Int. J. Aquat. Biol. (2020) 8(3): 184-193
ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.ij-aquaticbiology.com

Original Article

Antioxidant and antibacterial properties of protein hydrolysate from rocky shore crab, *Grapsus albolineathus*, as affected by progress of hydrolysis

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Abstract: Antibacterial and antioxidant activity of the rocky shore crab, *Grapsus albolineathus*, protein hydrolysate (CPH), with different degree of hydrolysis (DH) prepared using alcalase was investigated. The results showed that by increasing DH with reaction time up to 90 min, the DPPH radical scavenging activity of the hydrolysates raise, followed by a decrease in the next stages from 90 to 180 min. Interestingly, ABTS radical scavenging of the hydrolysates increase up to 120 min, and CPH_{120} show the highest activity with no significant difference with CPH_{90} and CPH_{180}. The degree of hydrolysis applied a significant influence on the antibacterial activity of crab hydrolysates against gram-positive bacteria, with a significant increase up to 90 min. The maximum zone of inhibitions was recorded against *Listeria monocytogene* for CPH_{90}:14.55 mm. The results suggest that the alcalase hydrolysis of rocky shore crab can produce bioactive peptides with potent antioxidant and antibacterial activities as affected by the degree of hydrolysis up to a certain level.

Article history:
Received 18 Julay 2019
Accepted 31 May 2020
Available online 25 June 2020

Keywords:
Crab protein
Hydrolysis
Antioxidant
Antibacterial

Introduction

Marine by-products comprise fishery wastes or low commercial species. Enzymatic hydrolysis is one of the best methods for protein recovery from such a marine protein sources (Petrova et al., 2018). The primary purpose of a hydrolysis process is to obtain high-quality protein with functional properties. An enzymatic hydrolysis process under optimized conditions could improve the functional properties and bioactivities of protein hydrolysates. Several studies have been proved alcalase 2.4L (from *Bacillus licheniformis*) as an effective enzyme for controlled protein hydrolysis due to high degree of hydrolysis in a short duration under moderate pH condition (Kristinsson and Rasco, 2000; Rajapakse et al., 2005; Ovissipour et al., 2009). Bioactivity of protein and peptides from fish and shellfish by-products are of great interest for pharmaceutical and sea food industries. The antioxidant activity of threadfin bream surimi wastes (Wiriyaphan et al., 2012) and unicorn leatherjacket skin (Karnjanapratum and Benjakul, 2015), ACE inhibitory and antihypertensive activities of tilapia by-product and mackerel skin gelatin (Khiari et al., 2013), anticancer activity of shrimp shell waste (Kannan et al., 2011) and gelatin hydrolysate of unicorn leatherjacket skin (Karnjanapratum et al., 2016), and antimicrobial activity of American lobster (Battison et al., 2008) and Atlantic mackerel (Ennaas et al., 2015) are some of works in this regard.

Natural compounds with high reactive oxygen species (ROS) reduction activity have attracted a great consideration since ROS are associated in cancer development. The great advantageous of the antioxidant peptides isolated from marine sources in reactive oxygen species (ROS) and free radicals scavenging and terminating the radical chain reaction were reported (Ngo and Kim, 2013).

In addition, during the past decades, the increment of antibiotic-resistant bacteria has been required for natural antimicrobial agents. Therefore, natural

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antimicrobial agents have attracted increasing attentions due to their potential role as biopreservative or therapeutics (Sperstad et al., 2011). Antimicrobial peptides (AMPs) are part of the innate immune systems of some animal species, as a first line of defense against infections and have been recently isolated and characterized from different organisms (Sila et al., 2014b). To date, several antimicrobial peptides (AMP_S) with low molecular weights and cationic nature which are active against diverse group of bacteria have been identified and isolated from marine organisms by enzymatic hydrolysis (Doyen et al., 2012; Ghanbari et al., 2012; Beaulieu et al., 2013; Sila et al., 2014a). However, the information regarding the functional properties of bioactive peptides from crabs as protein-rich by-products is limited.

Grapsus albolineatus is a relatively large-size crab species in the Persian Gulf, where it has a large population on the rocky intertidal shores. Therefore, purpose of the present study was to produce protein hydrolysate from *G. albolineatus*, as a non-commercial species, in the controlled hydrolysis conditions of the alcalase and to determine the effect of degree of hydrolysis (DH) on the antioxidant and antibacterial activity of hydrolysates produced.

Materials and Methods

Summarizing the experimental design is shown in Figure 1. In the current study, alcalase was provided from Novozymes (Denmark), and methanolwas from Merck (Darmstadt, Germany) companies. ABTS 2,2-azino-bis(3-ethylbenzothiazoline- 6-sulfonic acid) diammonium salt, DPPH (2,2-diphenyl-1-picryl hydrazyl), Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) and TCA (trichloroacetic acid) were purchased from Sigma (St. Louis, MO, USA). BHI (brain–heart infusion broth, life, Milan, Italy) and MRS (De Man, Rogosa and Sharpe, containing: peptone 10 g L^{-1}; yeast extract 4 g L^{-1}; glucose 20 g L^{-1}; beef extract 8 g L^{-1}; K_2HPO_4 2 g L^{-1}; sodium acetate trihydrate 5 g L^{-1}; ammonium citrate 2 g L^{-1}; $MgSO_4$, Merck, Germany) used as culture media. Bacterial strains in the study, including *Listeria monocytogenes* (PTCC 1306), *Staphylococcus aureus* (PTCC 1112), *Escherichia coli* (PTCC 1330) and *Lactobacillus sakei* (*PTCC* 1712) were preserved strains from the Persian Type Culture Collection, IROST, Iran.

Samples preparation: Live specimens of *G. albolineatus* (N=10, 55±2.63 g) were collected off rocky shore of the Qeshm Island, the Persian Gulf (Fig. 2). The samples were frozen at −20°C, and then stored in a sealed Styrofoam box containing ice and transported to the laboratory within 2 hrs.

Enzymatic hydrolysis: The total body of *G. albolineatus* was first minced using a grinder meat mincer (Parskhazar, Tehran, Iran) blades, then for inactivating the endogenous enzymes, the sample heat-treated at 85°C for 20 min. Afterwards, it was mixed with deionized water in a ratio of 1:2 and homogenized. Enzymatic hydrolysis was performed with alcalase (2% v/w) under the optimal condition of the enzyme (pH 8.5; 55°C). Crab protein hydrolysate (CPH) were taken at 30 (CPH_{30}), 60 (CPH_{60}), 90 (CPH_{90}), 120 (CPH_{120}) and 180 (CPH_{180}) min of hydrolysis and inactivated at 95°C (15 min) followed by 10 min centrifugation at 8000 g.

Estimation of soluble protein (SP): Soluble protein concentration during different stages of the hydrolysis was determined based on Lowry et al. (1951). BSA (Bovine Serum Albumin) was used as the protein standard. Absorbance was measured at 540 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Change in the amount of soluble protein was calculated as the ratio of soluble protein in the hydrolysate relative to the initial amount of soluble protein in non-hydrolyzed mixture by following equation:

Soluble protein Ratio = [Soluble protein in a hydrolyzed crab-soluble protein in non-hydrolyzed crab / Soluble protein in nonhydrolyzed crab] × 100.

The degree of hydrolysis (DH)L: The DH of all hydrolysates (CPH_{30}, CPH_{60}, CPH_{90}, CPH_{120}, and CPH_{180}) were measured according to the method described by Hoyle and Merritt (1994), using TCA (trichloroacetic acid) protein precipitation. Briefly, an equal volume of 20% TCA was added to the

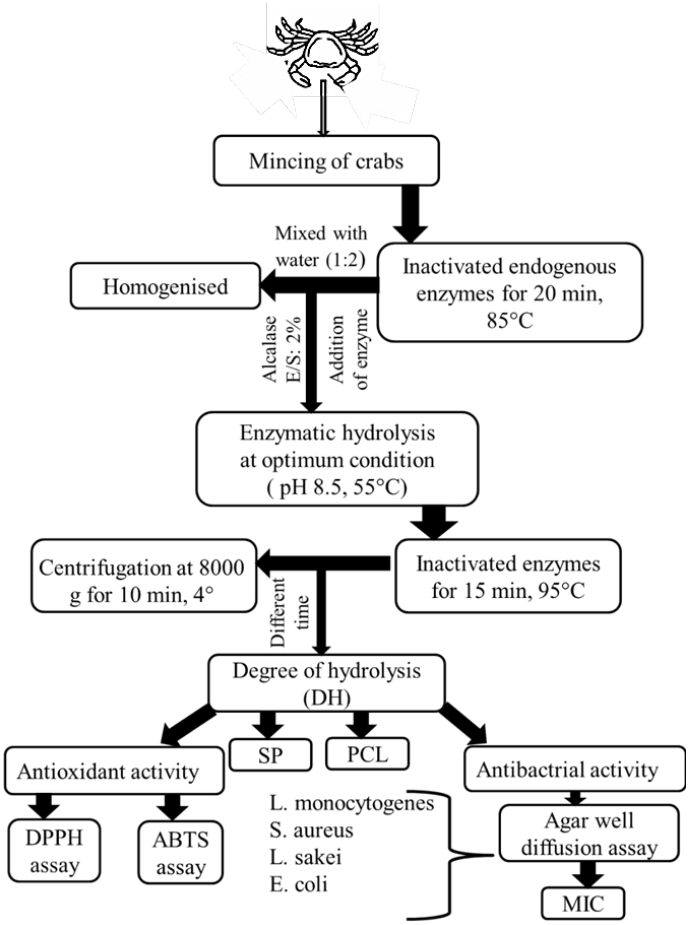

Figure 1. Flow diagram of the crab hydrolysis process and investigation of antioxidant and antibacterial activities.

hydrolysates followed by centrifugation at 6700×g for 20 min. The following equation was applied to calculate the DH:

DH (%) = [Soluble protein in TCA-sample mixture / Soluble protein in initial sample] × 100

Peptide chain length (PCL)*:* The average peptide chain length (PCL) introduced by Adler-Nissen (1986) was calculated from the degree of hydrolysis in the following equation.

$$PCL = 100/ DH$$

Antioxidant activity

DPPH assay: The DPPH radical scavenging activity was assayed according to the methods described by Binsan et al. (2008) with a slight modification. Briefly, 1.5 ml of samples (CPHs) were added to 1.5 ml of 0.15 mM methanolic solution of DPPH. The mixture was vigorously shaken and then incubated at 25°C in the dark for 30 min. The absorbance was read at 517 nm by a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The DPPH radical scavenging capacity was estimated according to the following equation:

$$\text{Radical-scavenging activity (\%)} = [(A_{Control} - A_{Hydrolysate} / A_{Control})] \times 100$$

The Trolox standard curve was used to determine the antioxidant capacity as μmol Trolox/mg protein sample.

ABTS assay: ABTS radical scavenging activity was determined by the slightly modified method of Re et al. (1999). For the present study, the test samples and ABTS solution were produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulphate and leaving the mixture in the dark for 12-16 hrs. Afterward, the solution was diluted with phosphate buffer (5 mM, pH 7.4) to an absorbance of 0.700±0.05 at 734 nm. Then 25 μl of sample was added to 1 mL of reagent and incubated at 25°C. The calculation of scavenging percentage is as described for the DPPH assay. The activity was calculated as μmol Trolox/mg protein sample using Trolox standard curve.

Antibacterial activity

Bacterial strains and growth media: The antibacterial activity of hydrolysate samples was studied against five gram-positive and gram-negative bacterial strains, including *L. monocytogenes* (PTCC 1306), *S. aureus* (PTCC 1112) and *E. coli* (PTCC 1330) grown in BHI and *L. sakei* (*PTCC* 1712) in MRS broth.

Antibacterial assay: Antibacterial activity was determined by the agar well-diffusion assay method and expressed by measuring the diameter of the inhibition zone (IZ) (Sreeramulu et al., 2001). IZ was measured in mm, and expressed as mean±SD. The culture medium (20 mL) was poured into Petri dishes (90 mm in diameter). A 20 μL of 24 h cultured suspensions of reference strains were spread on the plates uniformly, and wells of 6 mm in diameter were made. Sterile protein hydrolysate samples (40 μL) obtained from hydrolysis of the protein sample (1 mg dry weight /ml) during different times of hydrolysis, were transferred into the wells. The plates were incubated at 37°C for 24 hrs.

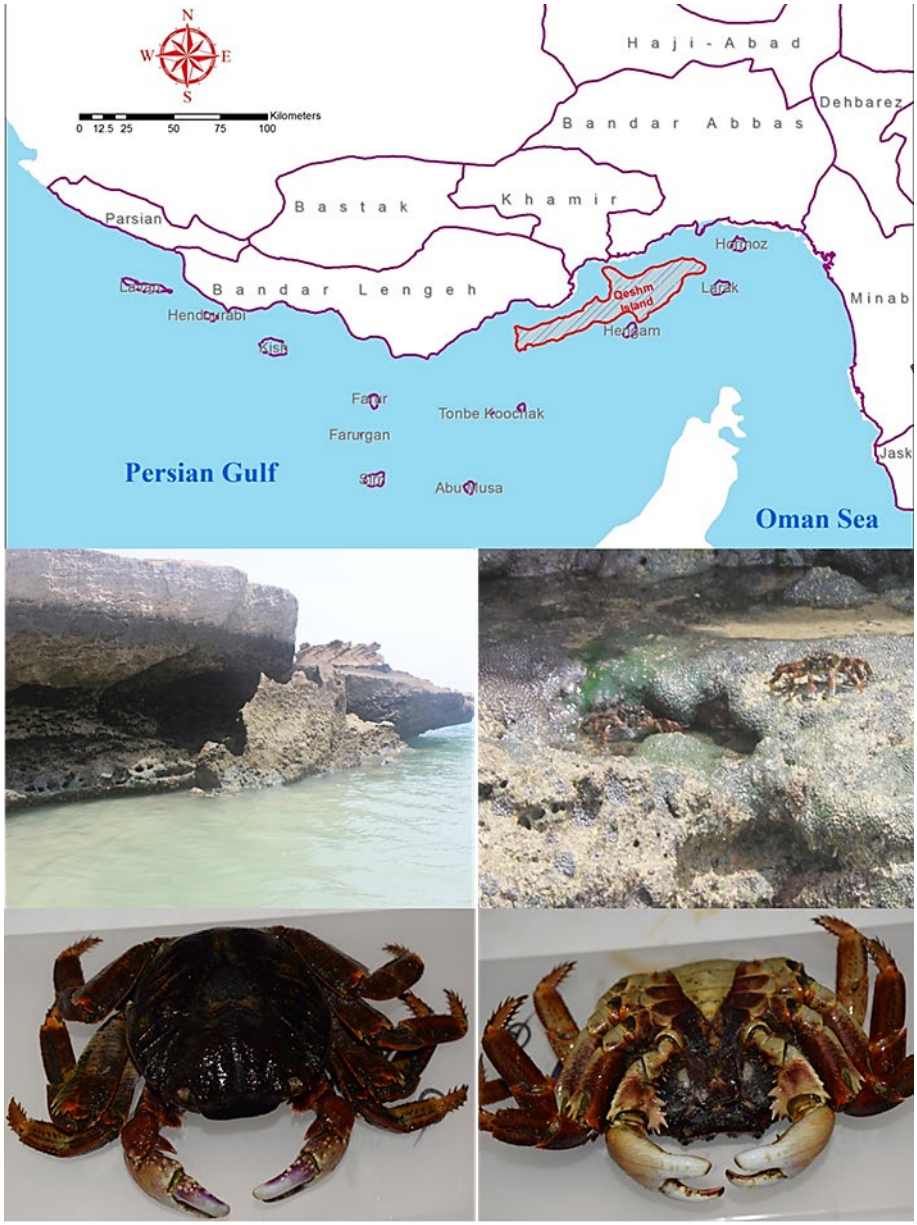

Figure 2. Rock crabs *Grapsus albolineathus*, from rocky shores of the Qeshm Island and nearby intertidal zones.

Bacteriocidal (MIC) studies: The minimum concentration (MIC) of the sample that prevents bacterial growth was determined for the protein hydrolysate with the most inhibition zone through the broth microdilution method in the 96-well microplates (Portella et al., 2009). The turbidometric method was used to determine the antibacterial activity of each concentration of the sample (Tafreshi et al., 2010). The indicator suspension was adjusted to a McFarland standard of 0.5. Then 50 µl of indicator strains (5×10^6) were added into 96-well plates containing 50 µl supernatant (serial dilution concentration of hydrolysate) and 100 µL nutrient broth. Bioactivity was calculated by determining the turbidity of the cell suspension after 24 hrs of incubation at 600 nm by a spectrophotometer (Turcotte et al., 2004). The lowest concentration of the samples showing no turbidity was recorded as MIC.

Statistical analysis: The data were analyzed using SPSS (Statistical Package for the Social Sciences version) 19.0 (Chicago, Illinois, USA). Analysis of Variance (ANOVA) and Duncan's Multiple Range Test was used to evaluate the differences between samples at $P\leq0.05$.

Table 1. Antibacterial activity of rocky shore crab protein hydrolysate with different DH.

Bacteria strains	CPH_{30}	CPH_{60}	CPH_{90}	CPH_{120}	CPH_{180}
Staphylococcus aureus	8.70 ±0.00b	9.16±0.25^{b}	10.68±0.51^{a}	10.83±0.68^{a}	**11.00±0.44^{a}**
Listeria monocytogene	11.66±0.55^{c}	12.50±0.00^{b}	14.50±0.44^{a}	14.16±0.51^{a}	**14.30±0.55^{a}**
Lactobacillus sakei	-	11.00±0.00^{a}	11.00±0.68^{a}	11.00±0.00^{a}	**11.16±0.25^{a}**
Escherichia coli	-	-	-	-	-

Inhibition zones: (mm), (-): no activity. Different letters indicate significant differences between groups. Mean ± SD, N = 3.

Results

Degree of hydrolysis (DH), peptide chain length (PCL) and soluble protein (SP): The DH at the end of hydrolysis was 38.12% with the PCL of about 2.62, and maximum protein soluble ratio of the hydrolysates was 45.83% (Fig. 3A, B). As described in Figure 3A, the DH values increased rapidly with increasing the hydrolysis duration from 15.11 to 32.2% and the PCL of the hydrolysates decreased correspondingly during the first 90 min ($P \leq 0.05$). This positive DH trend followed by a slight increase until 120 min (CPH_{120}), and then the enzymatic reaction reached the steady-state phase up to 180 min at DH 38.12 (CPH_{180}) ($P \geq 0.05$). The PCL trend followed by a sequential decrease in the rate up to 180 min ($P \geq 0.05$) (Fig. 3A). Figure 3B indicates that during the hydrolysis of rocky shore crab, SP increased with increasing of DH up to a certain level (at 90 min) ($P \leq 0.05$). By increasing time up to 180 min, the ratio was slightly stopped in the late phases of hydrolysis ($P \geq 0.05$).

Antioxidant activity: The DPPH radical-scavenging capacity of CPHs improved with increasing DH ($P \leq 0.05$), except for an increase from 32.2 (CPH_{90}) to 36.8% DH (CPH_{120}) ($P \geq 0.05$) (Fig. 4). The results showed that when DH increased up to 32.2%, the CPH_3 exhibited the strongest DPPH radical scavenging ($P \leq 0.05$). In the ABTS assay, the ABTS radical scavenging activity increased interestingly with increasing DH and reached a maximal value at DH 36.8% for CPH_{120} ($P \leq 0.05$), with no noteworthy difference with CPH_{90} and CPH_{180} ($P \geq 0.05$) (Fig. 5).

Antibacterial activity: The antibacterial effects of the five types of rocky shore crab hydrolysate with different DH were determined by the appearance of zone of growth inhibition around the well containing the hydrolysates (Table 1). The results showed that the

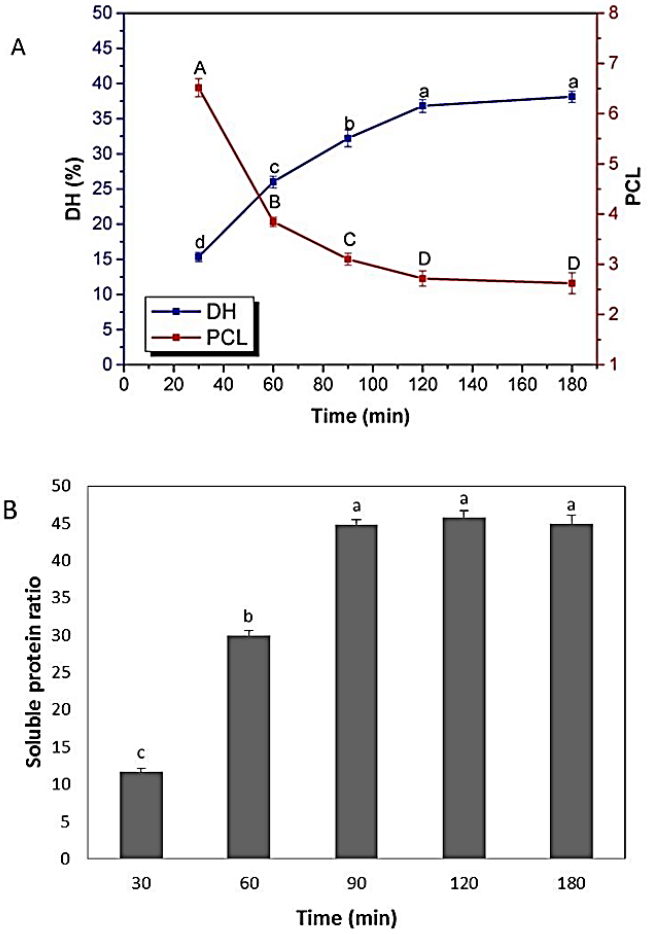

Figure 3. Effect of hydrolysis time on the degree of hydrolysis (DH) and peptide chain length (PCL) (a) Effect of hydrolysis time on the soluble protein ratio (SP) (b). Different letters indicate significant differences between groups, (mean ± SD, N = 3).

antibacterial activity of the rocky shore crab hydrolysates significantly increased by the progress of hydrolysis. The strongest antibacterial activity was obtained against *L. monocytogenes* with IZs of 14.50 mm by CPH_{90} followed by *S. aureus* with IZs of 11.16 mm by CPH_{180} and *L. sakei* with IZs of 11.00 mm by CPH_{180}. There was no significant difference with IZs of CPH_{90}, CPH_{120}, and CPH_{180} against these three bacteria ($P \geq 0.05$). The MIC value of CPH_{90} as the

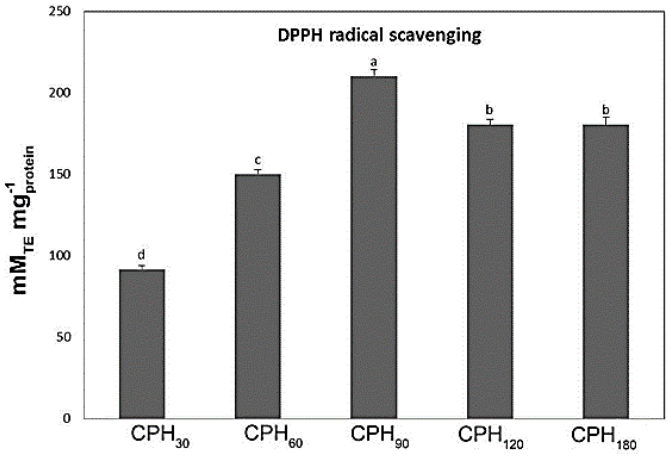

Figure 4. DPPH radical scavenging activity of rocky shore crab protein hydrolysate with different DH. Different letters indicate significant differences between groups, (mean ± SD, N = 3).

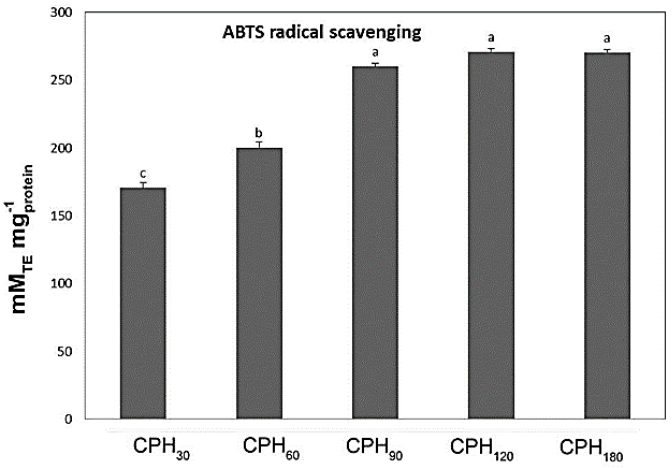

Figure 5. ABTS radical scavenging activity of rocky shore crab protein hydrolysate with different DH. Different letters indicate significant differences between groups, (mean ± SD, N = 3).

most potent hydrolysate against *L. monocytogenes* was evaluated to be about 2.75 mg/ml. The minimum activity was observed against *S. aureus* for CPH_{30} with IZs of 8.70 mm. There was no activity against *E. coli.* An increase in DH value up to 32.2% within the first 90 min was caused an improvement in the antibacterial activity value (for CPH_{30} to CPH_{90}) ($P \leq 0.05$) while in the next phases, with the slight increase in DH value up to 38% (for CPH_{90} to CPH_{180}) antibacterial activity did not change significantly ($P \geq 0.05$) (Table 1).

Discussions

Progress of enzymatic hydrolysis: One of the most imperative outcomes of hydrolysis is the capacity to part the soluble and insoluble proteins from each other in enzymatic processes (Guerard et al., 2002). Enzymatic hydrolysis is a process in which enzymes facilitate the cleavage of the peptide bonds and releasing the bioactive peptides that are inactive within the sequences of the parent proteins with the usually 2-20 amino acid residues in length (Ryan et al., 2011). Therefore, monitoring the progress of hydrolysis by the degree of hydrolysis (DH), the peptide chain length (PCL) and the amount of soluble protein (SP) could describe the hydrolysis process.

According to the results, the fast reaction within first 90 min of the hydrolysis implied that alcalase cleaved the most susceptible peptide bonds in the early phase of hydrolysis and the most available cleavage sites for alcalase to act were at this time. A sequential decrease in the progress of hydrolysis in the steady-state phase can be attributed to the competition between the substrate and the hydrolysates achieved, the reduction in peptide bonds with the capacity to being cleaved, and enzyme denaturation that lessens its activity (Adler-Nissen, 1986). Similar DH trends have been reported for other crustaceans alcalase hydrolysates (Dey and Dora, 2014; Gunasekaran et al., 2015; Antunes-Valcareggi et al., 2017). The increased soluble protein in the initial phase of the hydrolysis could be because of an increase in the concentrations of soluble peptides released by the alcalase digestion of the crab crude protein. Reduction in the number of peptide bonds available for hydrolysis could be attributed to the reduction in the rate of hydrolysis and stability in the percentage of SP during the later stages of hydrolysis.

Antioxidant activity: The wide specificity of proteases to peptide bonds could generate a vast range of smaller peptides and free amino acids with diverse activities. The results of radical scavenging activity assays (DPPH and ABTS) showed that the alcalase hydrolysates of rocky shore crab with different DH are enriched with antioxidant peptides, which can act as an electron donor and could convert the free radicals to stabilized products. In several cases, alcalase represented a good candidate for producing

antioxidant peptides based on the ability to cleave the peptide bonds from the interior of the peptide chain and releasing short or medium chain peptides containing hydrophobic amino acids such as Leu, Phe, Ile, Val, Trp, Met and Tyr. It was hypothesized that the presence of these hydrophobic amino acids in the protein sequence of hydrolysate could enhance the radical scavenging activity (Rajapakse et al., 2005; Song et al., 2008; Dryáková et al., 2010; Gallegos-Tintoré et al., 2011).

The results also demonstrated that an increase in DH and reduction in PCL resulted in more antioxidant activity of crab hydrolysates up to a certain level. Several studies preferred hydrolysates with the highest DH to warrant the existence of small peptides (Jeon et al., 1999; Kim et al., 2001; García-Moreno et al., 2014). This could be because of small size of the peptides obtained by progress of the hydrolysis may facilitate electron reduction and results in more antioxidant capacity. Zhang et al. (2015) pointed out that the DPPH radical scavenging capacity of oyster hydrolysates increased with the progress of protein hydrolysis by alcalase (DH: 30.12 to 39.89%) due to producing small and medium-sized peptides with active amino acid residues. In contrast, You et al. (2009) reported that although the DPPH and ABTS radical scavenging ability of papain hydrolysate of loach hydrolysis increased when the DH increased from 18 to 23%; this value decreased when the DH was increased from 23 to 33%. Prolonged hydrolysis and excessive increase in DH may result in the formation of highly soluble peptides or free amino acids, completely lacking the functional properties of the native proteins (Kristinsson and Rasco, 2000). Hence, the lack of a direct relationship between the hydrolysates radical scavenging activities and DH at the final stages of hydrolysis (90-180 min), could be attributed to the presence of free amino acids or peptides with different size, solubility and sequence compared to peptides in the first stages of hydrolysis, resulting in lower antioxidative activity.

Antibacterial activity: The antimicrobial activities of AMP are contributed to the charge, size, hydrophobicity, and solubility of their structure. While the peptide length required for spanning bacterial membrane is approximately under 22 amino acid residues, the adequate peptide solubility is critical for the antimicrobial peptides performance to suppress bacterial growth (Bahar and Ren, 2013). The results showed the interesting antibacterial potential of all alcalase hydrolysates of rocky shore crab against all gram-positive bacteria and no activity against gram-negative bacterium *E. coli*. This antibacterial activity is probably due to the release of some small and medium-sized peptides consisting of the hydrophobic amino acids upon alcalase hydrolysis. The hydrophobic characters of antibacterial peptides have been found as the main factor in peptide interactions with bacterial membranes, while they can directly kill bacteria or inhibit bacterial growth (Sperstad et al., 2011). The reason for no antibacterial effect of CPHs against *E. coli* can be due to the lack of active amino acid sequences with inhibitory effect towards the bacterial membrane composition.

The results also indicated that the antibacterial activity of the rocky shore crab hydrolysates significantly enhanced by the progress of hydrolysis up to certain level. This result suggested that cleavage of the peptide bonds alongside with subsequent decrease of PCL and SP in the initial stage of hydrolysis, exert a considerable influence on the antibacterial activity, but the slight progress of hydrolysis in the last phases could not improve this activity.

Similar to our result, the oyster alcalase hydrolysates showed inhibitory effect against the microorganisms viz. *L. monocytogenes, E. coli*, *S. aureus*, *P. aeruginosa*, *V. harveyi* and *V. parahaemolyticus*, whereas the highest DH hydrolysate (39.89%) showed a higher antimicrobial activity than lower DH hydrolysates with the maximum IZs of 13.44 mm against *V. parahaemolyticus* (Zhang et al., 2015). Sila et al. (2014b) indicated that five alcalase hydrolysates (DH: 2.8, 5.1, 6.6, 10.45 and 13%) from barbel muscle protein that show antibacterial activity, no antibacterial activity was detected with its hydrolysates with the highest DH (14.58 and 16.2%

after 2 hrs). In this case, the FII-3 sub-fractions, which obtained from purification and identification of (6.6% DH) hydrolysate showed the hydrophobic characteristics with a protective effect against pathogenic bacteria (*L. monocytogenes, E. coli*, *P. aeruginosa*, *K. Pneumonia.*, *S. aureus* and *M. Luteus*). According to the results, the progress of alcalase hydrolysis of rocky shore crab is led to increase the number of the soluble peptides with small and medium size and higher availability of hydrophobic regions. These structural changes of the protein improve the antibacterial activity of hydrolysates up to a certain level.

As conclusion, it can be mentioned that the hydrolysis of rocky shore crab enhances its antioxidant and antibacterial activities. We demonstrated that the progress of hydrolysis degree with time could modulate the biological activity of hydrolysate at a certain level, due to the presence of small and medium-sized peptides with specific amino acid composition, revealing the most antioxidant and antibacterial activity. Hence, the careful monitoring of the degree of hydrolysis based on enzyme specificity is crucial due to generating peptides with different molecular size and amino acid contents and resultant differences in bioactivity. Thus, alcalase hydrolysates of rocky shore crab may potentially serve as a suitable natural antioxidant and antibacterial source for future purification and identification.

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