HAYATI
Journal of Biosciences

Vol. 31 No. 1, January 2024 192-199
DOI:10.4308/hjb.31.1.192-199
ISSN: 1978-3019
EISSN: 2086-4094

Study of Antihypertensive Activity from Red Quinoa Seed Protein Hydrolysate Digested by Various Protease Enzymes

Dininurilmi Putri Suleman[2], Harijono[2], Jue-Liang Hsu[1,3,4*]

[1]*Department of Biological Science and Technology, National Pingtung University Science and Technology, Neipu, Pingtung 91201, Taiwan*
[2]*Department of Agricultural Product Technology, Faculty of Agricultural Technology, University of Brawijaya, Malang 65145, Indonesia*
[3]*Research Center for Animal Biologics, National Pingtung University of Science and Technology, Neipu, Pingtung 91201, Taiwan*
[4]*International Master's Degree Program in Food Science, National Pingtung University of Science and Technology, Neipu, Pingtung 91201, Taiwan*

ARTICLE INFO

Article history:
Received April 17, 2022
Received in revised form August 6, 2023
Accepted October 20, 2023

KEYWORDS:
ACE-I inhibitory activity,
Hydrolysate,
Red quinoa seed,
RP-HPLC,
Protein,
Proteases

ABSTRACT

Proteolytic enzymes are widely used to produce protein hydrolysates that contain bioactive peptides. Some of bioactive peptides are known inhibit the angiotensin-I-converting enzyme (ACE, EC 3.4.15.1) and act as human antihypertensive. Therefore, this study aims to produce protein hydrolysates via 16 hours of digestion process using *Chenopodium formosanum* (red quinoa) seed and the proteases, namely pepsin, trypsin, α-chymotrypsin, and thermolysin. The hydrolysates profiles and ACE-I inhibitory activity were analyzed using reversed phase-high performance liquid chromatography (RP-HPLC). The SDS-PAGE was also used to analyze the main storage protein in red quinoa seed, identified as being 11S seed storage globulin. Meanwhile, the ACE inhibitor activities of red quinoa seed protein (RQSP) produced by various proteases include the hydrolysate of pepsin 17.03% ± 3.88%, trypsin 42.67% ± 3.19%, α-chymotrypsin 72.71% ± 2.85% and thermolysin 77.67% ± 0.98%. These results show that red quinoa seed protein is a potential source of significant ACE inhibitor activity when hydrolyzed with α-chymotrypsin and thermolysin.

1. Introduction

Hypertension is a critical and widespread health problem that increases death risk, stroke, and heart failure. Some scientific evidence shows that drug treatments may be used to manage hypertension. This includes antihypertensive agents such as angiotensin-I-converting enzyme (ACE) inhibitors, which lower blood pressure (Heran *et al.* 2008). The renin-angiotensin system (RAS) and the kallikrein-kinin system (KKS) both work in conjunction with the ACE, a member of the zinc metallopeptidase family, to regulate blood pressure (Tu *et al.* 2018). In the RAS, ACE cleaves C-terminal dipeptide of angiotensin I, it produces the powerful vasoconstrictor angiotensin II. Furthermore, it also inactivates bradykinin which has depressor action in the KKS (Murray and FitzGerald 2007). The inhibitory activity results in a drop in angiotensin II levels, which lowers blood pressure and blood vessel tightness (Ondetti *et al.* 1977), showing antihypertensive activity. A previous study has shown that ACE inhibitory agents can be released from food protein (Yokoyama *et al.* 1992).

Currently, several ACE inhibitor synthetic drugs such as captopril, enalapril, and lisinopril, are being used for the clinical treatment of human hypertension (López-Fandiño *et al.* 2006). However, these medications frequently have negative side effects such allergic reactions, hypotension, coughing, potassium retention (hyperkalemia), reduced renal function, and fetal abnormalities (Kim and Wijesekara 2010). Food-derived ACE inhibitors are therefore excellent candidates for such products, offering a number of benefits such as safety, affordability, and the nutritional advantages of the proteins as sources of vital amino acids. ACE inhibitory activities have been identified in different food protein such as fish (Kohama *et al.* 1988), gelatin (Himaya *et al.* 2012), casein and whey (Ferreira *et al.* 2007; Tu *et al.* 2018) soybean (Hanafi *et al.* 2018), common bean (Luna-vital *et al.* 2015) and cereals (Liu

* Corresponding Author
E-mail Address: jlhsu@mail.npust.edu.tw

et al. 2013; Shamloo *et al.* 2015; Wang *et al.* 2017). Meanwhile, plant seeds, especially cereals are one of the most important sources of protein worldwide. It was also discovered that the cereal proteins are potential precursors of antihypertensive peptides (Anantharaman and Finot 1993).

Red quinoa (*Chenopodium formosanum*) is a cereal food protein grown in Taiwan, which is largely cultivated in Taitung and Pingtung districts (Chao and Hsueh 2016). Due to the nutrient content of its seeds, leaves, and stems, attention had been directed toward analyzing the potential for the biofunctional activity of various species of *Chenopodium*. This includes Vilcacundo *et al.* (2017) who identified the proteins from *Chenopodium quinoa* as possessing anti-diabetic properties, while Chirinos *et al.* (2018) discovered those from *Chenopodium pallidicaule* as possessing antioxidant and ACE inhibitor properties. This has led to the investigation of bio-functional activities derived from the *Chenopodium* genus. However, a study related to the bio-functional activity of red quinoa (*Chenopodium formosanum*) protein released by enzymatic digestion has not been carried out. Even though a variety of enzyme preparations have been utilized to make ACE inhibitors from food protein, alcalase, thermolysin, flavorzyme, and digestive enzymes such pepsin, trypsin, and α-chymotrypsin are the most popular (Mao *et al.* 2007). Protein hydrolysates are also effective in food application and feed ingredients for animals (Mizani *et al.* 2005). Therefore, this study aims to identify the potential of red quinoa seed (RQS) protein hydrolysate as released by various proteases, namely pepsin, trypsin, α-chymotrypsin, and thermolysin for the ACE inhibition.

2. Materials and Methods

2.1. Sample Preparation

Red quiona (*Chenopodium formosanum*) seed was purchased from National Pingtung University Science and Technology, Taiwan. The red quinoa seed was prepared by grinding it into powder using a grinder machine from Rong Tsong Precision Technology Co. (Taichung, Taiwan). The powder was put into a Falcon tube and placed in a vacuum chamber until used.

2.2. Obtaining Crude Protein Extract from RQS

The RQS powder was weighed into samples of 2 g, placed in a 50 ml Falcon tube, and 10 ml of 1% sodium dodecyl sulfate (SDS) in distilled water was added. The cell walls were disrupted with Brason Digital Sonifier® (Terra Universal Inc. LA, USA) at an amplitude of 30%. The pulse duration of 15 minutes with 20 seconds pulse on and 10 seconds pulse off was applied. Subsequently, the solution was separated by centrifugation (4,000 rpm, 15 minutes), and the supernatant was frozen at 80°C, and lyophilized.

The lyophilized sample was dissolved using 20% trichloroacetic acid (TCA) in acetone at a ratio of 3:1 (v/v). The solution was mixed by vortex and incubated at 4°C for 12 hours. After incubation, the samples were centrifugated at 4,000 rpm for 15 minutes to obtain the protein pellets. Subsequently, the pellets were lyophilized to obtain the crude seed protein and kept at 20°C for hydrolysis.

2.3. Obtaining Protein Hydrolysate from Crude Protein Extract

The RQS crude protein extract was subjected to hydrolysis with various protease enzymes, namely pepsin, trypsin, α-chymotrypsin, and thermolysin as described in Table 1. Each enzyme was prepared in 1 M HCl and sample protein concentration of 1/50 (w/w). The enzyme prepared at a concentration of 100 µg was added to 5 mg samples of protein. Based on the application of various buffers, trypsin, α-chymotrypsin, and thermolysin used 50 mM ammonium carbonate (pH 8.5), while pepsin used 20 mM NaCl (pH 2.0). The hydrolysis was performed for 16 hours at 37°C, except for thermolysin, which was kept at 60°C. Once the enzymatic reaction was completed, the hydrolysate was lyophilized (Table 1). The lyophilized hydrolysate was dissolved using 5% acetonitrile (ACN) in distilled water, vortexed, and generated through ultrafiltration with a range molecular weight cut-off (MWCO) of 3 kDa by centrifugation (15,000 rpm, 10 minutes at 4°C).

Table 1. Various proteases for red quinoa seed protein hydrolysis

Hydrolysate	Protease	Conditions
RQSP_Pep	Pepsin	37°C at pH 2.0
RQSP_Tryp	Trypsin	37°C at pH 8.5
RQSP_Cym	α-Chymotrypsin	37°C at pH 8.5
RQSP_Ther	Thermolysin	60°C at pH 8.5

*RQSP_Pep (Redquinoa seed protein digested by pepsin); RQSP_Tryp (Redquinoa seed protein digested by trypsin); RQSP_Cym (Redquinoa seed protein digested by α-chymotrypsin); RQSP_Ther (Redquinoa seed protein digested by thermolysin)

Acetonitrile (ACN) concentrations of 5%, 50%, 80%, and 95% ACN were used to desalt the hydrolysates below 3 kDa using a PepClean TMC18 spin column (Thermo Scientific, Rockwood, Tn, USA.). Out of these concentrations, 50% and 80% fractions were collected, combined, and lyophilized.

2.4. SDS-PAGE Analysis of Crude Protein

The BIO-RAD Laboratories' electrophoresis apparatus was employed in this study. Stephan *et al.* (2022), provided a description of the procedure utilized to prepare the gel. Acrylamide was employed in the application gel of the samples at a concentration of 12% in the running gel. The run took place in a 10.8 6.8 cm gel for 3 hours at a voltage of 100 V.

2.5. HPLC Analysis of RQS Protein Hydrolysate

The profile of hydrolyzed RQS was determined by RP-HPLC, where the lyophilized hydrolysate was initially resuspended in the mobile phase and dissolved in 5% acetonitrile and 0.1% ferulic acid in deionized water with a sample concentration of 10 µg/µl. The efficiency of production of RQS protein hydrolysate using various protease enzymes was confirmed on RP-HPLC chromatograms for 65 minutes at UV 214 nm with gradient elution of 0-30 minutes (10-36% B), 37–45 minutes (62–70% B), and isocratic elution at 50-60 minutes (80% B). Meanwhile, solvents A and B were 5% and 95% of acetonitrile, which contain 0.1% trifluoroacetic acid in deionized water, respectively.

2.6. Angiotensin I-converting Enzyme (ACE) Inhibitory Assay

The ACE inhibitory assay was measured by in vitro according to Cushman *et al.* (1977) as described by Pujiastuti *et al.* (2017) with slight modification. The ACE reaction was calculated by incubated 10 µl sampel, 30 µl HHL, and 20 µl of 0.05 mU/µl ACE in 200 mM borate buffer. The reaction was incubated at 37°C for 30 minutes without shaking and then shaken (200 rpm). Subsequently, the hipurric acid (HA) as the product released from HHL (Hippuryl-L-Histidyl-L-Leucine)) was analyzed by RP-HPLC equipped with a C18 column (4.6 mm × 250 mm; particle size 5 µm, Thermo Scientific Inc) and the detection of UV-Vis wavelength was at 228 nm for 30 minutes. The mobile phase were 5% of acetonitrile, which contain 0.1% trifluoroacetic acid in deionized water. The chromatographic peak area was quantified. The ACE inhibitory activity was calculated based on HA peaks areas as the following equation:

$$\text{ACE inhibition (\%)} = \frac{\text{A blank - A sample}}{\text{A blank}} \times 100\%$$

3. Results

3.1. Red Quinoa Seed (RQS) Yield Protein

This study produced a protein yield of 16% from 1 g of RQS powder, which was extracted and precipitated using TCA to obtain 0.16 g of crude protein. Trichloroacetic acid (TCA) is commonly used to concentrate protein samples or remove contaminants, including salts and detergents before downstream application such as SDS-PAGE or 2D gels. The crude protein was profiled using SDS-PAGE analysis to observe the distribution of the protein and estimate their molecular masses. Based on the results, the main protein was identified as 11S seed storage globulin (Table 2), with subunit molecular weights of 17 kDa, 20 kDa, 31 kDa, 34 kDa, and 53 kDa (Figure 1). As stated in the Uniprot database (https://www.uniprot.org/uniprot/Q06AW2), the 53 kDa is similar to the protein of 11S seed storage globulin A from Chenopodium Quinoa (Q06AW2_CHEQI). This study assumed that RQS major protein is 11S seed storage globulin protein.

3.2. Hydrolysate Profile of RQSP Using Various Protease

The defatted RQS was hydrolyzed with four different proteases under their optimum conditions. This was carried out to generate the RQS hydrolysate with ACE inhibitory activity, which was prepared as shown in Figure 2. The three potential ACE inhibitory hydrolysate fractions of RQS, namely trypsin, α-chymotrypsin, and thermolysin were profiled using RP-HPLC. Meanwhile, Figure 3 shows the effect of various proteases on the molecular weight of hydrolysate distribution of RQS protein. The peak distribution of peptides in the hydrolysate showed the characteristics of the hydrolysate produced during the hydrolysis process. The profiles of the three hydrolysates also indicated that their contents are mostly smaller peptides.

This showed that enzymatic hydrolysis was effective in producing small molecular mass compounds and potent bioactivity of the hydrolysate. The trypsin hydrolysate produced the lowest peak, therefore, only a small amount of peptide was

Table 2. The Observed band specificity of red quinoa seed protein (RQSP) crude protein

Observed band	Mascot score	Protein number	Observed protein in mascot search
17 kDa	113	gi|45510877	11S seed storage globulin [*Chenopodium quinoa*]
20 kDa	388	gi|45510877	11S seed storage globulin [*Chenopodium quinoa*]
31 kDa	196	gi|45510877	11S seed storage globulin [*Chenopodium quinoa*]
53 kDa	53	gi|45510877	11S seed storage globulin [*Chenopodium quinoa*]

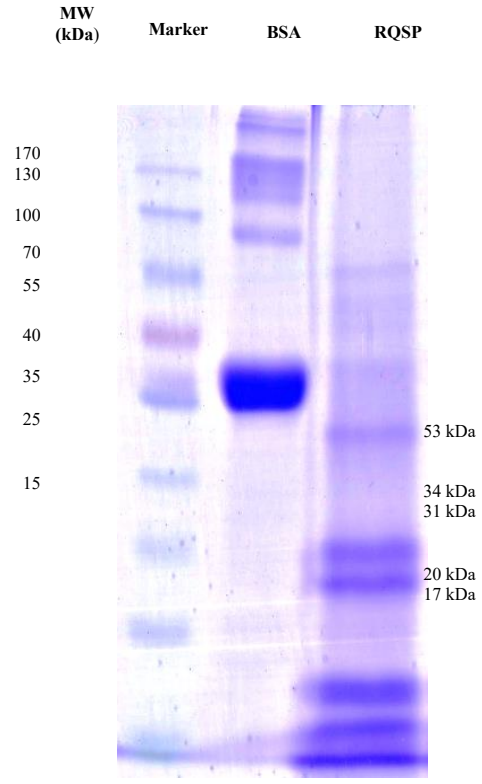

Figure 1. SDS-PAGE analysis of red quinoa seed protein (RQSP) crude protein

present. The α-chymotrypsin hydrolysate had a lower peak of peptides compared to the thermolysin hydrolysate, showing its richness in peptides. This showed that the thermolysin produced more peptides from RQS protein than pepsin, trypsin, and α-chymotrypsin. Therefore, the thermolysin is more effective at hydrolyzing the RQS proteins compare to other protease enzymes.

3.3. ACE Inhibitor Activity on RQS Protein Hydrolysate

The various proteases, namely pepsin, trypsin, α-chymotrypsin, and thermolysin usually filter the ACE inhibitor activity in RQS. The ACE inhibitor activity was calculated in three experiments using RP-HPLC. From Table 3, the results showed that pepsin, trypsin, α-chymotrypsin, and thermolysin

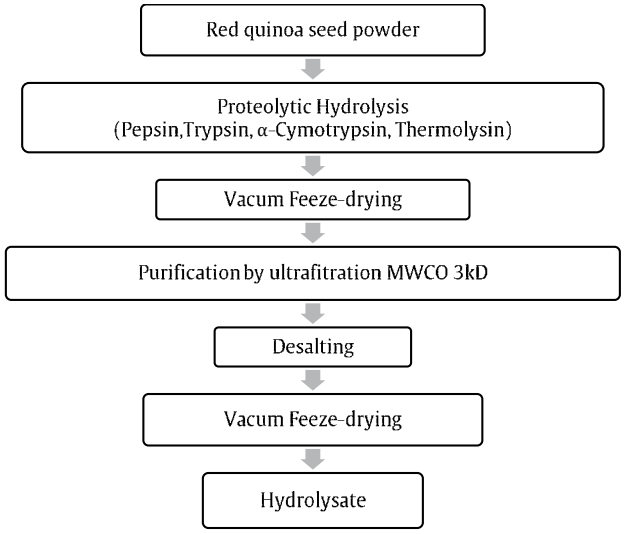

Figure 2. Flowchart for the preparation of RQSP hydrolysate with various protease

hydrolysates exhibit 17.03% ± 3.88%, 42.67% ± 3.19%, 72.71% ± 2.85%, and 77.67% ± 0.98% ACE-I inhibitory activity, respectively. Pepsin hydrolysate had the lowest activity, with trypsin and α-chymotrypsin showing the higher levels. Therefore, thermolysin is suggested as the best in the preparation of RQS protein hydrolysate fractions for ACE-I inhibition.

4. Discussion

Red quinoa (*Chenopodium formosanum*) belongs to the Amaranthaceae family and is a cereal plant widely cultivated by Aboriginal people in southern Taiwan, especially in the Taitung and Pingtung districts (Chao and Hsueh 2016). In recent years, much attention has been given to the plant after it was discovered to be a good source of antioxidants and other health-promoting substances. RQS contains 50% starch, and its protein content of 14% is twice that of rice and similar to wheat health (Murray and FitzGerald 2007). A previous report by Tsai (2011) identified RQS protein content of 17.7%, while this study obtained a yield of 16%. The RQS protein is a superior source of essential amino

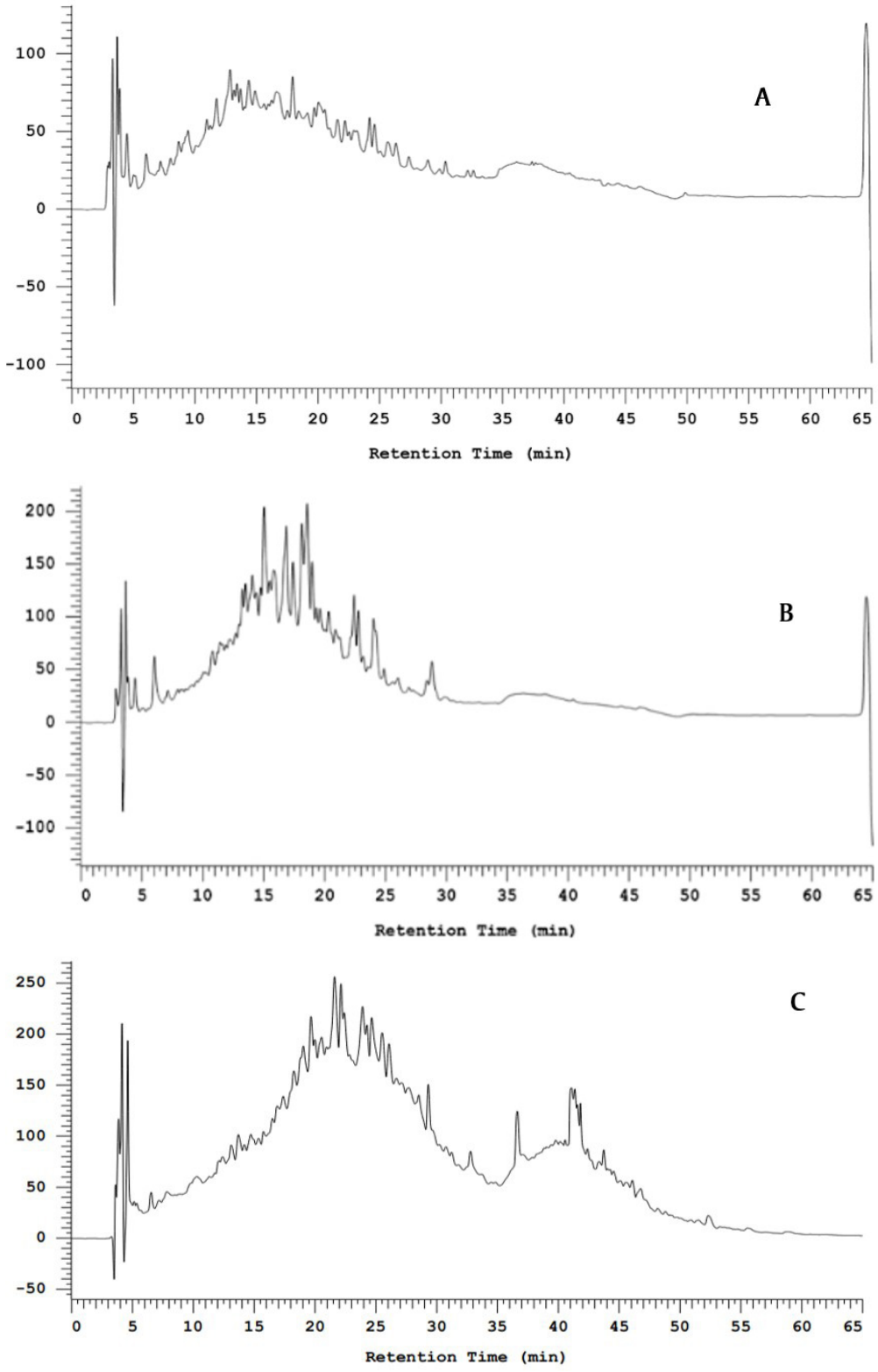

Figure 3. Reverse-phase chromatographic profile of (A) RQSP_tryp, (B) RQSP_ chym, and (C) RQSP_ther hydrolysate for 65 minutes

Tabel 3. Angiotensin I converting enzyme (ACE) inhibitory activity of various protease hydrolysate derived red quinoa seed protein

Hydolysate	Observed protein in mascot search			%ACE inhibitory activity
	I	II	III	
Captopril (control)	97.93	97.94	98.50	98.12±0.32
RQSP_Pep	16.53	21.14	13.43	17.03±3.88
RQSP_Tryp	41.77	40.03	46.22	42.67±3.19
RQSP_Cym	70.66	71.49	75.97	72.71±2.85
RQSP_Ther	77.61	78.90	76.48	77.67±0.98

*RQSP_Pep (Redquinoa seed protein pepsin hydrolysate); RQSP_Tryp (Redquinoa seed protein trypsin hydrolysate); RQSP_Cym (Redquinoa seed protein α-chymotrypsin hydrolysate); RQSP_Ther (Redquinoa seed protein thermolysin hydrolysate)

acids such as lysine, which provide bio-functional properties of benefit to human health (Murray and FitzGerald 2007). Tsai (2011), also revealed that RQS protein comprises various essential amino acids such as arginine, isoleucine, leucine, methionine, valine and phenylalanine. According to the *in silico* used mascot distiiler resulted RQS has four sub-proteins of 17 kDa, 20 kDa, 31 kDa, and 34 kDa. Another report showed that quinoa seed 11S globulin had a basic subunit at 17-20 kDa and 30-35 kDa (Martínez *et al.* 2019).

Protein are long polypeptide chains folded into the functional form with a hierarchical structure. Proteins contain bioactive properties that can be released from protein hydrolysate that produce by protease digestion. Several parameters that affect the bioactive properties of the protein hydrolysate are the method of production, enzyme applied for hydrolysis, proteases specificity, molecular weight, sequence of hydrolysate, and hydrolyzation conditions such as temperature, time, pH (Udenigwe and Aluko 2012). Protein hydrolysates are common intermediate products with easily digestible macronutrients (Mizani *et al.* 2005). Recently, biochemical hydrolysis by adding protease enzyme to produce hydrolysate with specific biological properties from food proteins has gained much attention. This is because the process provides the possibility of controlling the cleavage degree of protein in the substrate and the properties of the products (Shamloo *et al.* 2015). The use of commercially available microbial-derived food-grade protease to hydrolyze food proteins is advantageous because they are low-cost, safe, and have a high yield of product (Mao *et al.* 2007).

The protease digestion method is commonly used because it provides milder process conditions and produces higher-value products (Kose and Oncel 2015). Protease is constantly being introduced and more advantageous compared to the chemical processes which might increase hydrolysis specificity, increase purity, and reduce environmental effects (Tavano 2013). Furthermore, enzymes modify the amino acid sequence and the three-dimensional structure, generates amino acids and small peptides from the intact proteins and exhibit substrate specificity, enabling the creation of protein hydrolysates with more distinct, excellent solubility, hidden hydrophobic residues, and and enhances its nutritional value (Castro *et al.* 2011). On the other hand, small pepptides can present inhibitors, cofactors needs, or suffer autolysis (McGeagh *et al.* 2011).

Based on a previous report, protein hydrolysates are more effective than intact proteins or free amino acids (Wang and Zhang 2012). In a report by Tejano *et al.* (2019), *Chlorella sorokiniana* protein was hydrolyzed using pepsin, bromelain, and thermolysin. The results showed that thermolysin produced the highest hydrolysate fraction of < 5 kDa of 91.67±1.34% content. Meanwhile, the thermolysin in this study also produced the richest peptide distribution from RQS hydrolysate as shown in many peaks distribution of RP-HPLC chromatogram. This indicated that thermolysin is the most effective protease for releasing high peptide fractions compared to pepsin, trypsin, and α-chymotrypsin.

The thermolysin hydrolysate of RQS protein has gained much attention because of its high level of angiotensin-I-converting enzyme (ACE-I) inhibitory activity (77.67% ± 0.98%). It was also reported that potent ACE inhibitors usually contain hydrophobic amino acid residues at their C-terminal positions.

Therefore thermolysin, a thermostable neutral metalloproteinase enzyme produced by Bacillus thermoproteolytices, is being widely used for producing ACE inhibitors. This is because it specifically catalyzes the hydrolysis of peptide bonds containing hydrophobic amino acids. As proteases, thermolysin preferentially cleaves at the N-terminal side of the hydrophobic protein structure. It has been applied in protein hydrolyzation processes such in rice bran protein (Shobako *et al.* 2018), meat protein (Estell *et al.* 2019), collagen (Owen *et al.* 2007) for the release of ACE-I inhibitory. peptides. Some recent investigations also showed that thermolysin effectively cleaved cereal protein, especially oat and corn germ into a fraction with high ACE inhibitory activity (Cheung *et al.* 2009). This study found that RQS shows higher ACE-I inhibitory activity compared to other herbal plants such as *Curcuma domestica* Val. (24.15±3.21%), *Andrographis paniculata* (Burm.f.) Nees. (29.38±1.82%), and *Averrhoa bilimbi* L. (71.48±1.71%) (Muthia *et al.* 2017). This indicated that red quinoa (*Chenopodium formosanum*) is a herbal plant with high antihypertensive potential.

In conclusion, the potent antihypertensives in form of bioactive peptides encrypted in food-derived protein sources were released from RQS (*Chenopodium formosanum*) protein. The results showed that pepsin, trypsin, chymotrypsin, and thermolysin have the potential to produce ACE-I inhibitory protein hydrolysate. Among the proteases investigated, the hydrolysate prepared using thermolysin showed the most prosperous molecular weight distribution of peptides, therefore, it has the highest ACE-I inhibitory activity.

Acknowledgements

The Department of Biological Science and Technology, BT207 Proteomics Laboratory, National Pingtung University Science and Technology, Taiwan, and the Program Double Degree of Brawijaya University, Indonesia, provided financial support for this study.

References

Anantharaman, K., Finot, P.A., 1993. Nutritional aspects of food proteins in relation to technology. *Food Reviews International*. 9, 629-655. https://doi.org/10.1080/87559129309540981

Castro, H.C., Abreu, P.A., Geraldo, R.B., Martins, R.C., dos Santos, R., Loureiro, N.I., Cabral, L.M., Rodrigues, C.R., 2011. Looking at the proteases from a simple perspective. *Journal of Molecular Recognition*. 24, 165-181. https://doi.org/10.1002/jmr.1091

Chao, Y.Y., Hsueh, I., 2016. Insight into the growth traits in different color of djulis (*Chenopodium formosanum* koidz.). *Weed Sci. Bull.* 37, 31.

Cheung, I.W., Nakayama, S., Hsu, M.N., Samaranayaka, A.G., Li-Chan, E.C., 2009. Angiotensin-I converting enzyme inhibitory activity of hydrolysates from oat (*Avena sativa*) proteins by *in silico* and *in vitro* analyses. *Journal of Agricultural and Food Chemistry*. 57, 9234-9242. https://doi.org/10.1021/jf9018245

Chirinos, R., Ochoa, K., Aguilar-Galvez, A., Carpentier, S., Pedreschi, R. Campos, D., 2018. Obtaining of peptides with *in vitro* antioxidant and angiotensin I converting enzyme inhibitory activities from cañihua protein (Aellen). *Journal of Cereal Science*. 83, 139-146.

Cushman, D.W., Cheung, H.S., Sabo, E.F. Ondetti, M.A., 1977. Design of potent competitive inhibitors of angiotensin-converting enzyme. Carboxyalkanoyl and mercaptoalkanoyl amino acids. *Biochemistry*. 16, 5484-5491.

Estell, D.A., Liu, A.D., Hommes, R.W. Shaw, A., 2019. *Production of Thermolysin and Variants Thereof, and Use in Liquid Detergents*. Danisco US Inc, Patent.

Ferreira, I. M.P.L.V.O., Pinho, O., Mota, M.V., Tavares, P., Pereira, A., Goncalves, M.P., Torres, D., Rocha, C. Teixeira, J.A. 2007. Preparation of ingredients containing an ACE-inhibitory peptide by tryptic hydrolysis of whey protein concentrates. *International Dairy Journal*. 17, 481-487. https://doi.org/10.1016/j.idairyj.2006.06.023

Hanafi, M. A., Hashim, S.N., Chay, S.Y., Ebrahimpour, A., Zarei, M., Muhammad, K., Abdul-Hamid, A. Saari, N., 2018. High angiotensin-I converting enzyme (ACE) inhibitory activity of Alcalase-digested green soybean (Glycine max) hydrolysates. *Food Research International*. 106, 589-597. https://doi.org/10.1016/j.foodres.2018.01.030

Heran, B.S., Wong, M.M., Heran, I.K. Wright, J.M., 2008. Blood pressure lowering efficacy of angiotensin converting enzyme (ACE) inhibitors for primary hypertension. *Cochrane Database of Systematic Reviews*. 4, 1-7.

Himaya, S.W.A., Ngo, D.H., Ryu, B., Kim, S.K., 2012. An active peptide purified from gastrointestinal enzyme hydrolysate of Pacific cod skin gelatin attenuates angiotensin-1 converting enzyme (ACE) activity and cellular oxidative stress. *Food Chemistry*. 132, 1872-1882. https://doi.org/10.1016/j.foodchem.2011.12.020

Kim, S.K., Wijesekara, I., 2010. Development and biological activities of marine-derived bioactive peptides: a review. *Journal of Functional Foods*. 2, 1-9. https://doi.org/10.1016/j.jff.2010.01.003

Kohama, Y., Matsumoto, S., Oka, H., Teramoto, T., Okabe, M., Mimura, T., 1988. Isolation of angiotensin-converting enzyme inhibitor from tuna muscle. *Biochemical and Biophysical Research Communications*. 155, 332-337. https://doi.org/10.1016/S0006-291X(88)81089-1

Kose, A., Oncel, S.S., 2015. Properties of microalgal enzymatic protein hydrolysates: biochemical composition, protein distribution and FTIR characteristics. *Biotechnology Reports*. 6, 137-143. https://doi.org/10.1016/j.btre.2015.02.005

Liu, M., Du, M., Zhang, Y., Xu, W., Wang, C., Wang, K., Zhang, L., 2013. Purification and identification of an ACE inhibitory peptide from walnut protein. *Journal of Agricultural and food Food Chemistry*. 61, 4097-4100. http://doi.org/10.1021/jf4001378

López-Fandiño, R., Otte, J., Van Camp, J., 2006. Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity. *International Dairy Journal*. 16, 1277-1293. https://doi.org/10.1016/j.idairyj.2006.06.004

Luna-Vital, D.A., Mojica, L., de Mejía, E.G., Mendoza, S., Loarca-Piña, G., 2015. Biological potential of protein hydrolysates and peptides from common bean (*Phaseolus vulgaris* L.): a review. *Food Research International*. 76, 39-50. https://doi.org/10.1016/j.foodres.2014.11.024

Mao, X.Y., Ni, J.R., Sun, W.L., Hao, P.P., Fan, L., 2007. Value-added utilization of yak milk casein for the production of angiotensin-I-converting enzyme inhibitory peptides. *Food Chemistry*. 103, 1282-1287. https://doi.org/10.1016/j.foodchem.2006.10.041

Martínez, J.H., Velázquez, F., Burrieza, H.P., Martínez, K.D., Rubio, A.P.D., dos Santos Ferreira, C., del Pilar Buera, M., Pérez, O.E., 2019. Betanin loaded nanocarriers based on quinoa seed 11S globulin. Impact on the protein structure and antioxidant activity. *Food Hydrocolloids*. 87, 880-890.

McGeagh, J.D., Ranaghan, K.E., Mulholland, A.J., 2011. Protein dynamics and enzyme catalysis: insights from simulations. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*. 1814, 1077-1092.

Mizani, M.A.R.Y.A.M., Aminlari, M., Khodabandeh, M., 2005. An effective method for producing a nutritive protein extract powder from shrimp-head waste. *Food Science and Technology International*. 11, 49-54.

Murray, B.A. FitzGerald, R.J., 2007. Angiotensin converting enzyme inhibitory peptides derived from food proteins: biochemistry, bioactivity and production. *Current Pharmaceutical Design*. 13, 773-791.

Muthia, R., Suganda, A.G. Sukandar, E.Y., 2017. Angiotensin-I converting enzyme (ACE) inhibitory activity of several Indonesian medicinal plants. *Research Journal of Pharmaceutical Biological and Chemical Sciences*. 8, 192-199.

Ondetti, M.A., Rubin, B., Cushman, D.W., 1977. Design of specific inhibitors of angiotensin-converting enzyme: new class of orally active antihypertensive agents. *Science*. 196, 441-444. https://doi.org/10.1126/science.191908

Owen, J.P., Maddison, B.C., Whitelam, G.C. Gough, K.C., 2007. Use of thermolysin in the diagnosis of prion diseases. *Molecular Biotechnology*. 35, 161-170.

Shamloo, M., Eck, P., Beta, T. 2015. Angiotensin converting enzyme inhibitory peptides derived from cereals. *Journal of Human Nutrition and Food Science*. 3, 1057-1067.

Shobako, N., Ogawa, Y., Ishikado, A., Harada, K., Kobayashi, E., Suido, H., Kusakari, T., Maeda, M., Suwa, M., Matsumoto, M. and Kanamoto, R., 2018. A novel antihypertensive peptide identified in thermolysin-digested rice bran. *Molecular Nutrition and Food Research*. 62, 1700732.

Stephan, M., Costa, A., Azevedo, T.D.L., da Rosa, J.S., dos Santos, A.A., 2022. Electrophoresis (SDS-PAGE) as a method for screening species of Passiflora using seed proteins as molecular markers. *Journal of Agricultural Science and Technology*. 12, 40-47.

Pujiastuti, D.Y., Shih, Y.H., Chen, W.L. Hsu, J.L., 2017. Screening of angiotensin-I converting enzyme inhibitory peptides derived from soft-shelled turtle yolk using two orthogonal bioassay-guided fractionations. *Journal of Functional Foods*. 28, 36-47.

Tavano, O.L., 2013. Protein hydrolysis using proteases: an important tool for food biotechnology. *Journal of Molecular Catalysis B: Enzymatic*. 90, 1-11. https://doi.org/10.1016/j.molcatb.2013.01.011

Tejano, L.A., Peralta, J.P., Yap, E.E.S. Chang, Y.W., 2019. Bioactivities of enzymatic protein hydrolysates derived from *Chlorella sorokiniana*. *Food Science and Nutrition*. 7, 2381-2390.

Tsai, P.J., Chen, Y.S., Sheu, C.H., Chen, C.Y., 2011. Effect of nanogrinding on the pigment and bioactivity of djulis (*Chenopodium formosanum* Koidz.). *Journal of Agricultural and Food Chemistry*. 59, 1814-1820. https://doi.org/10.1021/jf1041273

Tu, M., Wang, C., Chen, C., Zhang, R., Liu, H., Lu, W., Jiang, L., Du, M., 2018. Identification of a novel ACE-inhibitory peptide from casein and evaluation of the inhibitory mechanisms. *Food Chemistry*. 256, 98-104. https://doi.org/10.1016/j.foodchem.2018.02.107

Udenigwe, C.C., Aluko, R.E., 2012. Food protein-derived bioactive peptides: production, processing, and potential health benefits. *Journal of Food Science*. 77, 11-24. https://doi.org/10.1111/j.1750-3841.2011.02455.x

Vilcacundo, R., Martínez-Villaluenga, C., Hernández-Ledesma, B., 2017. Release of dipeptidyl peptidase IV, α-amylase and α-glucosidase inhibitory peptides from quinoa (*Chenopodium quinoa* Willd.) during *in vitro* simulated gastrointestinal digestion. *Journal of Functional Foods*. 35, 531-539.

Wang, X., Chen, H., Fu, X., Li, S., Wei, J. 2017. A novel antioxidant and ACE inhibitory peptide from rice bran protein: Biochemical characterization and molecular docking study. *LWT*. 75, 93-99. http://dx.doi.org/10.1016/j.lwt.2016.08.047

Wang, X., Zhang, X., 2012. Optimal extraction and hydrolysis of *Chlorella pyrenoidosa* proteins. *Bioresource Technology*. 126, 307-313. https://doi.org/10.1016/j.biortech.2012.09.059

Yokoyama, K., Chiba, H., Yoshikawa, M., 1992. Peptide inhibitors for angiotensin i-converting enzyme from thermolysin digest of dried bonitot. *Bioscience, Biotechnology, and Biochemistry*. 56, 1541-1545. https://doi.org/10.1271/bbb.56.1541