Hindawi
Journal of Food Quality
Volume 2018, Article ID 9354829, 8 pages
https://doi.org/10.1155/2018/9354829

Research Article

Angiotensin-I-Converting Enzyme Inhibitory Activity and Antioxidant Properties of Cryptides Derived from Natural Actomyosin of *Catla catla* Using Papain

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Received 9 February 2018; Accepted 30 April 2018; Published 8 July 2018

Academic Editor: Khizar Hayat

Natural actomyosin (NAM) from the freshwater fish *Catla catla* was extracted and hydrolyzed using papain enzyme at different enzyme-to-substrate ratios (0.5%, 1.0%, 2.0%, 5.0%, and 10%) to obtain the cryptides with different degrees of hydrolysis (DH). Derived cryptides were evaluated for bioactive properties such as angiotensin-I-converting enzyme (ACE) inhibitory activity and antioxidant properties. The pattern of hydrolysis of NAM as a function of time revealed that major protein components such as myosin and actin were hydrolyzed within 10 min of hydrolysis. The cryptides obtained with the DH of 29.4% had significantly higher ACE inhibitory activity and linoleic acid peroxidation inhibitory activity ($P < 0.05$). A higher DPPH free radical-scavenging activity and ferric-reducing power were exhibited by the NAM cryptide mixture obtained with the DH of 17.38 and 26.2%, respectively. The natural actomyosin could be a potential precursor to produce the cryptides with therapeutical and antioxidant properties.

1. Introduction

In the last decade, the quantum of research on peptides derived from food proteins using enzymes has increased because of their health beneficial properties. Bioactive peptides or cryptides are peptide fragments that were encrypted in the primary sequences of proteins with different functions. Once they are released by hydrolysis *in vivo* or *in vitro* by proteases, cryptides confer positive health-promoting properties other than their basic nutritional role. Identifying a suitable protein source and the proteolytic enzyme for the preparation of cryptides is critical. It has been reported that hydrolysis of individual protein constituents yielded peptides with higher bioactivity than hydrolyzing the complex raw material [1]. The possible conformational changes of protein substrates and coexistence of multiple protein substrates may affect the accessibility and susceptibility of peptide bonds to proteolysis and subsequently the release of peptides of desired bioactivities [2]. This underscores the importance of using individual protein constituents during bioactive peptide preparation.

Bioactive properties of the fish protein hydrolysate (mixture of peptides) prepared using processing by-products and underutilized fish species have been reported, and a few have reached the commercial market. Knowledge on critical process parameters such as major and minor protein constituents in the raw material, enzyme-to-substrate ratio, pH, temperature, and enzyme specificity is essential to produce multifunctional peptides or different peptides, each contributing to a specific function [2]. One common measure widely used during proteolysis is the degree of hydrolysis which can be used as a tool to monitor the cryptide production on commercial scale. In spite of extensive research on the fish protein hydrolysate, the studies on fish protein model systems like actomyosin are scarce, and such studies are essential to have more insight into the hydrolysis process and properties of cryptides.

Studies have revealed that the proteins from aquatic sources are high-quality raw materials for the preparation of therapeutic cryptides [2]. However, studies on bioactive properties of peptides from individual protein constituents

of fish are limited. Natural actomyosin referred to the actomyosin preparation contains mainly myosin and actin in association with other regulatory proteins. The actomyosin complex from fish has been well studied with reference to the functional properties particularly the gel-forming ability, an important property in fish product development [3].

To date, ACE inhibitory and/or antihypertensive activity and antioxidant properties are probably the most intensively studied properties of bioactive peptides. Angiotensin-I-converting enzyme (ACE; EC 3.4.15.1) participates in the renin-angiotensin system and plays an important physiological role in regulating blood pressure. ACE is a peptidyl dipeptidase A and primarily cleaves a decapeptide (angiotensin-I) to an octapeptide (angiotensin-II) which is a potent vasoconstrictor. ACE also inactivates the dilatational function of bradykinin [4]. Therefore, inhibition of ACE activity is a major target in the prevention of hypertension.

Lipid oxidation is one of the major issues in the food industry, and the end products of lipid oxidation are potentially toxic to human health, which also affect the quality of food [5]. The oxidation of lipids leads to liberation of free radicals which are highly reactive and damage the biological macromolecules such as DNA, RNA, proteins, and enzymes. As a result, it causes cancers, neurological disorders, early ageing, Parkinson's and Alzheimer's diseases, and rheumatic and coronary heart diseases. Peptides/protein hydrolysates derived from fish proteins have the potential to minimize the oxidation of lipids during processing and storage of foods [6]. Synthetic antioxidants do possess higher antioxidative properties than the natural counterparts, but there is a concern about their safety on long-term usage. For investigating the antioxidant activity of derived peptides, selection of right assays is highly critical. Most commonly, the antioxidant potential is assayed through different types of assays. There are assays associated with lipid peroxidation, including the thiobarbituric acid assay (TBA). Other types of assays associated with the electron or proton donation mechanism include the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric-reducing antioxidant power (FRAP) assay. In the present study, the linoleic acid peroxidation model system, DPPH free radical-scavenging activity, and ferric-reducing antioxidant power were employed to evaluate the ability of peptides derived from natural actomyosin to donate the electron/proton.

With this background, the present study was aimed to prepare the cryptides from the natural actomyosin from the fish *Catla catla* using papain and to study their bioactive properties. Papain is the most studied cysteine enzyme due its commercial importance. Papain has been used to release the bioactive peptides from various food proteins [7] and is having broader specificity towards hydrolyzing the peptide bonds. In the present study, the hydrolysis pattern of NAM by papain was profiled. The bioactive properties such as ACE inhibitory activity and antioxidant properties of cryptides were evaluated as influenced by the extent of hydrolysis.

2. Materials and Methods

2.1. Fish. Fresh water carp, *Catla catla*, is harvested from the fish farm in College of Fisheries, Mangalore, Karnataka Province, India. The fish was washed in chilled water, eviscerated, and beheaded. Meat was separated manually and subjected to water washing using chilled potable water ($4 \pm 1^{\circ}$C). The quantity of water used for washing was 1 : 3 (meat : water, w/v). The slurry was agitated for 3 min and allowed to settle for 7–10 min. Water was decanted and filtered through the muslin cloth. The excess water was removed manually by squeezing the mince by placing between coarse cloths. Water washing was carried out to remove the sarcoplasmic protein fractions and lipids. Water-washed meat was used to prepare the natural actomyosin (NAM).

2.2. Chemicals. Papain (from the latex of *Carica papaya,* ≥3 U/mg), angiotensin-I-converting enzyme (ACE) (lyophilized powder from the rabbit lung), *N*-[3-(2-Furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG), DPPH (2,2-diphenyl-1-picrylhydrazyl) free radicals, iron (II) chloride, linoleic acid, tyrosine, sodium dodecyl sulphate, acrylamide, bis-acrylamide (*N*,*N*'-methylene-bis-acrylamide), 2-mercaptoethanol, Trizma base (tris[hydroxyl methyl]aminomethane), Coomassie Blue G and protein molecular weight markers (wide range, MW 205 kDa to 65 kDa), TEMED (N,N,N^1,N^1-tetramethylethylenediamine), and bromophenol blue were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade.

2.3. Preparation of Natural Actomyosin (NAM) from C. catla. NAM was prepared according to the method of Chaijan et al. [8] with a slight modification. Water-washed meat of catla (100 g) was homogenized in 500 ml of chilled phosphate buffer (pH 7) containing 0.6 M KCl for 4 min using a homogenizer at 9000 rpm (ULTRA-TURRAX T25, IKA Labortechnik, Staufen, Germany). The homogenization was carried out with a short span of 20 s followed by a stoppage for 20 s. The total time for actual homogenization was 2 min. The homogenate was kept in ice for 30 min to settle and subjected to centrifugation at 9000 ×g for 30 min at 4°C in a refrigerated centrifuge (Sorvall Legend XTR centrifuge, Thermo Fisher Scientific, New Hampshire, USA). The supernatant obtained was added slowly to ninefold of chilled double-distilled water (<5°C) and allowed to stand for 1 h in an ice bath to precipitate. The pellet was collected by centrifuging at 9000 ×g for 30 min at 4°C. The pellet obtained was referred as NAM. Total nitrogen content of the NAM pellet was analyzed by the Kjeldahl method as described in AOAC (2002) and multiplied by a factor of 6.25 to quantify the protein content. NAM was stored in refrigerator and used for hydrolysis within 48 h.

2.4. Preparation of Cryptides from Natural Actomyosin Using Papain. The NAM pellet of 100 g (3 g of protein) was dispersed in 200 ml of chilled double-distilled water and homogenized at 9000 rpm using the homogenizer for 2 min. Papain at different concentrations was used for hydrolysis. The concentration of the enzyme was based on protein content of NAM. The concentrations of the enzyme used

were 0.5%, 1.0%, 2.0%, 5.0%, and 10%. The NAM homogenate was preincubated at 50°C for 3 min prior to the addition of enzyme at different concentrations. The homogenate without papain was served as control. The reaction mixture was incubated for 1 h at 50°C, and the pH was 6.5 ± 0.2. After incubation, the reaction was terminated by keeping the mixture in a boiling water bath for 15 min. The slurry was filtered, and the supernatant obtained was referred as NAM cryptides, stored under refrigerated conditions, and used for the analysis within 48 h.

2.5. SDS-PAGE Profile of NAM. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the method described by Laemmli [9]. The samples (75 μg of protein) were loaded into the wells of the polyacrylamide gel (10% running and 4% stacking). The run was carried out on a constant-voltage mode of 30 V using the power pack (model PS-3000, Hofer Pharmacia Biotech Inc., Halliston, USA) till the samples reached the end of the stacking gel. Furthermore, the voltage was raised to 90 V, and the run was terminated when the dye front reached the bottom of the gel. A standard molecular weight marker of wide range was loaded into a separate well of the gel. After the run, the gel was stained in Coomassie Brilliant Blue G-250 (0.025% in 40% methanol and 7% acetic acid) for 30–40 min. The gels were destained using the acetic acid-methanol mixture (7% acetic acid and 2% methanol) till the protein bands were clearly visible. The molecular weight of the bands obtained in the sample was approximated by measuring the relative mobility of the standard protein markers.

2.6. Pattern of Cryptide Liberation from NAM by Papain. The NAM prepared from catla was subjected to proteolysis with papain using the following conditions: E/S ratio of 2.5 : 100, temperature of 50°C, pH of 6.5 ± 0.2, and duration of hydrolysis of 1 h. Aliquot samples were drawn from the reaction chamber and used for SDS-PAGE analysis to profile the generation of cryptides. The SDS-PAGE (10 and 15% gel) pattern was obtained after 10, 20, 30, 40, 50, and 60 min of hydrolysis. The NAM without enzyme incubated for 60 min at 50°C was used as control.

2.7. Monitoring the Extent of Proteolysis

2.7.1. Degree of Hydrolysis. Degree of hydrolysis was calculated as the ratio of α-amino nitrogen liberated from the NAM and total nitrogen content of NAM taken for the hydrolysis. The α-amino nitrogen was determined by formol titration according to the method as described by Taylor [10], and the total protein nitrogen was determined by the Kjeldahl method [11]. The following formula was used to calculate the degree of hydrolysis:

$$\text{DH } (\%) = \frac{\text{AAN} \times \text{TVS}}{\text{WM} \times \text{TN}} \times 100, \quad (1)$$

where AAN is the α-amino nitrogen (mg/ml of the supernatant), TVS is the total volume of the supernatant (ml), TN is the total nitrogen content (mg/g of NAM), and WM is the weight of NAM taken for hydrolysis (g).

2.8. Tyrosine Measurement. The extent of hydrolysis was also monitored by measuring the liberated tyrosine. The supernatant (150 μl) obtained after hydrolysis was diluted to 3 ml using double-distilled water, and the absorbance was measured at 280 nm using a double-beam UV-Vis spectrophotometer (Labomed, Inc., Los Angeles, CA, USA). A standard curve of L-tyrosine was used to quantify the liberated tyrosine from NAM and expressed as μM of tyrosine liberated/g of protein.

2.9. Bioactive Properties of NAM Cryptides Derived Using Papain

2.9.1. Angiotensin-I-Converting Enzyme Inhibitory Activity. The angiotensin-I-converting enzyme inhibitory (ACE) activity of NAM cryptides was determined according to the method described by Raghavan and Kristinsson [12] with the modifications described by Elavarasan et al. [13]. A known concentration of NAM cryptide solution (1 mg/ml) was prepared and used for the ACE inhibition assay. ACE enzyme (100 μl of 30 mU enzyme), 200 μl of cryptide solutions, and substrate (2 ml of 0.5 mM FAPGG substrate) were mixed, and the absorbance at 340 nm was continuously monitored with a double-beam spectrophotometer in kinetic mode option. The absorbance at 340 nm was monitored for 20 min at 25°C. The slope of the curve was used to calculate the percentage of ACE inhibition. A sample containing the FAPGG substrate and the ACE enzyme was used as control. ACE inhibitory activity of NAM cryptides was calculated as follows:

$$\%\text{ of ACE inhibition} = 1 - \frac{\text{slope of the sample curve}}{\text{slope of the control curve}} \times 100, \quad (2)$$

where sample is the mixture of the substrate, enzyme, and hydrolysate or inhibitor, and control is the mixture of the enzyme and substrate.

2.10. Antioxidant Properties

2.10.1. Diphenyl-1-picrylhydrazyl (DPPH) Free Radical-Scavenging Activity. The DPPH free radical-scavenging activities of NAM cryptides was determined according to the method described by Yen and Wu [14]. The solution of NAM cryptides at a known concentration (1 mg/ml) was prepared by dissolving them in double-distilled water. A known volume of 1.5 ml was added to 1.5 ml of 0.1 mM DPPH in 99.50% ethanol and mixed thoroughly by vortexing using a cyclomixer at high speed. The solution was stored at room temperature in dark for 30 min. The absorbance was measured at 517 nm using a double-beam spectrophotometer. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. DPPH radical-scavenging activity was calculated as follows:

$$\text{DPPH free radical} - \text{scavenging activty (\%)} = 1 - \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100. \quad (3)$$

Appropriate control was maintained along with double-distilled water. The analysis was carried out in triplicate.

2.11. Ferric-Reducing Power Assay. The ferric-reducing power of NAM cryptides was determined by the method as described by Oyaizu [15]. An aliquot of 1 ml of the sample (1 mg/ml) was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% (w/v) potassium ferric cyanide. The mixture was incubated at 50°C for 30 min, and the reaction was stopped by addition of 2.5 ml of 10% (w/v) trichloroacetic acid. Finally, 2.5 ml of solution from the mixture was drawn and mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% (w/v) ferric chloride solution. The solution was incubated for 10 min, and the absorbance was measured at 700 nm using a double-beam spectrophotometer. Higher absorbance of the reaction mixture indicated higher reducing power. The test was carried out in triplicate.

2.12. Linoleic Acid Peroxidation Inhibition Activity. The linoleic acid peroxidation inhibition (LAPI) activity of NAM cryptides was measured according to the method described by Osawa and Namiki [16]. NAM cryptide solution at a known concentration (3 mg/ml) was mixed with 10 ml of 50 mM phosphate buffer (pH 7.0). To this, a solution of 0.13 ml of linoleic acid and 10 ml of 99.5% ethanol was added. The total volume was then adjusted to 25 ml with distilled water. The mixture was incubated in a 30 ml assay tube with a screw cap at 40 ± 1°C for 5 days in a hot air oven. The tubes were wrapped with aluminum foil and brown paper to prevent the entry of light. The degree of oxidation of linoleic acid was measured using the ferric thiocyanate method [17]. To 0.1 ml of the reaction mixture, 4.7 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocyanate, and 0.1 ml of 20 mM ferrous chloride solution in 3.5% HCl were added. After 3 min of incubation, the colour was measured at 500 nm using a double-beam spectrophotometer. The phosphate buffer (50 mM; pH 7.0) served as control. The ability of NAM cryptides to inhibit the peroxide formation in linoleic acid was calculated using the following formula:

$$\text{Lipid peroxidation inhibition (\%)} = 1 - \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100. \quad (4)$$

2.13. Data Analysis. Experiments were carried out in triplicates, and data were subjected to analysis of variance (ANOVA). The significant difference in mean values was analyzed using Duncan's multiple range mean comparison test using statistics programme (SPSS.16.0 for windows, SPSS Inc., Chicago, IL).

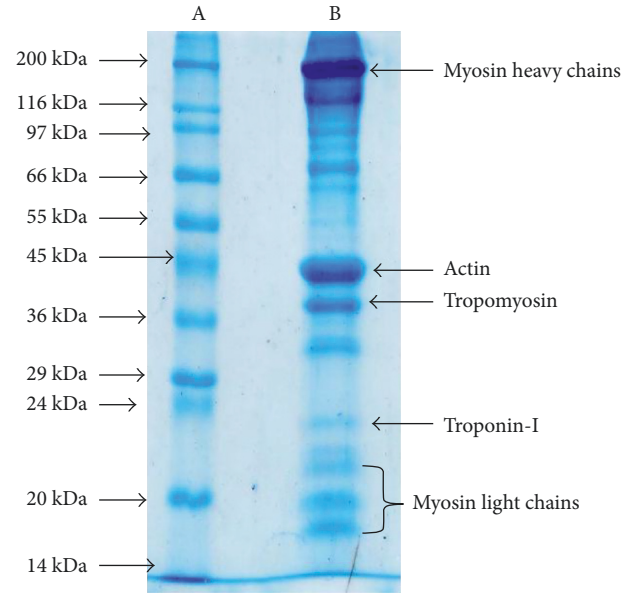

Figure 1: SDS-PAGE profile of natural actomyosin from *Catla catla* (lane A: standard molecular weight markers; lane B: natural actomyosin preparation).

3. Results and Discussion

3.1. SDS-PAGE Pattern of NAM. The SDS-PAGE pattern of NAM from *C. catla* is presented in Figure 1. The pattern revealed multiple bands with the prominent one being the component of 200 kDa which is the myosin heavy chain. The NAM comprises subunits of actin and myosin and other components such as tropomyosin and troponin. The SDS-PAGE pattern of actomyosin from *C. catla* is similar to that of actomyosin from other fish species [18, 19]. Earlier studies on purification of actomyosin reported that, along with actomyosin, other myofibrillar proteins such as the tropomyosin-troponin complex are also extracted during purification [19, 20].

3.2. Pattern of Cryptide Liberation from NAM by Papain. The SDS-PAGE (10% gel) profile of cryptides released from NAM is given in Figure 2. The major protein components in NAM were myosin heavy chains (MHCs), actin, tropomyosin, troponin, and myosin light chains. No degradation was found in the muscle protein profile during the incubation period of 60 min at 50°C without the addition of papain (Supplementary Figure 1). Yongsawatdigul and Park [21] reported no evidence of myosin heavy chain (MHC) or actin degradation in the actomyosin isolated from Pacific whiting in the temperature range of 20–80°C. However, the intensity of the MHC band decreased slightly as incubation time increased. In the sample where the papain was added, major muscle protein fractions including myosin heavy chains and actin were found to be cleaved within 10 min (Figure 2). The intensity of the band below the dye front increased with increasing time of proteolysis, indicating that the hydrolysis by papain resulted in the formation of low-molecular-weight cryptides. The SDS-PAGE pattern of

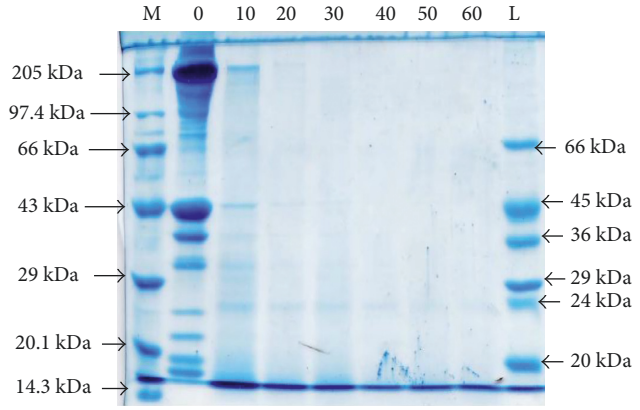

Figure 2: SDS-PAGE (10% gel) pattern of natural actomyosin during hydrolysis at 50°C for 60 min by the papain enzyme (lane M: wide-range molecular weight markers; lanes 0, 10, 20, 30, 40, 50, and 60: time of hydrolysis (min); lane L: low-molecular-weight markers).

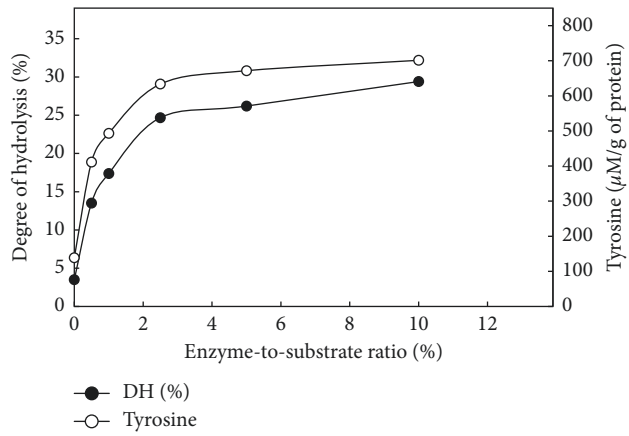

Figure 3: Hydrolysis of natural actomyosin (NAM) from the freshwater fish *Catla catla* using the papain enzyme at different enzyme-to-substrate ratios.

cryptide generation in 15% gel showed intensive bands diffused in the approximate molecular weight mass region of less than 6.5 kDa (Supplementary Figure 1). A peptide chain with the approximate molecular weight mass of 26 kDa was detected in both 10 and 15% gel. Ha et al. [22] reported the stability of C-reactive protein (140 kDa), α-actinin (90 kDa), tropomyosins (35 kDa), and troponins (22 and 17.8 kDa) from topside myofibril extracts against the activity of commercial papain preparation. Crude papain has been reported to cleave the myofibrillar proteins from the chicken muscle rapidly [23]. The subsets B and C of given Supplementary Figure 1 (SDS-PAGE profile of peptides released during hydrolysis of natural actomyosin) show the hydrolysis changes in the sample where papain was not added. A peptide fraction around 26 kDa, suspected to be troponin, was found to be resistant to hydrolysis by papain. Similarly, the subset C of Supplementary Figure 1 also indicated the degradation of the major protein fractions myosin and actin within 20 min of hydrolysis reaction.

3.3. Hydrolysis of NAM by Papain. Natural actomyosin was hydrolyzed using papain at different enzyme-to-substrate ratios, namely, 0.5%, 1.0%, 2.5%, 5.0%, and 10.0%. The degree of hydrolysis and the liberated tyrosine content are presented in Figure 3. The degree of hydrolysis and the liberated tyrosine content increased with the increase in enzyme-to-substrate ratio. There exists a good correlation between the liberated tyrosine content and degree of hydrolysis. The results indicated that lower E/S yielded a higher degree of hydrolysis. The high degree of hydrolysis at low E/S indicated that a large number of peptide bonds were hydrolyzed. Thereafter, the degree of hydrolysis increased marginally, mainly due to a decrease in available sites for cleavage. The degree of hydrolysis obtained for different E/S ratios varied from 3.5 to 29.4%. Higher degree of hydrolysis will yield more of low-molecular-weight cryptides. Papain has been reported to be more efficient in hydrolyzing the myofibrillar proteins [24]. The NAM cryptides obtained with different degrees of proteolysis were evaluated for their bioactive properties. Although papain is nonspecific in action, the preference for cleavage of the peptide bond is more between arginine and lysine. The specificity of papain for cleavage of the X-Y bond is as follows: where X is a nonspecific amino acid, but arginine and lysine are preferred; the phenylalanine-X-Y bond where residues following phenylalanine are preferred; and Y is a nonspecific amino acid residue. The protein sequences of fish species were retrieved from the UniProt database, and the presence of the number of arginine, lysine, and phenylalanine was documented. The results are presented in Table 1. The myosin heavy chain contains more number of arginine, lysine and phenylalanine. Hence, more number of peptides is expected from the myosin heavy chain.

3.4. Bioactive Properties of NAM Cryptides

3.4.1. ACE Inhibitory Cryptides. ACE inhibitory activity of NAM cryptides derived using papain is presented in Figure 4. ACE inhibitory activity of NAM cryptides increased with the increase in degree of hydrolysis. The results clearly indicate that the papain enzyme releases the cryptides from NAM with the sequence that can inhibit the ACE enzyme. It is well known that the biological properties of cryptides to a larger extent are influenced by their molecular structure and length, which in turn are affected by degree of hydrolysis. During hydrolysis, a wide variety of larger, medium, and smaller cryptides are generated depending on enzyme specificity. Increasing the degree of hydrolysis produces low-molecular-weight cryptides. Low MW cryptides are better ACE inhibitors than high MW cryptides [12]. Based on the specificity of the papain enzyme, we expect the peptides released to have the lysine or arginine in the C-terminal and phenylalanine in the penultimate position of peptides. A potent ACE inhibitory dipeptide V-R from the Atlantic salmon skin hydrolysate prepared using papain has been identified [25]. The theoretical search for this region in the retrieved amino acid sequences of proteins indicated that

Table 1: Number of preferred amino acid residues for cleavage sites in the sequence of myosin, actin, tropomyosin, and troponin.

Protein	Accession number	Total number of residues	Lysine	Arginine	Phenylalanine-X
Myosin heavy chain	UniProtKB-A8R0Q4 (A8R0Q4_HYPMO)	1933	210	99	56
Actin	UniProtKB-S4U1R3 (S4U1R3_HYPMO)	377	19	18	13
Troponin T	UniProtKB-A0A0F8B0M6 (A0A0F8B0M6_LARCR)	170	20	25	5
Troponin I	(UniProtKB-Q90366 (Q90366_CLUHA)	176	29	7	2
Troponin C	UniProtKB-B9VJM4 (B9VJM4_SINCH)	160	8	7	10
Tropomyosin	UniProtKB-A0A0D5MCW6 (A0A0D5MCW6_CTEID)	284	38	14	1
Myosin LC-1b	UniProtKB-Q90332 (Q90332_CYPCA)	193	16	6	8
Myosin LC-1a	UniProtKB-Q90331 (Q90331_CYPCA)	193	16	6	8
Myosin LC-3	UniProtKB-Q90333 (Q90333_CYPCA)	151	11	5	8
Myosin RLC	UniProtKB-Q9I892 (Q9I892_CYPCA)	169	15	5	11

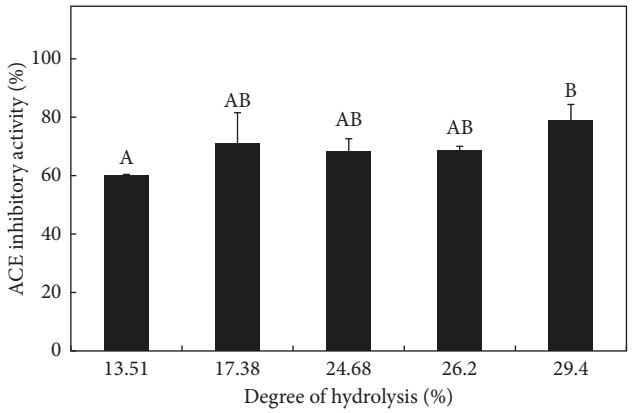

Figure 4: Angiotensin-I-converting enzyme (ACE) inhibitory activity of cryptides obtained from natural actomyosin with different degrees of hydrolysis at a peptide concentration of 1 mg/ml. Error bars represent the standard deviation from triplicate determinations. Different capital letters on the error bars indicate that the results are significantly different ($P < 0.05$).

this peptide could be sourced to myosin heavy chains (238-239, 669-670, 1604-1605, 1819-1819, and 1839-1840), actin (211-212), and troponin T (38-39). This sequence is absent in myosin light chains and tropomyosin. The quantitative structure-activity relationship studies on di- and tri-ACE inhibitory peptides confirmed that the presence of amino acid residues with bulky side chains and hydrophobic side chains in the carboxyl terminal was preferred for dipeptides, while that for tripeptides, the most favorable residues were aromatic amino acids. The amino acid residues with positive charge in the middle position and hydrophobic amino acid residues in the N-terminal region were preferred [26].

3.5. Antioxidant Properties of NAM Cryptides. The antioxidant properties of cryptides released from natural actomyosin by the action of papain including DPPH free radical-scavenging activity, ferric-reducing antioxidant power, and linoleic acid peroxidation inhibition are shown in Figures 5(a)–5(c).

The DPPH free radical-scavenging activity of NAM cryptides prepared using papain increased with the increase in DH up to 17.38%, and a further increase in DH up to 29.4% showed no significant difference in radical-scavenging activity (Figure 5(a)). An increase in the degree of hydrolysis produces greater numbers of low-molecular-weight cryptides [27]. The results obtained suggest that the NAM cryptides that were electron/proton donors could react with free radicals to convert them to more stable products. These cryptides could be useful in terminating the radical chain reaction-mediated oxidation process. The appropriate DH needs to be achieved to produce the NAM cryptides with maximum functions as radical scavengers.

The FRAP of NAM cryptides increased with the increase in DH up to 26.2% and showed a significant reduction at DH 29.4% (Figure 5(b)). Cryptides derived from loach protein using papain showed an increase in FRAP in the earlier stage of hydrolysis (DH-23%), and further hydrolysis (DH-33%) decreased the reducing power [28]. FRAP generally measures the reducing ability against ferric ions. Cryptides with a higher reducing power have better abilities to donate electrons.

The antioxidant assays such as DPPH free radical-scavenging activity and ferric-reducing antioxidant power evaluate the antioxidant properties by different mechanisms, and different specific structural requirements are associated with each mechanism of antioxidant action [29]. This may not reflect the complex mechanism through which cryptides may act as antioxidants to retard or inhibit lipid oxidation. Therefore, the ability of NAM cryptides to retard the lipid peroxidation was investigated using a linoleic acid model system. NAM cryptides showed an increase in peroxidation inhibition activity with the increase in degree of hydrolysis of NAM (Figure 5(c)). A linear relationship between the degree of hydrolysis and the antioxidant properties of cryptides from a small yellow croaker derived by papain has been reported [30]. Loach protein cryptides prepared using papain showed maximum free radical-scavenging activity at the degree of hydrolysis of 23% [31]. A potent antioxidant cryptide L-N-K has been purified from the *Sphyrna lewini* muscle protein hydrolysate derived using papain [29]. The lipid peroxidation inhibition activity of peptides depends on the hydrophobic nature [32]. Five antioxidant peptides, namely, DSGVT (actin), IEAEGE (unknown), DAQEKLE (tropomyosin), EELDNALN (tropomyosin), and VPSIDDQEELM (myosin heavy chain), have been purified from the porcine myofibrillar hydrolysates prepared using papain

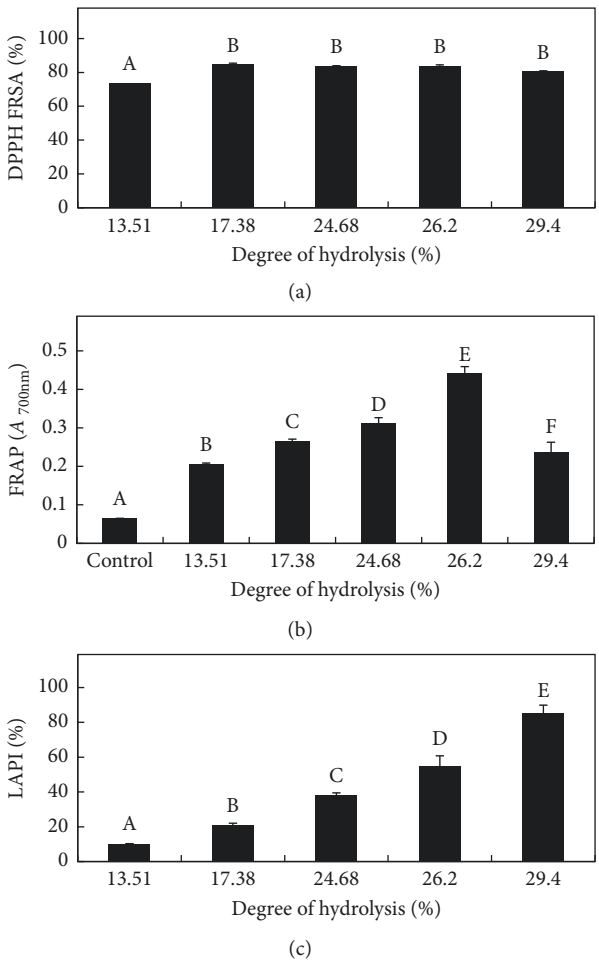

Figure 5: Antioxidant properties of cryptides obtained from natural actomyosin with different degrees of hydrolysis. Error bars represent the standard deviation from triplicate determinations. Different capital letters on the error bars indicate that the results are significantly different ($P < 0.05$). (a) DPPH free radical-scavenging activity at a peptide concentration of 1 mg/ml (DPPH FRSA); (b) ferric-reducing antioxidant power (FRAP) at a peptide concentration of 1 mg/ml; (c) linoleic acid peroxidation inhibition activity (LAPI) at a peptide concentration of 3 mg/ml.

[33]. Quantitative structure-activity relationship studies on peptides with antioxidant properties indicated that the properties of amino acids at C-terminal regions are more important than those at the N-terminal regions for antioxidant activity. Bulky hydrophobic amino acids at the C-terminal were related to the antioxidant activity of cryptides in free radical systems [34]. The amino acid composition, structure, and hydrophobicity of peptides influence the antioxidative properties. In addition to this, the molecular weight of peptides can also influence the antioxidant properties [35].

4. Conclusion

The papain enzyme released the cryptides mainly from myosin and actin (as revealed by the SDS-PAGE profile) with angiotensin-I-converting enzyme inhibitory and antioxidant properties such DPPH free radical-scavenging activity, ferric-reducing antioxidant power, and linoleic acid peroxidation inhibition activity. The present study indicated that the fish actomyosin is a potential precursor for the production of therapeutic cryptides using papain hydrolysis and their health beneficial properties depend on the extent of hydrolysis. Further study is needed to identify the sequence of cryptides.

Data Availability

The data used to support the findings of this study are accessible through request to the authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The financial support provided by the European Union, Brussels, under FP-7, SECUREFISH (Grant no. 289282) for conducting the research work is gratefully acknowledged.

Supplementary Materials

Supplementary Figure 1: SDS-PAGE profile of peptides released during hydrolysis of natural actomyosin (M-Marker; NAM-Natural Actomyosin; 0,10,20,30, 40, 50, 60-Time of hydrolysis; UL-Ultra low molecular weight markers): (A) natural actomyosin, (B) control sample during hydrolysis, (C) peptide pattern in 10% gel, and (D) peptide pattern in 15% gel. (*Supplementary Materials*)

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