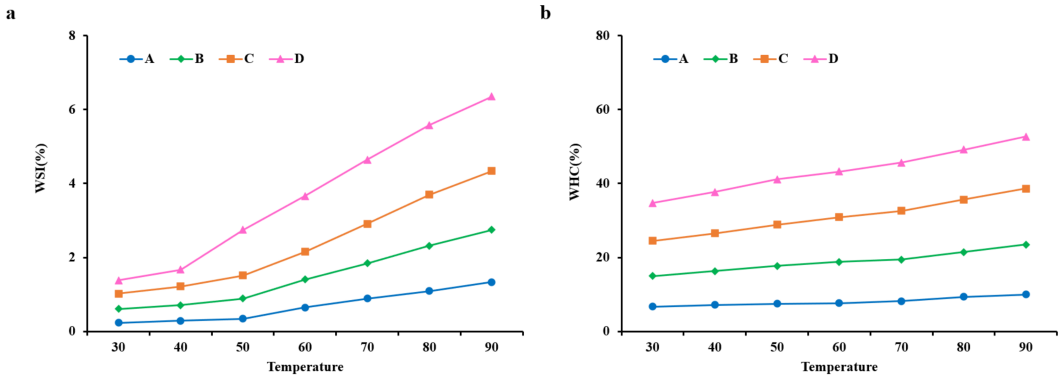


Figure 4. (**a**) WSI, and (**b**) WHC of rice protein.

3.5. Sulfhydryl and S-S Contents

Figure 5 shows that samples A, B, C, and D had the FSH contents of 3.26 ± 0.11, 4.38 ± 0.24, 5.27 ± 0.33, and 6.39 ± 0.64 mmol/g, respectively, and the S-S contents of 28.15 ± 1.35, 24.32 ± 1.55, 20.18 ± 0.95, and 17.63 ± 1.26 mmol/g, respectively. The S-S content of sample A was markedly higher than that of other samples due to the exposure of hidden groups, for instance, the hidden thiol groups, disulfide bonds, and hydrophobic groups inside protein molecules, during protein unfolding [16]. The oxidation of free S-H into S-S may also function in the reduction in free S-H content and the increases in S-S bonds [16]. A previous study concluded that the hydrogen bonds were seen to be destabilized due to the HPP treatment, while the SH groups and S-S bonds showed the dissociation and refolding of proteins during the treatment [23]. In this study, the FSH content of the samples increased. By contrast, the S-S contents markedly decreased from samples A to D. S-S bonds play an important role in maintaining the spatial three-dimensional structure of peptides and proteins. Hence, the assessment of the FSH group and S-S contents of proteins plays a vital role in the evaluation of the higher-level structure of proteins for elucidating protein biological functions.

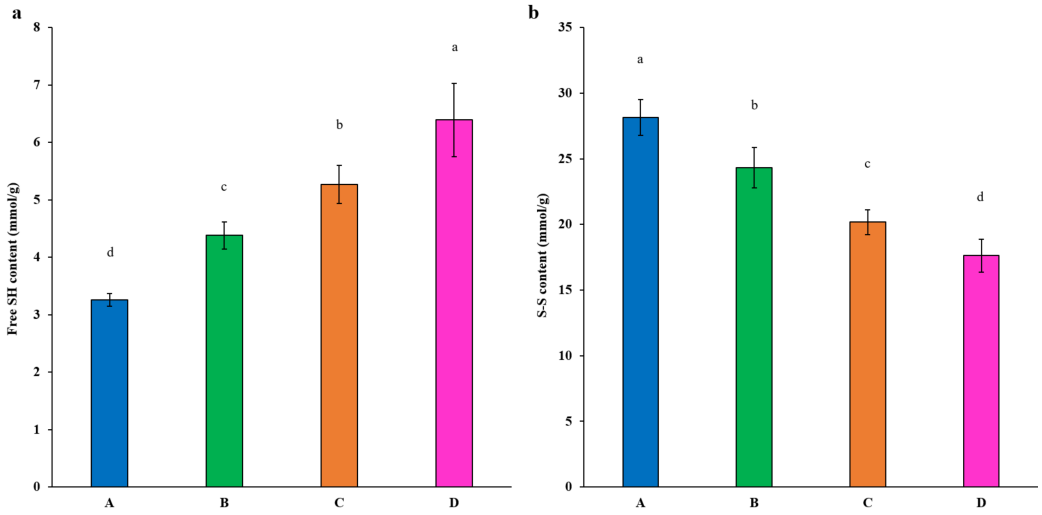


Figure 5. (**a**) Sulfhydryl and (**b**) S-S contents of rice protein. Lowercase letters in the same column indicate significant differences according to Duncan's multiple range tests ($p < 0.05$).

3.6. In Vitro Antioxidant Capacity

The results of the antioxidant activities of rice samples are depicted in Figure 6. DPPH and ORAC assays were used to evaluate the antioxidant capacity of the rice proteins produced through different isolation methods. In the DPPH test, sample D (26.33 ± 2.29 μmol TEAC/g) exhibited the highest free radical scavenging activities, fol-

lowed by sample C (22.31 ± 1.58 µmol TEAC/g), B (19.25 ± 1.37 µmol TEAC/g), and A (17.38 ± 2.15 µmol TEAC/g). Meanwhile, sample D (106.52 ± 9.35 µmol TEAC/g) showed the highest ORAC activities, no significant difference was found between samples C (93.29 ± 4.18 µmol TEAC/g) and B (85.53 ± 6.47 µmol TEAC/g), and sample A (76.35 ± 6.82 µmol TEAC/g) had the lowest content. High-pressure treatments improved accessibility for proteolysis and ensured a better hydrolysis rate and the maximum release of antioxidant peptides (51.26% at 500–1000 Da) [23]. The antioxidant properties of the legume protein hydrolysates generated by the HPP-assisted enzymatic treatment can be attributed to the low molecular weight of small peptide fractions. Moreover, the HPP-assisted enzymatic treatment increases surface areas and enables the release of phenolic compounds with a hydrogen-donating capacity that can therefore act as antioxidants [24]. Small peptides with molecular weight < 3 kDa have potential antioxidant properties that can prevent oxidative stress-related chronic diseases. Apart from the difference in the peptide contents of protein hydrolysates obtained from different legumes, the type of enzyme used and the different parameters of HPP may produce protein hydrolysates with dissimilar peptide contents from the same source [23].

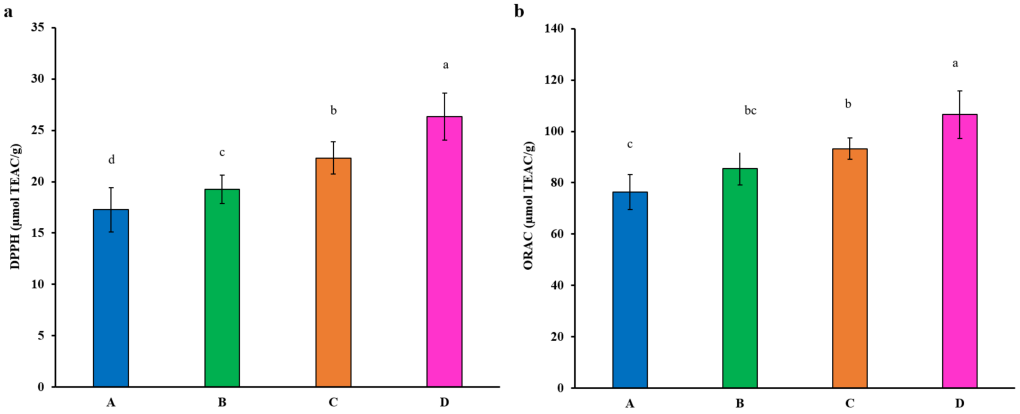


Figure 6. Antioxidant capacity of rice protein samples, (**a**) DPPH, and (**b**) ORAC assays. Lowercase letters in the same column indicate significant differences according to Duncan's multiple range tests ($p < 0.05$).

3.7. Rice Protein Protects C2C12 Myotubes against DEX-Induced Atrophy

The C2C12 mouse muscle myoblast cell line is a subclone of the mouse myoblast cell line established from the normal adult C3H mouse leg muscle. These cells differentiate rapidly and generate extensively contracting myotubes that express characteristic muscle proteins. Meanwhile, BCAAs can prevent and/or improve age-related muscle atrophy, such as sarcopenia. Therefore, in this study, the BCAA was used as the positive control group. Table 2 summarizes the cell viability of C2C12 myotubes treated with different concentrations of rice protein samples. At the concentration of 50 mg/mL, all samples, except for sample D (96.8% ± 2.9%), showed no significant difference from the standard BCAA. Moreover, all samples showed no significant differences at the concentration of 100 mg/mL. At the concentration of 200 mg/mL, samples A (100.3% ± 1.2%) and C (100.9% ± 0.6%) showed no significant difference from the standard BCAA (103.0% ± 0.4%). The cell viabilities under treatment with samples B and C at the concentration of 200 mg/mL were 96.3% ± 0.4% and 87.4% ± 0.2%, respectively. In the follow-up experiments, rice protein was used at a concentration of 200 mg/mL for the intervention.

Figure 7a illustrates the cultured C2C12 myotubes, where the blank represents normal C2C12 cells; the CON group represents the cells under 5 mM DEX induction and BCAA treatment; the DEX group is the C2C12 cells induced by DEX; and (A), (B), (C), and (D) are the DEX-induced C2C12 cells treated with 200 mg/mL samples A, B, C, and D, respectively. In the morphological analysis, periodic acid Schiff (PAS) staining was applied to visualize C2C12 myotubes. Figure 7b presents a typical PAS stain of C2C12-derived myotubes. PAS-

positive staining was restricted to myotubes and excluded nonfused myoblasts. Moreover, it helped distinguish between myotubes of different sizes clearly. Nuclei were counterstained with hematoxylin. Note the high number of nuclei in the large myotubes and the strong contrast of the PAS stain restricted to myotubes, in contrast to the unstained nonfusing myoblasts. Many studies have confirmed that DEX decreases the diameter and myosin heavy chain expression of C2C12 myotubes, which are recognized as the representative phenotype modifiers of muscle atrophy [34]. Figure 7c,d indicate that supplementation with rice protein prevented the DEX-induced reduction in myotube diameter. The length of the DEX-induced C2C12 myotubes had decreased to 30.3 ± 0.7 nm relative to that of the blank (77.2 ± 0.9 nm). After treatment with BCAA (con) and samples A, B, C, and D, the length of the myotubes increased to 78.0 ± 0.7, 57.8 ± 2.0, 66.4 ± 1.5, 56.3 ± 8.6, and 53.3 ± 0.5 nm, respectively.

Table 2. Cell viability of C2C12 myotubes treated with different concentrations of rice protein samples.

	Cell Viability (% of CON)				
	Mean ± SD				
	50 mg/mL	**100 mg/mL**	**200 mg/mL**	**500 mg/mL**	**1000 mg/mL**
BCAA	100.7 ± 0.8	101.1 ± 2.9	103.0 ± 0.4	84.9 ± 1.3	75.8 ± 1.1
A	101.4 ± 1.3	100.9 ± 2.8	100.3 ± 1.2	103.5 ± 0.6	98.7 ± 0.1
B	101.4 ± 1.4	100.6 ± 0.1	96.3 ± 0.4	87.5 ± 1.0	81.6 ± 0.0
C	101.9 ± 0.0	102.6 ± 0.7	100.9 ± 0.6	87.3 ± 0.5	75.6 ± 4.0
D	96.8 ± 2.9	101.5 ± 5.0	87.4 ± 0.2	69.3 ± 0.2	47.0 ± 0.0

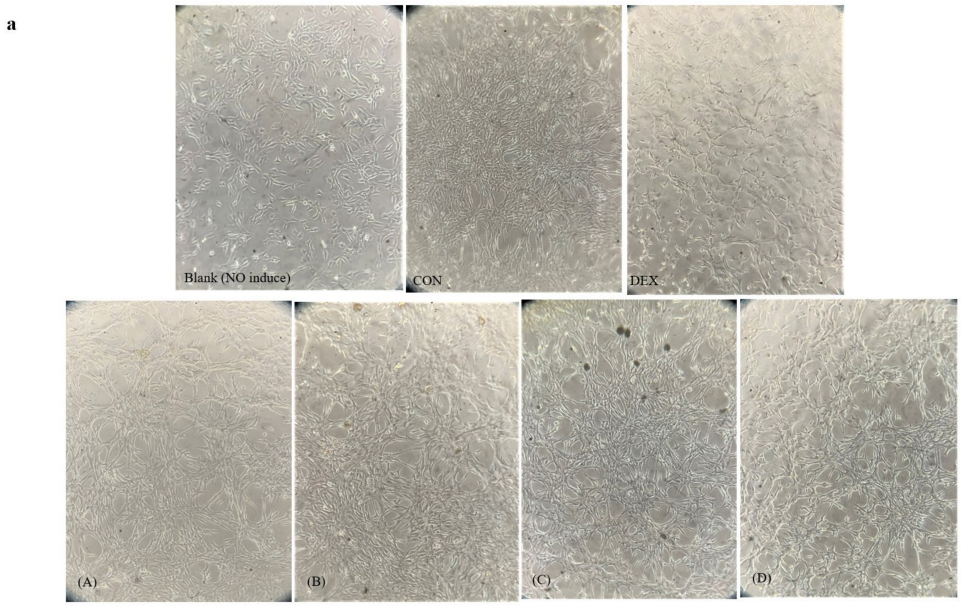


Figure 7. *Cont.*

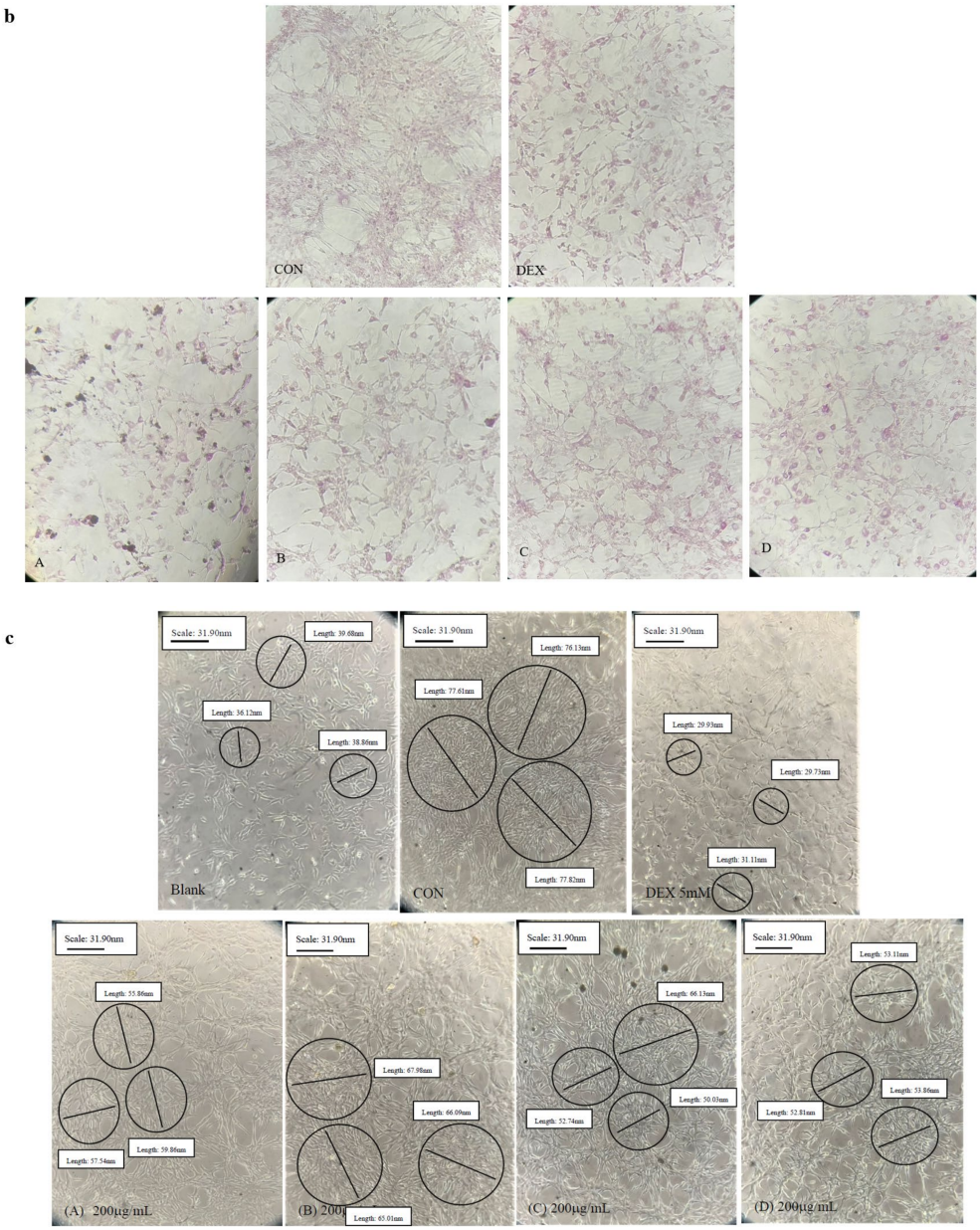


Figure 7. *Cont*.

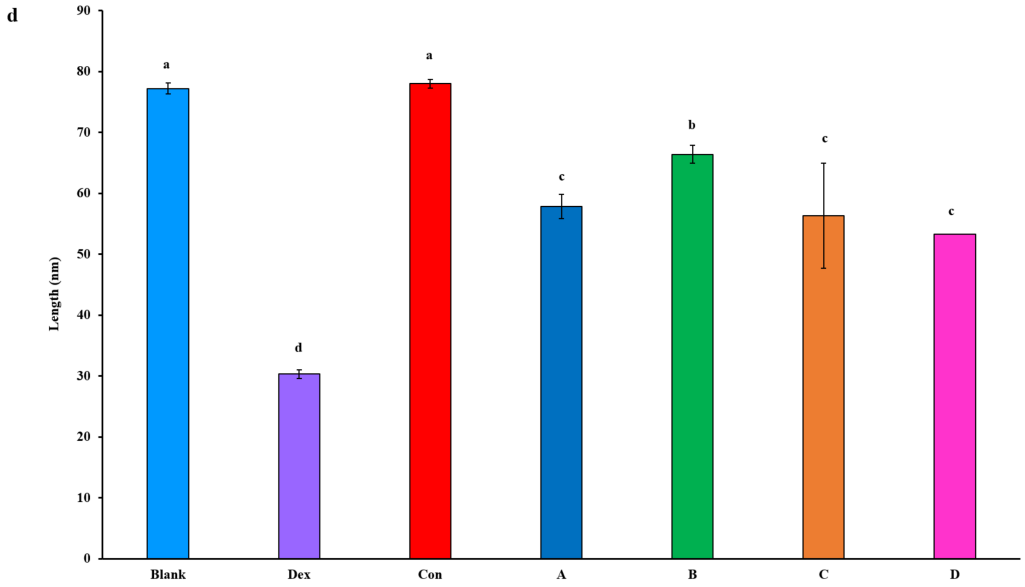


Figure 7. (**a**) Morphological observation of C2C12 myotubes; (**b**) PAS staining of C2C12 myotubes; (**c**,**d**) quantification of C2C12 myotubes. Blank = normal C2C12 cells; CON = DEX-induced and treated with BCAA; DEX = C2C12 cells induced by DEX; (**A**–**D**) = DEX-induced C2C12 cells and treated with 200 mg/mL of samples A, B, C, and D. Lowercase letters in the same column indicate significant differences according to Duncan's multiple range tests ($p < 0.05$).

The mechanical stretching of the skeletal muscle stimulates nitric oxide (NO) production and is a crucial stimulator of satellite cell proliferation. The results in Figure 8 showed that sample D effectively reduced NO production by 46.6%. Therefore, it reduced NO production more efficiently than the BCAA, which reduced NO production by 42.4%.

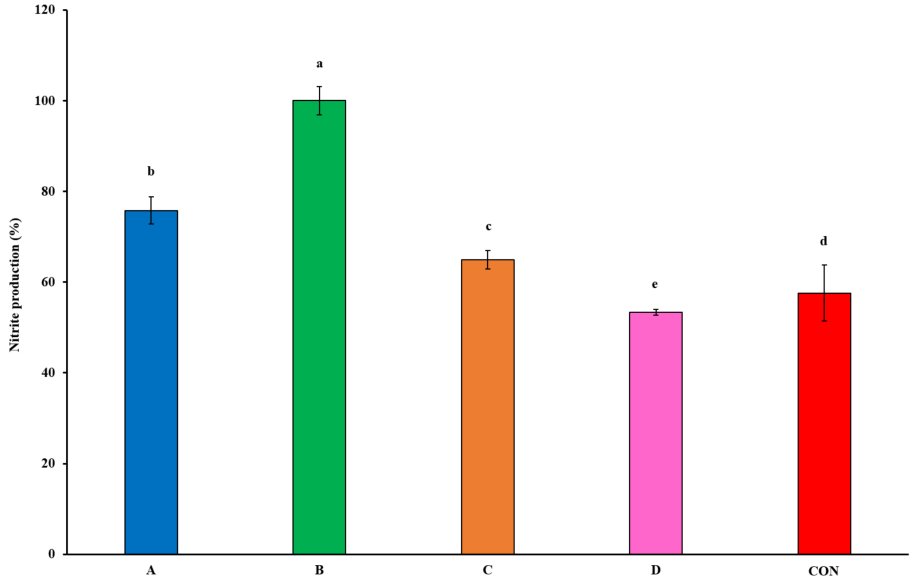


Figure 8. NO production in C2C12 myotubes. Lowercase letters in the same column indicate significant differences according to Duncan's multiple range tests ($p < 0.05$).

Skeletal muscle tissue is the major protein/amino acid reservoir in the human body. It is not only crucial for locomotion, but it also represents the largest metabolically active tissue in the body and acts as the glucose disposal site and fuel reservoir for other organs under fasting and pathological conditions, as illustrated by the hepatic supply of amino

acids for gluconeogenesis. Nutrients (amino acids) and nutrient-derived hormones, such as insulin, play an important role in controlling the balance between muscle protein synthesis and muscle protein breakdown [35].

A previous study concluded that the BCAAs Leu and Val suppressed the loss of body weight in NMRI mice bearing cachexia-inducing tumors (MAC16), significantly increasing the wet weight of the skeletal muscle by increasing the protein synthesis and decreasing degradation [36].

4. Conclusions

The inhibitory activities of the rice protein samples against DEX-induced muscle atrophy in C2C12 myotubes were investigated. Sample D, which was subjected to enzyme and HPP treatment, effectively improved amino acid composition and increased protein, BCAA, and peptide contents. It also had higher WHC and WSI, as well as a significantly higher antioxidant capacity in terms of DPPH and ORAC, than the other samples. In the C2C12 model of DEX-induced muscle atrophy, the sample processed through enzymatic hydrolysis and HPP not only increased the myotube length, but also effectively decreased NO production. These findings can be used to create novel rice protein products with superior WHC and WSI, and increase the protein, BCAA, peptide contents, and functional properties. Furthermore, these results suggested that rice protein produced through enzymatic hydrolysis and HPP treatment may provide protective effects against muscle atrophy, making it a potential candidate for the development of anti-sarcopenia healthy functional foods. Yet, there are some other enzymes which play a variety of roles in regulating physiological functions in C2C12 cells, such as sirtuin 1 (SIRT1), and it is possible the SIRT1-related signaling pathway in protein proteolysis, autophagy, and apoptosis may need to be further studied to understand the molecular mechanism of rice protein in the anti-sarcopenia effect.

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