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Purification and Characterization of Antioxidant Peptides and Enzymatic Hydrolysates from Rice Bean *(Vigna umbellata)*

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ABSTRACT

Higher intake of plant protein is associated with lower risk in degenerative diseases caused by oxidative stress, thereby placing enormous demand in the identification of plant-based sources of protein like legumes. Rice bean (*Vigna umbellata*) is known to be a great source of low-cost and good nutritional quality protein for utilization in food products. This study characterized the soluble and hydrolyzed proteins from rice beans and assessed their antioxidant properties *in vitro*. The total soluble proteins from the rice bean were extracted using 35 mM potassium phosphate buffer (pH 7.2 M, 0.40 M NaCl) affording a concentration of 1.23 mg/mL. The isolated proteins were purified by a combination of gel filtration chromatography, ammonium sulfate precipitation and dialysis. SDS-PAGE was performed to monitor the changes in the protein profile after every purification step. Enzyme hydrolysis was also done using pepsin and a mixture of trypsin, chymotrypsin and thermolysin. The antioxidant activity of the protein extract and digest after 4 h of hydrolysis were determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay, and found to be 35.11% and 19.68%, respectively. The results indicate that rice beans can be utilized as a rich source of protein with antioxidant properties and can be a rich source of functional foods.

Keywords: *Vigna umbellata, antioxidant activity, protein, hydrolysate*

INTRODUCTION

The lack of antioxidant defense system promotes oxidative stress which is correlated to cognitive and biological degeneration such as physical frailty, psychological impairment, and cognitive decline (Tan et al., 2018; Wang et al., 2018). Consequently, there had been a demand for the determination of dietary proteins from plant sources as it had been previously reported to display

significant antioxidant activity (Tan et al., 2018), thereby offering beneficial effects in repressing oxidative stress (Zou et al., 2016).

Increased interest in plant proteins led to evaluating legumes. Legumes are not only cheap and popular food for many people, but also have a higher content of proteins than vegetables and fruits (Creighton, 1993). The nutritional benefits of some legumes such as yellow string beans (*Phaseolus vulgaris* L.) containing essential amino acids, are attributed to their high-quality proteins and peptides (Matemu et al., 2021; Karaś et al., 2012). Many studies on the potential antioxidative activities of legume-derived protein hydrolysates and peptides showed a link between reducing the risks of cancer and cardiovascular diseases and their potent action against oxidation and inflammation (Gupta et al., 2018; Wen et al., 2022; Matemu et al., 2021; Karaś et al., 2012). Bioactive peptides in legume-derived hydrolysates are abundant such as our previous discovery of antihypertensive peptides from mung bean (Viernes et al., 2012).

Biofunctional peptides and hydrolyzed proteins may be produced by many methods such as enzymatic hydrolysis, chemical hydrolysis or microbial fermentation (Matemu et al., 2021; Esfandi et al., 2019). The enzymatic hydrolysis is the acceptable method in the food industry. Aside from the type of enzyme and enzyme-to-substrate ratio, there are other factors affecting the resulting peptide sequence and their biological properties such as substrate concentration, incubation duration, temperature, pH, and source of proteins (Tkaczewska et al., 2020; Matemu et al., 2021; Esfandi et al., 2019). Thus, in different enzymatic hydrolysis conditions, the biological activity, such as antioxidative activity, of purified proteins would also vary.

Rice bean (*Vigna umbellata*) was previously identified as a good source of protein, with a well-balanced amino acid composition for human consumption (Sritongtae et al., 2017). However, its commercial importance and demand remain undervalued (Katoch, 2012). Rice bean is particularly rich in methionine, lysine, tyrosine and valine (Katoch, 2012; Sritongtae et al., 2017). Based on the study by Sritongtae et al. (2017), the chemical composition of selected 16 rice bean genotypes were reported as follows: crude protein (23.17 – 25.57%), lipids (1.92 – 3.42%), dietary fiber (4.11 – 5.56%), carbohydrates (52.23 – 55.65%), ascorbic acid (15.14 – 29.19 mg/100 g), and niacin (3.48 – 4.28 mg/100 g). While the phenolic compounds of rice beans have been tested for antioxidant activity (Yao et al., 2012), little work has been performed on the determination of the antioxidant potential of *V. umbellata* proteins. Therefore, it is essential to isolate, purify, characterize, and evaluate the *V. umbellata* protein extract for antioxidant activity.

METHODS

Materials and Equipment. Rice bean (*V. umbellata*) seeds were obtained from the Institute of Plant Breeding, University of the Philippines Los Baños. Sodium dodecyl sulfate (SDS) (JT Baker), Coomassie brilliant blue R-250 (Sigma Aldrich), bovine serum albumin (BSA), and enzymes such as pepsin (Merck), trypsin (Sigma Aldrich), chymotrypsin (Sigma Aldrich), and thermolysin (Sigma Aldrich), were obtained from the Biochemistry Laboratory, University of the Philippines Los Baños and used as received.

Sample preparation. The seeds were air-dried at room temperature and manually dehulled using scalpel. The dehulled seeds were ground using a laboratory grinder and defatted with *n*-hexane (1.0 g ground seeds:10 mL hexane) for an hour in an ice bath with constant stirring. The resulting mixture was allowed to stand for 3 mins. The solvent was removed by decantation. The residue was then air-dried and stored at 4 °C until use.

Crude protein extraction and characterization. Two sets of dried, ground, and defatted sample were extracted using two buffers: 50 mM Tris-HCl (pH 7.2) and 35 mM phosphate buffer (pH 7.2) with 0.4 M NaCl, according to the method of Kortt (1986) with minor modifications. In a 14 g

sample, a 300 mL buffer was added and stirred in an ice bath for 1 h. The mixtures were filtered using Whatman No. 1 filter paper. The crude extracts were collected and stored at 4 °C until use.

The protein concentration of the crude extracts were determined using Bradford (1976) assay in triplicates. The crude extracts were also subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a Bio-Rad mini gel electrophoresis apparatus according to the method of Laemmli (1970). Samples were first mixed with the sample buffer at 2:1 v/v ratio. The run was carried out in 4% stacking gel and 12.5% separating gel at 200 V for 30 min. The gel was stained using 0.05% Coomassie Brilliant Blue R-250 for 30 min and destained using a solution of methanol and glacial acetic acid until the bands were distinct.

Protein purification. To partially purify the crude extract, gel filtration chromatography (GFC) was conducted using Sephadex G-150 column. The column was eluted at a flow rate of 1.5 mL/min. Thirty fractions were collected. The absorbance of the collected fractions were monitored at 280 nm using the Bio-Rad Spectrophotometer. Fraction sequence was plotted against absorbance reading to reveal peaks that correspond to a particular type with specific molecular weight. From 30 fractions collected, the protein concentrations of fractions 1, 4 and 6 were evaluated using SDS-PAGE at 110 V for 2 h.

GFC fractions 1-14 were pooled and precipitated by ammonium sulfate precipitation (ASP) at 30%, 60%, and 90% saturation (Scopes, 1994). The precipitate and supernatant at each saturation point were separated by centrifugation at 10,000 rpm for 10 min at 4 °C. Following the globulin precipitate collection of Gupta et al. (2018) and Viernes et al. (2012) specific for extraction of major storage protein of beans, the precipitate was then dissolved in 1 mL distilled water and dialyzed in a bag with pore size of 14 kDa against distilled water with 10 mM β-mercaptoethanol. The ASP precipitate and supernatants together with the dialysate were subjected to SDS-PAGE at 110 V for 2 h for characterization.

Enzymatic protein digestion. The dialyzed sample was subjected to enzymatic hydrolysis using pepsin, trypsin, chymotrypsin, and thermolysin. An 8.0 mL pepsin (1.0 mg/mL) was added to 8.0 mL of the protein sample. The pH was adjusted to pH 2.0. Digestion was allowed to proceed for 2 h at room temperature before adjusting the pH to 7.0. A mixture of trypsin, chymotrypsin, and thermolysin was then added to the mixture. Digestion was continued and a 2.0 mL aliquot was collected after 1, 2, and 4 h of digestion. Enzymes were inactivated by heating the mixture at 100 °C for 5 min. The efficiency of the enzymatic digestion was monitored using SDS-PAGE at 150 – 180 V for 1 h.

Antioxidant activity. The electron donating ability of 0.5 mg/mL crude extract, dialysate, and hydrolysates were measured by bleaching a purple solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. Ascorbic acid was used as standard. The absorbance of the samples and standards at 517 nm was determined. The percent inhibition was calculated using the formula below. DPPH assay was done in triplicates.

% Scavenging Activity = [(Absorbance blank – Absorbance sample)/Absorbance blank] x 100

RESULTS AND DISCUSSION

Protein extraction and characterization. A technique that can be employed for legume exploitation in preparing rich-in-protein materials is wet protein extraction method (Kortt, 1986). The major storage protein of *Vigna umbellata* was isolated using two buffers: 35 mM phosphate buffer (pH 7.2) with 0.4 M NaCl and 50 mM Tris-HCl (pH 7.2).

The crude extracts isolated using each buffer were assayed to get the concentration using Bradford method. The protein concentration of the crude extracts in phosphate buffer (1.23 mg/mL) and Tris-HCl (1.58 mg/mL) were compared (Figure 1) using Independent Samples T-test. At $\alpha = 0.05$, it was found that there is no significant difference (p-value = 0.415) between the two. Thus, both buffers can be used for the extraction of proteins from *V. umbellata* considering the quantity of protein extracted.

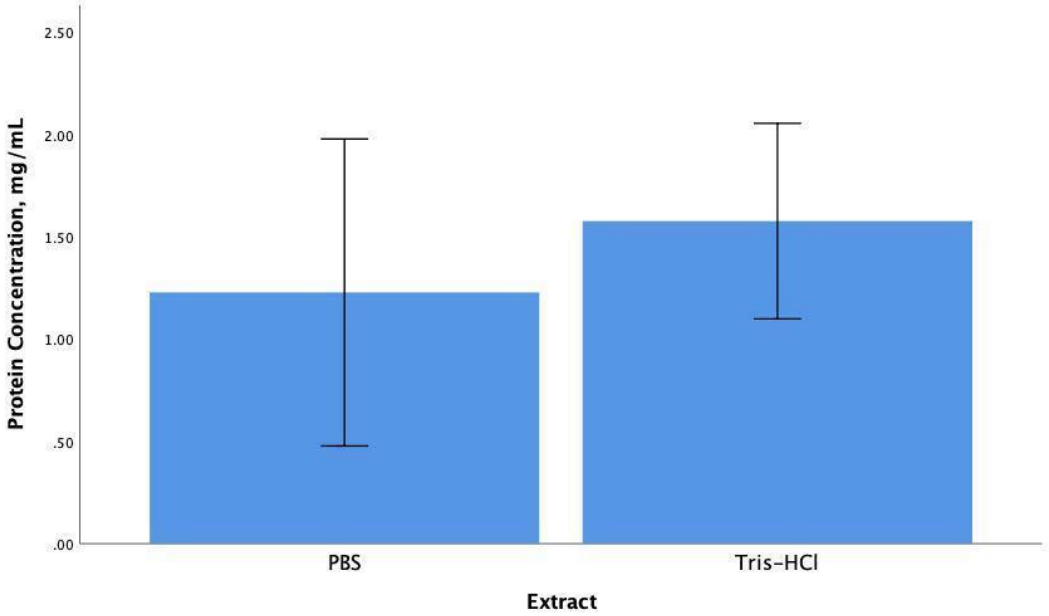

Figure 1. Protein concentrations (mg/mL ± std. dev.) of samples extracted using phosphate buffer (PBS) and Tris-HCl. Error bars display the standard deviation of three independent repeats.

Major storage proteins from *V. umbellata* were extracted in the rice bean crude extract, which was further removed of minor storage proteins via ammonium sulfate dialysis. Afterwards, globulins were selectively precipitated while albumins remained solubilized.

SDS-PAGE was done to compare the crude extracts based on the quality of the protein isolated. Bands were compared against the BSA standard (~66.5 kDa). As shown in Figure 2a, bands corresponding to higher molecular weights are present in crude extracts in the phosphate buffer (PBS T1 – T3). The result also showed that the extracted protein using phosphate buffer had higher molecular weight and quality (i.e., more intact). Thus, the phosphate buffer was selected as the final extracting solvent for the isolation of proteins from *V. umbellata*.

Based on the molecular weight of ~66.5 kDa detected in the SDS PAGE, the major storage protein present in rice beans is likely vicilin. Vicilin found in many beans such as mung and winged beans, has been reported to cover wide range of molecular masses from 14 to 66 kDa in legumes (Quiroga et al., 2012; Ribeiro et al., 2014; Gupta et al., 2018; Viernes et al.; 2012)

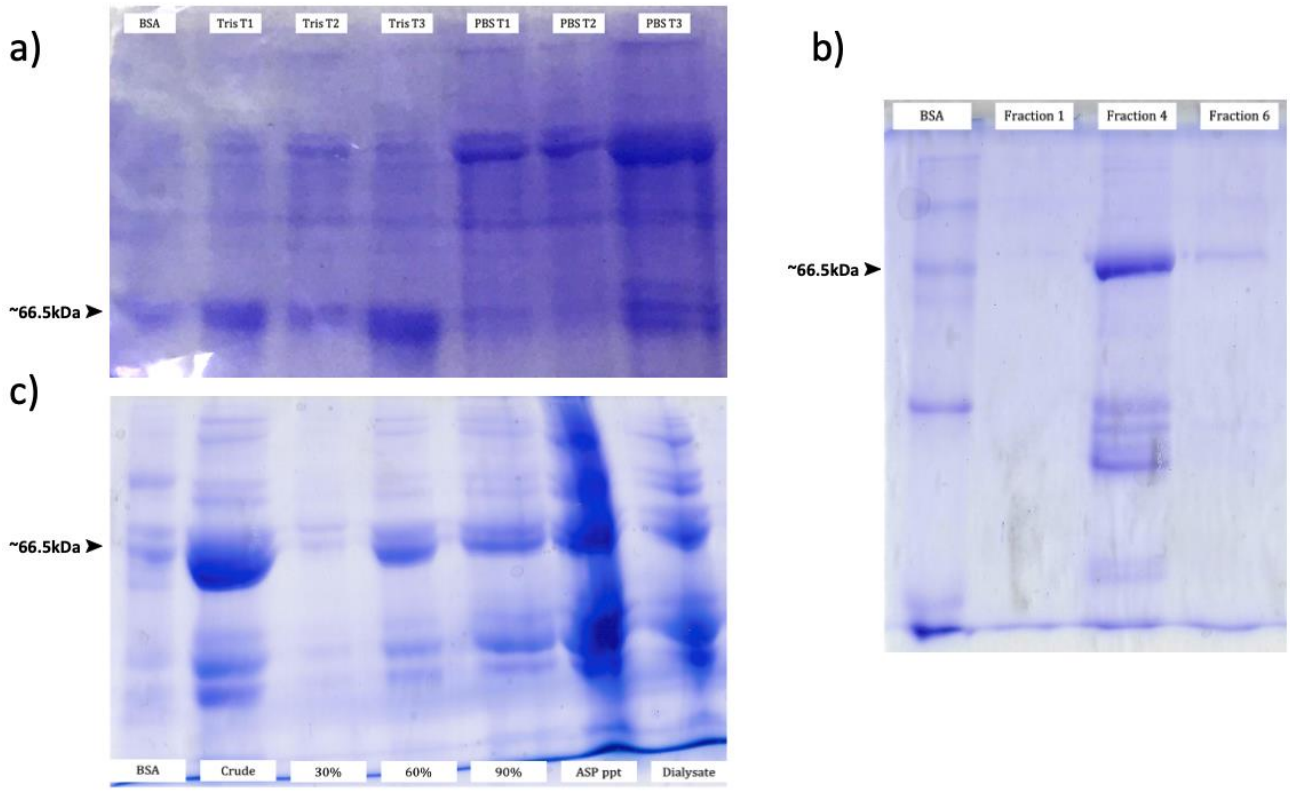

Figure 2. SDS-PAGE gel of (a) proteins extracted using Tris-HCl (Tris T1–T3) and phosphate buffer (PBS T1–T3) against BSA standard (b) GFC Fractions 1, 4 and 6 against BSA standard, and (c) supernatant obtained from selective precipitation (30%, 60%, and 90%), precipitate (ASP ppt), and dialysate of 90% against BSA standard and crude protein.

Protein purification. The crude protein extract was purified by a combination of gel filtration chromatography, ammonium sulfate precipitation and dialysis. GFC is a type of size-exclusion chromatography wherein the analytes are separated based on their size (i.e., hydrodynamic volume) (Moldoveanu and David, 2013). For the stationary phase used here, Sephadex G-150, it has an exclusion limit of 150 kDa; thus, proteins with molecular weights larger than 150 kDa are "excluded" from entering the gel matrix while proteins with lower molecular weights can be separated based on their separation between the inside and outside of the gel matrix (Malhotra and Kumar, 1989). Thirty GFC fractions were collected and the absorbance of the collected fractions were monitored at 280 nm. The elution profile (Figure 3) showed three peaks at Fractions 1, 4 and 6.

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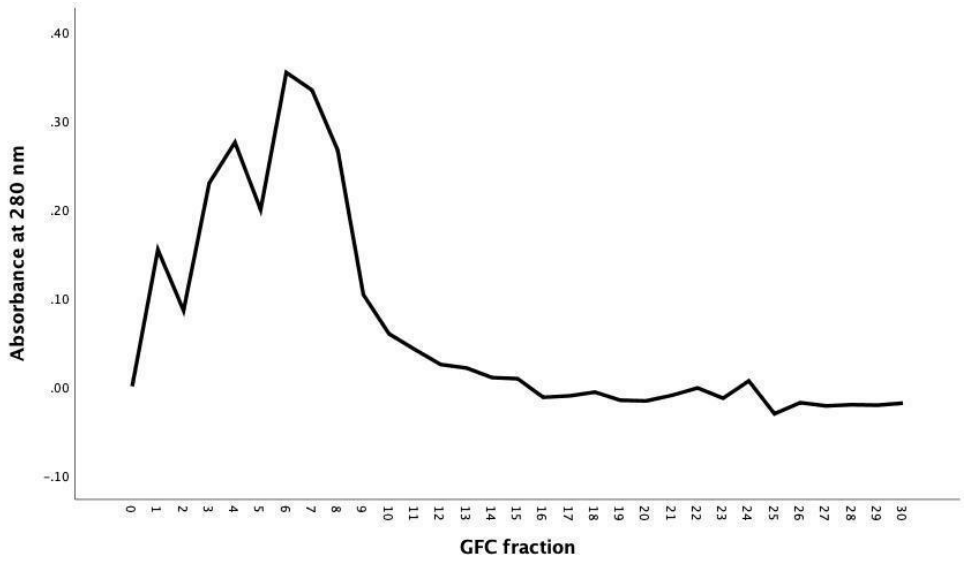

Figure 3. GFC elution profile of the crude protein extract using phosphate buffer. One major peak was observed from the gel filtration chromatography of the precipitated globulin fraction, which corresponds to the purified globulin fraction.

The GFC fractions that gave high absorbances at 280 nm were evaluated using SDS-PAGE. As shown in Figure 2b, fractions 4 and 6 have molecular weights equivalent to BSA. In addition, fraction 4 contained several bands that had much lower molecular weights. However, due to unavailability of a proper protein molecular weight marker, the molecular weights of these additional bands in fraction 4 were not determined. The GFC profile obtained was consistent with other studies extracting major storage proteins from beans (Gupta et al., 2018; Viernes et al., 2012). Although Fraction 1 supposedly contained a peak according to the GFC elution profile, which corresponds to a purified globulin fraction rather than total soluble proteins of rice bean, it did not show any band. Since the staining reagent used, Coomassie G-250 blue dye, binds to positively-charged side chains in amino acids (e.g. lysine, arginine), the proteins in Fraction 1 possibly do not contain these residues. This result is also consistent with the studies of Viernes et al. (2012) and Gupta et al. (2018), which means digestion was fairly complete as the major storage protein, vicilin was converted to very small molecular weight peptides that are not resolved and retained by SDS-PAGE.

For the next step, the fractions 1-14 were pooled together and further purified by selective precipitation by ammonium sulfate or "salting out". In this way, proteins are "salted-out" due to the increasing salt content, such as ammonium sulfate, whose ions compete with the proteins for solvation (Wingfield, 2001). When this happens, the proteins in solution precipitate can be separated using centrifugation.

The optimum ammonium sulfate saturation, which removed most of the minor storage proteins while albumins remain solubilized, was found to be 90% as can be seen on Figure 2c. Furthermore, even with addition of 60% ammonium sulfate, the majority of globulins were already precipitated out. This agrees with a previous study by Viernes et al. (2012), where protein purification was optimum upon addition of 60% ammonium sulfate. After salting-out, dialysis was performed to remove the salt ions in the supernatants, since these could interfere with subsequent analyses.

Enzymatic protein digestion. Prior to further bioassay analyses, the crude protein was digested using several proteases – pepsin, trypsin, chymotrypsin, and thermolysin. The reason for doing so is that antioxidant peptides from food proteins have been documented from a variety of food

sources (Sarmadi and Ismail, 2010). As such, the crude protein was digested with common enzymes: a) pepsin, which cleaves at the C-terminal of aromatic amino acids; b) trypsin, at the C-terminus of basic amino acids; c) chymotrypsin, at the C-terminal of aromatic amino acids; and d) thermolysin, which cleaves at the N-terminal side of leucine, phenylalanine, valine, isoleucine, alanine, and methionine. Using the combination of these peptides, we can obtain peptides with low molecular weights. When the 1 h, 2 h, and 4 h-digests were subjected to SDS-PAGE, there were no protein bands observed. This means that the protein isolated was converted to very small molecular weight peptides that are not resolved and retained by SDS-PAGE.

Antioxidant activity. The antioxidant activities of the crude protein, enzyme digests, and dialysate were determined and compared with the antioxidant activity of ascorbic acid using DPPH scavenging assay. In this assay, the ability of the samples to scavenge and stabilize the DPPH radical in solution gives rise to its antioxidant activity (Plank et al., 2012). Based on the results shown in Figure 4, the crude protein extract (35.11%) had the highest scavenging activity followed by 4 h-digest of enzymatic hydrolysis (19.68%). The reason why the crude protein extract had higher activity than the enzyme digest is because of its higher concentration upon treatment. In order to make this comparison more valid, the protein concentrations should be the same. Nevertheless, this result is noteworthy since antioxidant activity of the crude extract and 4 h-digest have no significant difference with the antioxidant activity of ascorbic acid (29.45%), and due to this being the first report of antioxidant protein and peptides from *V. umbellata.* Previous reports of the bioactivity of *V. umbellata* peptides include antimicrobial (Carvalho et al., 2001), angiotensin-I converting enzyme inhibition (Segura Campos et al., 2010), and dipeptidyl peptidase IV (Rocha et al., 2014), among others.

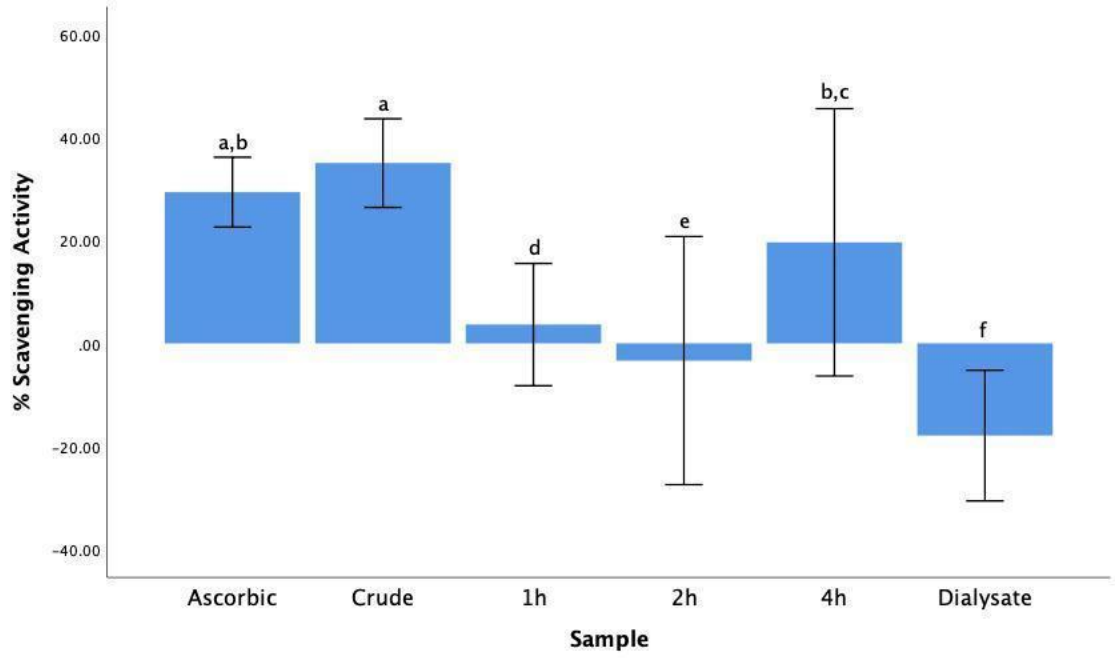

Figure 4. Antioxidant activities (% scavenging activity ± std. dev.) of the crude protein extract, enzyme digests (1h, 2h, and 4h) and dialysate against ascorbic acid standard as determined by DPPH scavenging assay. (Means with same letters are not significantly different at $\alpha = 0.05$; Error bars display the standard deviation of three independent repeats.)

CONCLUSIONS

V. Umbellata have been previously reported to contain a variety of peptides that have beneficial bioactivities. In this study, the protein and peptides of *V. umbellata* were extracted, purified, characterized, and subsequently assayed for *in vitro* antioxidant activity using the DPPH scavenging assay. Based on the results, the crude protein extract and enzyme digest after 4 h of hydrolysis provided the highest antioxidant activity. In depth analyses of antioxidant mechanisms as well as characterization of the antioxidant peptides are suggested.

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