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PRODUCTION OF PROTEIN HYDROLYSATES FROM SEA CUCUMBER (HOLOTHUROIDEA) INNARDS BY PAPAIN HYDROLYSIS

Ta Ngoc Ly[1, *], Nguyen Thi Anh[2]

[1]*University of Technology and Sciences, The University of Da Nang, 54 Nguyen Luong Bang, Lien Chieu, Da Nang, Viet Nam*

[2]*Environmental Conservation Organization in Central Viet Nam, 178 Trieu Nu Vuong, Hai Chau, Da Nang, Viet Nam*

[*]Email: *tnly@dut.udn.vn*

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Abstract. Sea cucumber innards (SCI) are the main waste of the sea cucumber processing industry. SCI protein hydrolysates were prepared using Papain and kinematic parameters of SCI-Papain hydrolysis reaction were studied. The hydrolysates were subsequently analyzed for their antioxidant potential and amino acid composition for the first time. The results showed that the highest degree of hydrolysis (DH) was achieved at an enzyme/substrate ratio of 0.06/75 (w/w) and a hydrolysis time of 180 mins. The kinematic parameters of SCI-Papain hydrolysis reaction were investigated using the Lineweaver-Burk model, which Km and Vmax were calculated to be 0.21 g/L and 1.69 mgN/min, respectively. SCI hydrolysates have high nutritional components when detecting 16/22 amino acids, including 8/9 essential amino acids. SCI hydrolysates with a DH of 15 % exhibited the highest 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, equal to 62.8 %. This finding suggested the potential of using SCI protein hydrolysates as therapeutic bioactive ingredients in functional food development.

Keywords: Sea cucumber innards, Papain, enzymatic hydrolysis, kinetic, DPPH.

Classification numbers: 1.2.1, 1.3.1.

1. INTRODUCTION

Sea cucumbers are marine animals from the phylum echinoderm and class *Holothuroidea*. Of the more than 1250 species worldwide, many are gathered for human consumption or grown in aquaculture systems [1]. With increased knowledge about their nutritional value and benefits, the consumption of sea cucumbers has increased worldwide. The current high global demand for sea cucumbers has led to an overexploitation of many wild populations.

Sea cucumbers are generally regarded as a premium seafood since they usually have a high protein to lipid ratio and contain high levels of beneficial polyunsaturated fatty acids, collagens, vitamins and minerals [2]. The lipid, fatty acid, collagen, and amino acid compositions are most frequently associated with the functional food properties of sea cucumbers. The range of active

compounds chemically identified from sea cucumbers is quite diverse and includes polysaccharides such as glycosaminoglycans including neutral glycans, fucosylated chondroitin sulfates and sulfated fucans, peptides, phospholipids and glycolipids, including glycosphingolipids, polyunsaturated fatty acids, phenols, and saponins [3].The therapeutic and medicinal benefits of sea cucumbers have been intensively studied due to their anticancer, antioxidant, anti-inflammatory, and antimicrobial properties, and wound healing activities [4][1].

During processing, the innards of sea cucumbers are usually discarded as waste. It is estimated that sea cucumber organs account for 45 % of body weight. This waste includes the internal organs, including respiratory track, gonad, and intestines. Sea cucumber innards are abundant in various nutrients and promising for valorization. Little research has been done specifically on the waste products from sea cucumber industry. Recently, Senadheera *et al*. discovered that the SCI contained a high concentration of protein and lipids, a small amount of carbohydrates in the form of glycogen, and very little cholesterol [1]. Regarding mineral content, the coproduct contained relatively high levels of calcium, selenium, and zinc, and very high levels of iron, potassium, sodium, and phosphorus [5]. The SCI contained high levels of essential amino acids, particularly lysine and leucine. It was also rich in many of the non-essential amino acids, notably glutamic acid, aspartic acid, glycine, alanine, and arginine [1]. Another reported that both air and freeze-dried sea cucumber viscera had total fatty acid composition like fresh viscera with high levels of omega-3 polyunsaturated fatty acids, especially eicosapentaenoic acid. The dried samples were abundant in essential amino acids (46 - 51 %) [6]. The high content of nutritious components in SCI suggests the potential of valorizing into high-value products. However, unlike the by-products of many types of fish that are intensively valorized for added value, not much research has been done on the valorization of SCI. Currently in Viet Nam, SCI are entirely discarded as waste. Considering the various bioactive compounds, more research is needed to valorize them for industrial production of high value nutritional products.

The use of enzymes as an alternative to current mechanical methods to recover high-quality protein from sea cucumber processing waste is attracting considerable attention. This process beholds several advantages, such as it requires only milder conditions with a short reaction time, better predictability control for hydrolysis and does not involve organic solvents or toxic chemicals.

The present study aimed to determine the hydrolysis condition of SCI protein by Papain including enzyme/substrate ration (E/S) and hydrolysis time, which affects the degree of hydrolysis (DH).

This work also involved solving the complexity of hydrolysis reactions from the perspective of kinetics for which the K_m and V_{max} are identified. This is the first study in the field of utilizing by-products of the sea cucumber industry, the innards, to produce protein hydrolysates using Papain and to investigate their amino acid composition and antioxidant capacity.

2. MATERIALS AND METHODS

2.1. Materials

Sea cucumber innards were supplied by Viet Truong Seafood Processing and Import - Export Co., *Ltd*. Sea cucumber innards were stored in polyethylene bags at -20 ^{0}C until used for SCI hydrolysate production.

Papain was supplied by Novaco Pharmaceutical Company. The Papain activity was measured, with its specific activity equal to 1800 UI/g protein.

2.2. Methods

2.2.1. Analytical Methods

The samples were thawed at room temperature and mixed with distillate water (1:1) then blended for 2-3 mins. The homogenate obtained was adjusted to pH 8.0 using phosphate buffer, followed by pre-incubation at 37 °C for 5 mins to attain the temperature equilibrium with occasional stirring. Hydrolysis reaction was initiated by adding Papain solution and was carried out at 62 °C for 30 mins. At the end of the hydrolysis process, the enzymes are inactivated by adding TCA for 15 mins, the mixture was then filtered through a muslin cloth and centrifuged at 10,000 rpm for 15 mins to remove the fine solids. The supernatant obtained was determined for N amino acid content.

2.2.3. Measurement of Degree of Hydrolysis (DH)

The DH refers to the percentage of free amino terminal groups cleaved from proteins during hydrolysis and was determined using OPA (O-phthaldialdehyde) according to the method of Nielsen *et al*. [7] with minor modifications. The content of the alpha-amino groups of the samples was determined as concentration of L-serine from a standard curve. The DH was then calculated as the ratio of alpha-amino nitrogen to total nitrogen content using the following equation:

$$DH\ (\%) = \frac{L_i - L_0}{L_{total} - L_0} X\ 100$$

where L_i is the amount of released free amino groups resulting from hydrolysis at a time "i", L_o is the amount of free amino groups in the original sample before the hydrolysis, and L_{total} is the total amount of free amino groups in the original sample.

2.2.4. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

The 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity was measured according to the method described by Akar *et al.* [8] with minor modification. 100 µL aliquot of sample (1 % w/v) was mixed with 100 µL of 0.25 mM DPPH that was dissolved in 100 % ethanol. After incubation in the dark at 25 °C for 30 min, the absorbance of the mixture was determined at 517 nm using a UV-Vis spectrophotometer. The following equation was used to calculate the percentage DPPH scavenging capacity:

$$\text{DPPH scavenging capacity (\%)} = \left[1 - \frac{(As - Ab)}{Ac}\right] \times 100$$

where A_s is the absorbance of the tested SCI hydrolysates, A_b is the absorbance of the blank, and A_c is the absorbance of the DPPH solution. All experiments were conducted in triplicate.

2.2.5. Effect of enzyme substrate ratio on protein hydrolysis

SCI hydrolysis was studied at different enzyme substrate (E-S) ratio in batch mode, 300 g/L protein solution was diluted to various concentrations then subjected to constant enzyme loading

(0.06 g/L). Proteolysis was carried out at 62 °C for 30 mins, changes in degree of hydrolysis were determined and compared to evaluate the effect of enzyme - substrate loading on the progress of the reaction.

2.2.6. The Determination of Km and Vmax of Papain

Enzymatic kinetics can be described by the Michaelis-Menten model. The enzyme kinetic was measured according to the method described by Beg [9] with minor modification. In this experiment, reaction mixtures were designed varying the substrate concentration from 1.0 mg/mL to 50 g/L to which 1 mL of solution containing 0.06 g Papain was added. The reaction mixtures were then incubated at 62 ^{o}C for 10 mins and then protease activity was determined. Kinetic parameters K_m and V_{max} were calculated from Lineweaver Burk plots.

2.2.7. Analysis of amino acid composition

Amino acid composition of SCI hydrolysate was determined using HPLC method following the Vietnamese National Standards TCVN 8764:2012. Before performing the chromatography, the hydrolysate was set to room temperature. The mixture was well shaked and filtered to obtain an appropriate amount through a 0.2 μm filter. A Water HPLC system was used for the separation of individual amino acids. The detector was set up at a fixed wavelength (254 nm). The amino acids were identified and quantified by comparing their retention times with those of amino acids present in standard calibration curves. The calibration curves were created using an amino acid standard H (Thermo Scientific) containing 2.5 μmol mL^{-1} for each amino acid in 0.1M HCl. The calibration curve for each amino acid was constructed by plotting the mean peak area for each concentration. Then, the content of each amino acid in a sample was determined by interpolation of the respective peak area of the amino acid.

3. RESULTS AND DISCUSSION

3.1. Characterization of samples

The SCI waste used in this study had a high moisture content (69.2 ± 1 % w/w), which is consistent with the literature demonstrating that sea cucumbers have a high water content. On a dry matter basis, SCI waste was rich in proteins. SCI waste consisted of viscera, gonad, respiratory trees, and circulatory system causing it to have high protein content (81.25 ± 0.6 % w/w). The ash and lipid contents in the samples were reported to be low, 3.21 ± 0.02 % w/w and 3.65 ± 0.24 % w/w, respectively). Mamelona *et al*. [10] reported 92.3 % moisture, 0.7 % ash, 2.0 % lipids, and 4.5 % proteins in the fresh viscera of *Cucumaira frondosa* from Québec, QC, Canada. Zhong *et al*. reported that the body wall of *Cucumaira frondosa* from Newfoundland waters contained 87.4 % moisture, 2.97 % ash, 0.50 % lipids, and 8.34 % proteins [3]. Compared with the body wall, viscera have similar moisture, ash and protein content, but much higher lipid content, possibly due to better fat storage capacity of internal organs.

3.2. Effect of substrate concentration on DH

In the previous study, we optimized the hydrolysis conditions by using the Surface Response Method with 3 factors of pH (4 ÷ 8), temperature (30 ÷ 90 °C), and Papain concentration (0.001 ÷ 0.1 g/mL). The results showed that the highest degree of hydrolytic was 28.07 % at pH 8.0, temperature 62 0C, and Papain concentration 0.06 g/mL. However, enzyme substrate (E-S) ratio which contributes to overall hydrolysis of proteins was not identified; hence

in this study, the SCI hydrolysis reaction was investigated at different E-S ratios in batch mode. The DH of SCI hydrolysis at various initial substrate concentrations is shown in Figure 1. The DH increased as the substrate concentration decreased from 300 g/L to 75 g/L. The E-S ratio (0.06/75) showed the highest hydrolysis up to 14.39 % DH. Substrate concentrations below this point decreased DH. At a constant enzyme concentration (0.06 g/L), high concentrations of the substrate (higher than 75 mg/L) will act as a dead-end inhibitor. On the other hand, at lower concentrations of the substrate, the substrate concentration is the limiting factor, thus the enzyme reaction rate will increase with increasing substrate concentration. Increasing the initial concentration of the substrate, keeping the enzyme concentration constant, did not favor the recovery of by-products, which seems to indicate that the enzyme/substrate ratio is a significant process variable. This means that when a sufficient concentration of the substrate is available, increasing enzyme concentration will increase the rate of enzymatic reaction.

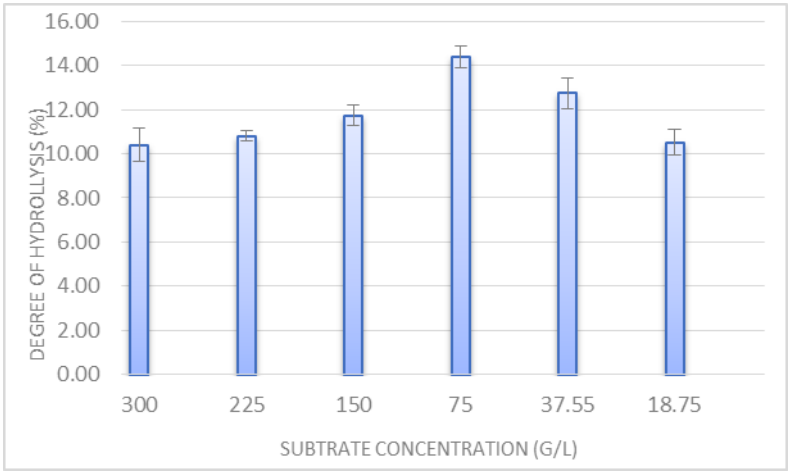

Figure 1. Effect of substrate concentration on degree of hydrolysis.

3.3. The SCI-Papain hydrolysis curves

As shown in Figure 2, the time-course relationship of SCI-papain DH is characterized by a high initial reaction rate, followed by a rate reduction that tends to a constant value with increasing time. The DH reached half of its maximum value in the first 30 min of hydrolysis and peaked at 180 min, with a DH of 48.2 %. After that point, the hydrolysis curves state a constant value. The downward trend of the hydrolysis curves is attributed to the reduced concentration of the effective peptide bonds, substrate or product inhibition, and enzyme inhibition or inactivation. Senadheera *et al*. reported that the DH values of the body wall, internal organs and flower sea cucumber hydrolysates prepared with the combination of Alcalase and Flavourzyme were 18.3, 10.7 and 16.4 %, respectively [1]. Alcalase, trypsin, and flavourzyme hydrolysates prepared from the viscera of sea cucumber exhibited a higher degree of hydrolysis, coming to 19.08, 32.38, and 15.94 %, respectively [11]. Here, by using Papain, an enzyme from a family of related proteins with both *exo* and *endopeptidase* activities [12], we obtained a higher degree of hydrolysis than the single enzyme**.** The observed trend showed that single enzyme application is not effective in bringing about extensive hydrolysis, so that Papain is a preferable choice for food protein. The above results suggested that the hydrolysis reaction under the defined optimal conditions takes only 3 hours to complete, which is a short time, helping to reduce the cost of hydrolysis.

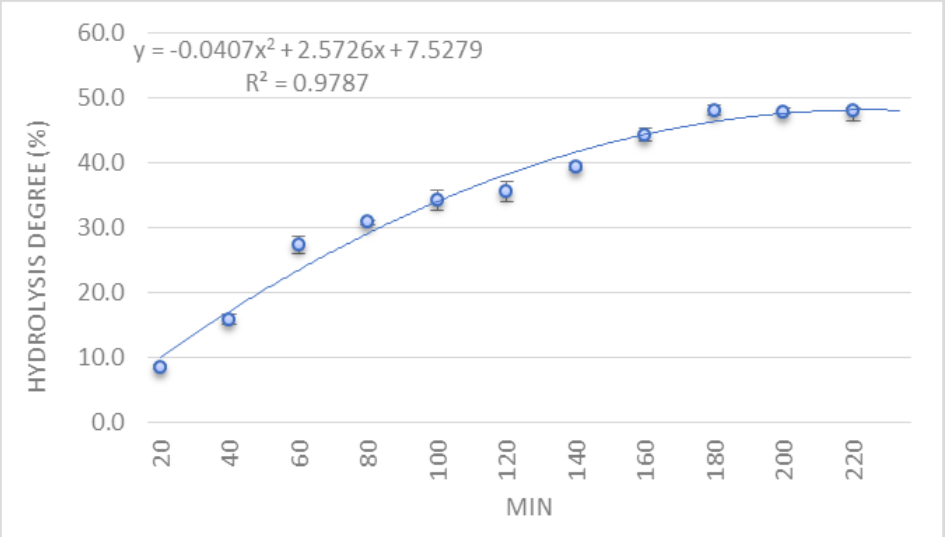

Figure 2. Effect of hydrolysis time on degree of hydrolysis of SCI protein

3.4. Determination of K_m and V_{max} of SCI hydrolysis reaction by Papain

Linear regression analysis was applied to assess the relationship between product concentration and hydrolysis time, showing that this is a direct linear relationship. The determination coefficients close to 1 for the three initial substrate concentrations indicated that the product concentration had a general tendency to increase as the initial substrate concentration and hydrolysis time increased. According to the time-course hydrolysis curves given in Figure 4, the values of parameters K_m and V_{max} corresponding to different experimental conditions were calculated. The analysis found that the coefficient of determination (R-squared) is 0.98, respectively. The good linear relationship between the variables demonstrated the validity of the proposed reaction model of the SCI-papain system.

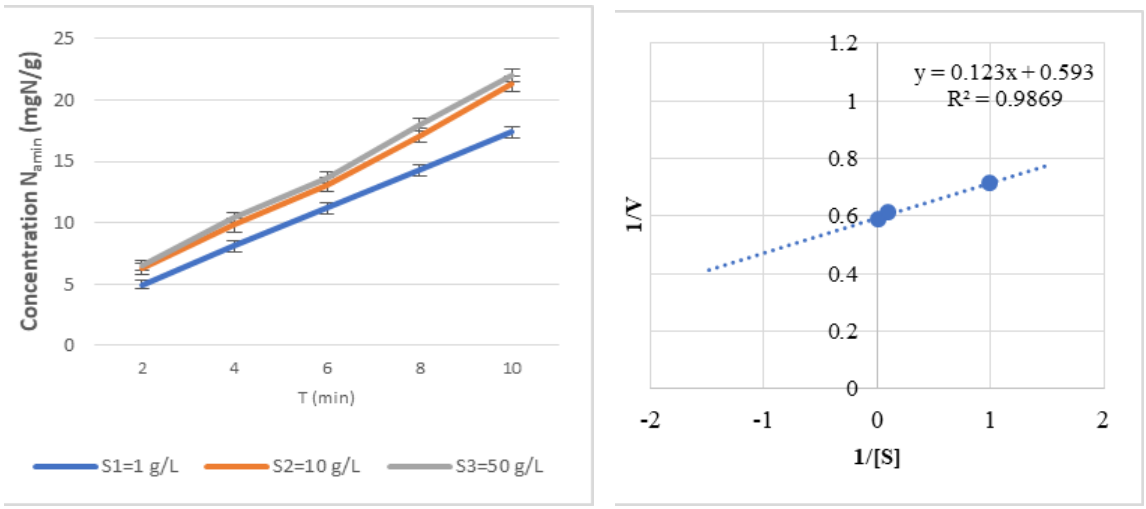

Figure 3. Correlation between the concentration of N_{amin} release and hydrolysis time (left) and Lineweaver-Burk plots for SCI hydrolysis by Papain (right).

The reaction rate increased quickly when increasing the substrate concentration from 1 to 10 g/L, and when continuing to increase the concentration to 50 g/L, the rate increase was not significant. The substrate concentration from 1 to 10 g/L was suitable for enzyme activity, so the product concentration was high. When the concentration raised too high (50 g/L), competition for space occurs, reducing the reaction rate. We obtained the equation of the line that will be used to calculate the values of K_m and V_{max} of Papain by using the Michaelis-Menten equation transformed into its opposite or the Lineweaver-Burk into the equation as below:

$$Y = 0.123\ x + 0.593 \text{ or } 1/V = 0{,}123\ (1/[S]) + 0{,}593$$

K_m and V_{max} were calculated: $V_{max} = 1.69$ mgN/$_{min}$ and $K_m = 0.21$ g/L

K_m and V_{max} are two important parameters in kinetic study of enzyme. The velocity of an enzymatic reaction will increase with increasing substrate concentration, but will reach a fixed value after further increasing the substrate concentration. In the specific enzyme concentration value, V is almost linear with increasing substrate concentration. K_m is the Michaelis - Menten constant that characterizes the affinity between the enzyme and the substrate, the smaller the Km value, the greater the affinity between the enzyme and the substrate. The V_{max} value plays a role in selecting the appropriate enzyme and substrate concentration in the practical application. In a study of Elsson *et al*., kinetic parameters of Papain hydrolysis on casein substrates were determined (K_m = 0.249 g/L, V_{max} = 1.514 ppm) [13], compared with our results, SCI is a preferable substrate for Papain.

3.5. The amino acid composition of SCI hydrolysates by Papain

In the present study, sixteen amino acids were identified, including eight essential amino acids and eight non-essential amino acids (Table 1). The products typically have a ratio of essential amino acids to eight non-essential amino acids of 0.5, indicating the high quality of marine proteins and their potential as a source of balanced dietary proteins. Glutamic acid was the predominant amino acid in SCI hydrolysate, which is consistent with the amino acid profile reported by Mamelona *et al*. [10] for fresh *Cucumaria frondosa* viscera and dried cucumber viscera [6]. Glutamic acid was also the most abundant amino acid in sea cucumber body wall [3]. The most abundant essential amino acid in the SCI hydrolysate was Valine, followed by Lysine and Leucine. Only low levels of Arginine, Proline and Tyrosine were detected in the SCI hydrolysate.

Glutamic acid and its derivative glutamine have been reported with various health benefits, including anticancer activity, cell proliferation, wound healing, improvement of protein metabolism, enhancement of immune system, and prevention of bacterial translocation. Valine helps stimulate muscle growth and regeneration and is involved in energy production. It is an essential amino acid important for nervous system, immune and cognitive function. Leucine is often considered preferable to other branched-chain amino acids because it’s broken down and absorbed more rapidly, allowing it to be used more readily than other types, such as isoleucine and valine. Currently, sea cucumber is one of the most important sources of bioactive peptides. The high composition of essential amino acids in the SCI hydrolysate reflects the premium quality of the protein from this source, indicating its potential use in functional foods or pharmaceutical industry.

Table 1. Amino acid profile of SCI protein hydrolysates by Papain

Number	Amino acids	Papain- SCI hydrolysate (mg/g Sample)
1	Alanine	19.245
2	Arginine	0.660
3	Aspartic acid	1.155
4	Glutamic acid	31.815
5	Glycine	14.145
6	Histidine *	1.920
7	Isoleucine *	1.995
8	Leucine *	3.210
9	Lysine *	4.170
10	Methionine *	1.125
11	Phenylalanine *	2.220
12	Proline	0.780
13	Serine	2.475
14	Threonine *	3.645
15	Tyrosine	0.900
16	Valine *	6.960
Total		96.420

* Essential amino acids

3.6. DPPH free radical scavenging activity

DPPH free radical scavenging activity of SCI hydrolysate was tested at different DH of 5, 10, 15, 20, 25 and 30 %. The results for the DPPH scavenging activity are shown in Figure 4. The DPPH free radical scavenging activities of SCI hydrolysate were dependent on DH. The degree of hydrolysis indicated quadratic effects of the DPPH scavenging activity. The scavenging activity of DPPH increased with increasing DH from 5 % to 15 % and then gradually decreased. This could be due to the difference in the sequence of peptides released during hydrolysis. In this study, the samples underwent more hydrolysis and released smaller peptides exhibiting lower DPPH free radical scavenging activity. The effect may also be attributed to the specific amino acid sequences in the hydrolysates, typically hydrophobic amino acids. A previous study performed with marine sources revealed that the molecular weights of peptides influenced the antioxidant activity of the hydrolysates [14]. The radical scavenging ability of protein hydrolysates depends mainly on various factors such as the size and amino acid composition of the peptides, and the specificity of the protease. As reported by Safari et al., the hydrolysates derived from sea cucumber body exhibited DPPH scavenging activity that increased with increasing the protein hydrolysis time [15]. The sea cucumber collagen hydrolysates also exhibited excellent radical-scavenging activity [16]. Yan *et al.* reported that sea cucumber viscera hydrolysates prepared with alkaline proteases such as Alcalase and

Flavourzyme possess higher antioxidant activities than other tested enzymes including papain, bromelain and pepsin [11]. The results showed that the deep hydrolysis by Papain could lose the antioxidant activity, so choosing the right hydrolytic enzyme and hydrolysis time to reach the right hydrolysis level is an important factor in food processing.

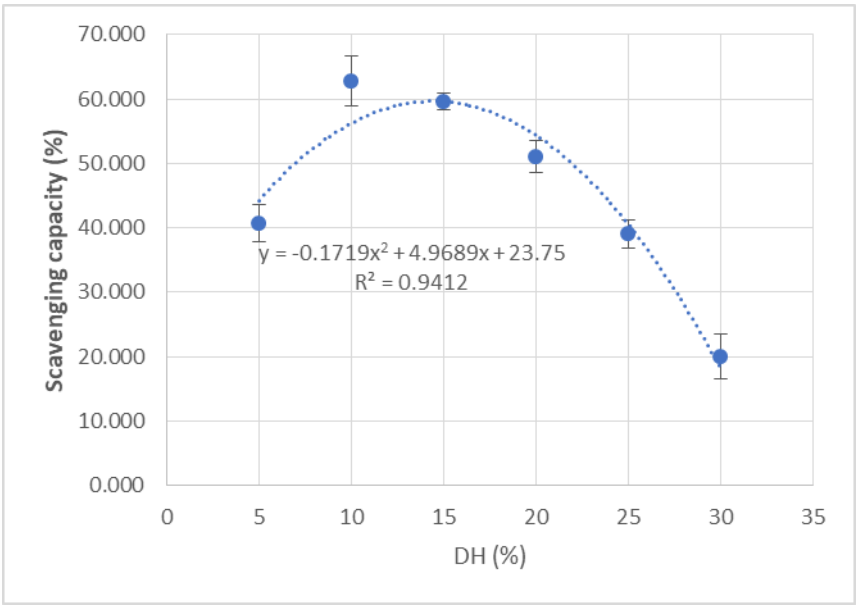

Figure 4. DPPH scavenging capacity of SCI hydrolysate at different hydrolysis degrees

4. CONCLUSIONS

In this study, kinetic parameters of Papain-SCI hydrolysis reaction were determined. The optimized levels of the E/S ratio and time to degree of hydrolysis were found to be 0.06/75 (w/w) and 180 min, respectively. The present study identified a large amount of nutrients (Glutamic, Valine, Leucine) in SCI hydrolysate, and addressed their potential for further processing into functional foods. Enzymatic hydrolysis improves the quality and functional characteristics of by-products and has been employed to obtain hydrolyzed proteins with better nutritional characteristics and bioactive compounds. Therefore, the hydrolysates prepared from the processing by-products of sea cucumber can be used as a natural value-added ingredient with antioxidative properties and functionalities. In addition, sea cucumber protein hydrolysates can serve as a good source of dietary protein due to their rich profile of essential amino acids. This study creates a paradigm for future research on hydrolyzing sea cucumber innards for the production of value-added products.

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CRediT authorship contribution statement. Author 1: Methodology, Investigation, Writing manuscript. Author 2: Formal analysis. Methodology.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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