Kinetic characterization of *Channa striatus* muscle sarcoplasmic and myofibrillar protein hydrolysates

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Abstract This study was conducted to evaluate the kinetic characteristics of proteolytic activity of proteases on *Channa striatus* protein fractions. Degree of hydrolysis (DH), amino acid composition and kinetic parameters of sarcoplasmic and myofibrillar proteins were investigated when incubated with proteinase K and thermolysin, separately. After 30 min incubation with proteases, a decrease in DH of sarcoplasmic protein was observed whereas, hydrolysis of myofibrillar protein with proteases took 2 h with an increase in DH. The major amino acids were glutamic acid (16.6%) in thermolysin- myofibrillar hydrolysate followed by aspartic acid (11.1%) in sarcoplasmic protein fraction with no enzyme treatment and lysine (10%) in thermolysin-myofibrillar hydrolysate. The apparent Michaelis constant of proteinase K was lower than thermolysin for both sarcoplasmic and myofibrillar proteins. However, rate of turnover and enzyme efficiency suggested that sarcoplasmic and myofibrillar proteins are suitable substrates for proteinase K and thermolysin hydrolytic reaction, respectively.

Keywords *Channa striatus* · Proteolytic activity · Degree of hydrolysis · Kinetic parameters · Michaelis constant

Introduction

Most people have a concern for their diet from a health aspect. In the food guide pyramid, meat is categorized as a protein food group with high biological value in many countries. Meat is also an excellent source of some valuable nutrients such as minerals and vitamins (Biesalski 2005). Although consumption of meat and meat products is mostly avoided due to reducing the risk of cancer or obesity, meat plays a critical role due to nutritional value and maintenance of human health (Arihara 2006).

Due to increasing concerns about health in recent years, much attention has been paid to the tertiary functions of foods. Tertiary functions are the roles of natural substances of food components in preventing diseases by modulating physiological systems (Sairam et al. 2010). Meat protein-derived peptides proved to have functional properties. Although the activities of these peptides in the sequences of proteins are latent, they are released by proteolytic enzymes (Arihara 2006). Biochemical production of protein hydrolysates with functional properties may be carried out by employing controllable enzymatic methods (Nazeer et al. 2011). Several researches have investigated physiologically functional properties of enzymatic hydrolysates of food proteins (e.g., milk caseins, soy protein, fish muscle protein, meat muscle protein, etc....) such as antihypertensive (ACE inhibitory); antioxidative; opioid agonistic and antimicrobial activity (Murray and FitzGerald 2007; Nagai et al. 2006).

Fish is an important component of the human diet and an enormous industry exists to provide a huge variety of consumer products in which fish is a major component. Freshwater fishes are long associated with daily life in Malaysia. *Channa striatus*, a genus of the Channidae family of snakehead fishes, known locally to the Malays as Haruan, is a freshwater, air-breather and carnivorous fish

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indigenous to many tropical and subtropical countries including Malaysia. Traditionally, this fish is believed to promote wound healing and alleviates post-operative pain and discomfort. It contains a good composition of amino acids and fatty acids hypothesized to be active components in the initiation of wound repair (Mat Jais 2007).

Sarcoplasmic proteins are water soluble proteins, mainly composed of oxidative enzymes, myoglobin, the glycolytic enzymes responsible for glycolysis, and lysosomal enzymes (Nakagawa et al. 1988) while myofibrils predominantly consist of myosin heavy chain (MHC, 200 kDa) and actin (42 kDa) and other minor proteins, such as α -actinin (100 kDa) and tropomyosin (34 kDa). The chemistry and biochemistry of these proteins has been studied both in vivo and in vitro by scientists from many different fields due to their important role in meat and meat products (Skaara and Regenstein 1990).

Different enzymes produce different peptides with different characteristics during proteolysis due to their specificity and affinity towards the protein substrate. The specificities of proteases contribute to the length and amino acid sequence of peptides, which may influence antioxidant activities of protein hydrolysates (Qian et al. 2011). The present study was carried out to study proteolytic activity and kinetic parameters of *C. striatus* muscle protein hydrolysates with respect to bioactive potential in further studies.

Materials and methods

Materials Amino acid standards were purchased from Pierce (Rockford, IL, USA). Folin-Ciocalteu's phenol reagent was obtained from Merck. Protein standards from Bio-Rad, Thermolysin from *Bacillus thermoproteolyticus* rokko (EC 3.4.24.27; activity 42 units mg/protein), Proteinase K from *Tritirachium album* (EC 3.4.21.64; activity 40 units mg/protein), AccQ-Fluor reagent kit and AccQ-Tag Eluent, internal standard α -aminobutyric acid (AABA) and other chemicals used in this study were purchased from sigma (Sigma-Aldrich Chemical Co., USA).

Sampling Adults Haruan (*C. striatus*), sized 1–1.5 kg, were bought from a nearby farm. The fish were brought alive to the laboratory in batches. The fish fillets were obtained by cutting the fish lengthwise along the backbone to gain the maximum amount of flesh without any backbone. Filleting was done manually and kept in ice box. The fillets were then washed several times under running tap water to remove blood, dirt and slime.

Extraction of sarcoplasmic and myofibrillar protein Sarcoplasmic and myofibrillar protein were extracted from *C. striatus* meat according to the method described by Hay et

al. (1973). A 20 g of excised muscle was minced and homogenized in 5 volumes of the 0.25 M sucrose, 1 mM disodium ethylenedinitrilo tetraacetate (EDTA), 0.05 M Tris [tris (hydroxymethyl) aminomethane] pH 7.6 extracting solution by three separate bursts of 15 s duration with a 45 s interval between each burst. This procedure was repeated 3 times. The extract was stirred gently for 1 h at 4 °C and sedimented at $2,500 \times g$ for 10 min by centrifugation (Thermo Scientific, High Speed Centrifuge, Sorvall HS23, Germany). The supernatant, known as the source for sarcoplasmic protein fraction, was dialyzed and lyophilized.

The sediment, mainly consisting of myofibrils, was suspended in 5 volumes of 0.05 M Tris pH 7.6, 1 mM EDTA extracting solution and passed through a 3 layered cheese cloth to remove connective tissue. Purification of crude myofibrillar protein was achieved by washing the myofibrils successively with (1) 0.15 M KCl, 0.03 M Tris pH 7.6; (2) 1 mM EDTA pH 7.6; (3) double-deionized distilled water; and (4) 0.15 M KCl, 0.03 M Tris pH 7.6. The myofibrillar protein was stirred for 10 min at 4 °C before each wash followed by sedimentation at $2,500 \times g$ for 10 min. The rather densely packed myofibrils were finally suspended in 0.15 M KCl, 0.03 M Tris pH 7.6.

Hydrolysis of sarcoplasmic and myofibrillar proteins *C. striatus* muscle extracts (sarcoplasmic and myofibrillar protein fractions) were adjusted to optimal pH by 0.1 N HCl and temperature for each enzyme; such as thermolysin (pH 7.4; 37 °C) and proteinase K (pH 7.5; 37 °C), separately. Each protein fraction was suspended in distilled water with ratio of 1:100 w/v and incubated with each enzyme for 0, 0.5, 1 and 2 h at 37 °C, separately. The enzymatic hydrolysis was stopped by boiling for 5 min or heating at 98 °C for 10 min. The hydrolysate was centrifuged at $3000 \times g$ for 20 min and the soluble aqueous fraction decanted and lyophilized. The ratio of each enzyme to meat protein fractions (sarcoplasmic and myofibrillar) was 1:100, w/w.

Protein solubility The soluble contents of sarcoplasmic and myofibrillar proteins were determined by the Folin-Lowry method (Lowry et al. 1951). An aliquot of 0.5 mL of the sample was mixed with 0.7 mL of an alkaline-copper reagent and incubated for 20 min at room temperature. The mixture was added to 0.1 mL of the Folin-Ciocalteu's phenol reagent at 2-fold dilution with deionized water and allowed to stand for 30 min or longer at room temperature. The absorbance at 750 nm was measured with a spectrophotometer (Model UV-160A, Shimadzu, Japan). The soluble protein content was quantified using bovine serum albumin as standard.

Degree of hydrolysis The effect of hydrolysis time on DH was estimated using the method introduced by Kim et al.

(1996). At the end of each hydrolysis time of 0, 0.5, 1 and 2 h at 37 °C, an aliquot of 10 mL of the aqueous suspension of enzyme-treated fractions was removed and mixed with 10 mL of 20% trichloroacetic acid (TCA) to obtain 10% TCA-soluble nitrogen and 10% TCA-insoluble nitrogen and then centrifuged at $15,770\times g$ for 15 min at 4 °C. The supernatant was decanted and analyzed for soluble nitrogen using kjeldahl method (Kjeltec TM2100, Foss, Denmark). The percent DH is expressed as follows:

$$\%DH = (10\%TCA - \text{solubleNinsample} / \text{totalNinsample}) \times 100$$

Measurement of peptide content The peptide contents of hydrolysates were measured by method of Church et al. (1983) with some modifications using o-phthalaldehyde (OPA) spectrophotometric assay. Fifty milliliters of fresh OPA reagent was prepared by mixing 25 ml of 100 mM sodium tetra hydroborate, 2.5 mL of 20% (w/w) sodium dodecyl sulfate, 40 mg of OPA solution (dissolved in 1 ml of methanol) and 100 mL of β -mercaptoethanol and then adjusted the volume to 50 mL with deionized water. Fifty microliters of each hydrolysate, containing 5–100 μ g protein, was mixed with 2 mL of OPA reagent and incubated for 2 min at ambient temperature. The absorbance at 340 nm was measured with spectrophotometer. Casein trypton in phosphate buffer (pH 7.4) was used as standard to quantify the peptide content. Highly concentrated samples were diluted in phosphate buffer.

Preparation of sarcoplasmic and myofibrillar protein fractions for SDS-PAGE The isolated sarcoplasmic and myofibrillar protein fractions and their hydrolysates were prepared for Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) analysis according to the method described by Ming et al. (2009). Samples were mixed with 30 mL treatment buffer (125 mM Tris, 4% SDS, 20% glycerol) and heated in a 50 °C water bath for 20 min and then centrifuged at $16000 \times g$ for 30 min at 4 °C. Protein concentration was determined with lowry method and diluted to 4 mg/ml using treatment buffer containing 10% MCE and 0.001% bromophenol blue. Samples were well mixed and heated at 50 °C for 10 min and then stored at –80 °C for subsequent SDS-PAGE.

SDS-PAGE analysis

a. Gel system SDS-PAGE analysis was performed according to the technique developed by Schagger and von Jagow (1987). Ten and 20% polyacrylamide separating gels

(Acrylamide: N,N'-bis-methylene acrylamide = 32:1 (w/w), gel buffer (3 M Tris-HCl, pH 8.45, containing 0.3% SDS (w/v)); 0.05% (v/v) TEMED, 10% (w/v) Ammonium persulfate and a 4% polyacrylamide stacking gel were used to in a tris-tricine system to detect the sarcoplasmic and myofibrillar protein hydrolysate patterns, respectively. Proteins standard used for obtaining the standard curve were myosin (200.0 kDa), β -galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

b. SDS-PAGE Forty and 80 μ g per well of sarcoplasmic and myofibrillar protein hydrolysate samples were loaded on the 10 and 20% gel (protean dual slab cell (Bio-Rad), 14 cm wide $\times$ 14 cm tall $\times$ 1.5 mm thick) and run using discontinuous tris-tricine buffer, containing 1X anode buffer (2 M tris, pH 8.9) and 1X cathode buffer (1 M Tris, 1 M Tricine, 1% SDS, pH 8.25) at a constant current setting of 25 mA/gel and a constant voltage of 150 and 90 for 2 and 4 h, respectively. Proteins were visualized by 0.02% (w/v) Coomassie blue G250 staining and destaining by soaking in several changes of 40% (v/v) methanol–10% (v/v) acetic acid until a clear background resulted.

Amino acid analysis Acid hydrolysis, performic acid and alkaline hydrolysis of *C. striatus* muscle hydrolysates were performed using Waters AccQ.Tag amino acid analyzer (Waters Corporation, Ireland). Acid analysis of hydrolysate samples was performed according to the Alaiz et al. (1992). Each sample was hydrolysed with 5 mL 6 N hydrochloric acid (HCl) in a closed test tube, shaken for 20 min and then kept in oven for 24 h at 110 °C. Internal standard α -aminobutyric acid (AABA) was added to the hydrolyzed samples and filtered through a nylon 0.2 mm cellulose acetate membrane filter (Whatman No.1). The total content of cysteine and cystine was determined by oxidizing the protein with performic acid and then samples were hydrolyzed with 6 N HCl. Alkaline hydrolysate was performed to determine the amount of tryptophan using 4.3 N LiOH. H₂O as described by Thiansilakul et al. (2007). Each hydrolysate was then hydrolyzed with 6 N HCl and filtered.

Derivatization of amino acid was done at 55°C in a heating block for 10 min. Separations of amino acid was carried out on an C₁₈ AccQ-Tag amino acid analysis column (150 $\times$ 3.9 mm, Waters, USA). The column temperature was set at 37 °C with a flow rate set at 1 mL/min. The UV detector was operated at 248 nm (for peak identification), and the fluorescence detector was with a 250 nm excitation and a 395 nm emission wavelength (for amino acid quantification). The hydrolysed sample was then analysed using an

automatic amino acid analyser (Waters Corporation, USA) equipped with Waters 717 Autosampler.

Kinetic analysis of protein hydrolysate The spectrophotometric OPA assay was applied for kinetic analysis of *C. striatus* sarcoplasmic and myofibrillar protein fractions using the method introduced by Salami et al. (2008) with minor modification. Various concentrations of sarcoplasmic and myofibrillar protein samples (0.2–10 mg/mL) were prepared in 30 mM Tris-KCl buffer, pH 7.6, at 37 °C. The reaction was initiated by adding 50 µg of each enzyme to 1 mL of sarcoplasmic and myofibrillar protein. At zero time, and after 10 and 30 min of incubations for sarcoplasmic and myofibrillar protein, respectively, aliquots of 50 µL were withdrawn and mixed with 2 mL of OPA reagent. The kinetic parameters (Michaelis constant (K_m), maximum velocity (V_{max}) and catalytic constant (K_{cat})) of hydrolysis of sarcoplasmic and myofibrillar protein by thermolysin and proteinase K were determined according to the double reciprocal (Lineweaver–Burk) plots. Each measurement was done three times and the results shown are the average of data from three experiments.

Statistical analysis Each treatment was conducted in triplicates. The results were presented as mean $\pm$ standard deviation and subjected to an analysis of variance (SPSS 14, SPSS Inc., 2005) in order to determine significant statistical differences among samples. Data analysis was

carried out using One-way Analysis of Variance (ANOVA) with Duncan Multiple Range Test (DMRT) and General Linear Model (GLM) using Multivariate test. The differences in means between the samples were determined at 5% confidence level ($P < 0.05$).

Results and discussion

Degree of hydrolysis, soluble protein and peptide content - Degree of hydrolysis is a measure of the extent of hydrolytic degradation of a protein. It is considered the most practical and convenient means for measuring the hydrolysis process. The effect of time on DH, soluble protein and peptide content of sarcoplasmic and myofibrillar protein of *C. striatus* hydrolyzed with thermolysin (ST and MT), proteinase K; (SP and MP) and the mixture of enzymes (STP and MTP) are shown in Table 1. Results indicate faster hydrolysis of sarcoplasmic than of myofibrillar protein with proteinase K, thermolysin and the mixture of these enzymes. Due to faster digestion, enzymatic hydrolysis of sarcoplasmic protein with proteases was performed over 30 min, while hydrolysis of myofibrillar protein with the same proteases took over 2 h. The DH of sarcoplasmic and myofibrillar treated with proteinase K and thermolysin after 0.5 and 2 h incubation was 89.5 and 89.8%, respectively. According to DH results, the extent of proteinase K hydrolysis of sarcoplasmic protein was slightly

Table 1 Effect of hydrolysis time on degree of hydrolysis, soluble protein and peptide content of sarcoplasmic and myofibrillar protein hydrolysates

Each data represents average value and $\pm$ SD of triplicate determinations.

^aS, sarcoplasmic protein; SP, sarcoplasmic hydrolysed with proteinase K; ST, sarcoplasmic hydrolysed with thermolysin, STP, sarcoplasmic hydrolysed with mixture of thermolysin and proteinase K.

^bM, Myofibrillar protein; MP, Myofibrillar protein hydrolysed with proteinase K; MT, Myofibrillar protein hydrolysed with thermolysin; MTP, Myofibrillar protein hydrolysed with mixture of thermolysin and proteinase K.

Samples	Time of incubation (h)	DH (%)	Protein solubility (mg/g)	Peptide content (mg/g)
S ^a			32.2 $\pm$ 1.00	16.9 $\pm$ 1.11
ST	0.5	86.3 $\pm$ 1.01	340.4 $\pm$ 2.11	178.2 $\pm$ 3.30
	1	72.4 $\pm$ 1.42	125.8 $\pm$ 1.82	180.2 $\pm$ 3.31
	2	69.4 $\pm$ 3.20	115.6 $\pm$ 2.91	185.5 $\pm$ 2.50
SP	0.5	89.5 $\pm$ 4.31	380.1 $\pm$ 2.10	183.4 $\pm$ 2.62
	1	78.3 $\pm$ 2.22	228.3 $\pm$ 4.31	192.7 $\pm$ 1.31
	2	72.1 $\pm$ 3.22	150.3 $\pm$ 4.43	193.1 $\pm$ 2.03
STP	0.5	90.4 $\pm$ 2.53	410.2 $\pm$ 2.82	191.8 $\pm$ 1.23
	1	79.7 $\pm$ 4.10	280.7 $\pm$ 1.91	196.4 $\pm$ 4.41
	2	75.2 $\pm$ 2.11	210.6 $\pm$ 2.00	196.9 $\pm$ 2.32
M ^b			85.4 $\pm$ 2.00	82.2 $\pm$ 4.31
MT	0.5	32.2 $\pm$ 2.00	332.1 $\pm$ 3.21	180.4 $\pm$ 4.80
	1	50.2 $\pm$ 1.11	475.5 $\pm$ 3.3	210.8 $\pm$ 2.30
	2	89.8 $\pm$ 3.43	558.3 $\pm$ 2.61	224.2 $\pm$ 2.01
MP	0.5	29.1 $\pm$ 3.31	382.2 $\pm$ 1.70	146.4 $\pm$ 1.03
	1	45.6 $\pm$ 2.80	430.1 $\pm$ 1.00	150.3 $\pm$ 1.11
	2	86.4 $\pm$ 3.12	510.6 $\pm$ 2.42	171.6 $\pm$ 2.34
MTP	0.5	37.1 $\pm$ 1.22	350.2 $\pm$ 1.43	210.5 $\pm$ 4.15
	1	50.8 $\pm$ 3.31	480.5 $\pm$ 3.72	225.2 $\pm$ 2.87
	2	91.1 $\pm$ 4.59	570.6 $\pm$ 2.71	274.7 $\pm$ 1.00

higher than thermolysin hydrolysis. In this study after initial increase in DH, a decrease in rate of hydrolysis of sarcoplasmic protein with proteases was observed when incubated more than 0.5 h. Mackie (1982) stated that after an initial rapid phase of hydrolysis; the rate of hydrolysis tends to decrease, entering a stationary phase. Despite of having more cleavage sites of sarcoplasmic protein for proteinase K and of myofibrillar protein for thermolysin, the extent of hydrolysis of each protein depends on cleavage specificity of enzyme and accessibility of peptide bonds to each protease. When sarcoplasmic and myofibrillar protein were hydrolyzed with the mixture of enzymes, additional increase in DH% was observed which is due to accessibility of more cleavage sites for enzymes.

There was a steady increase in DH% of myofibrillar protein by the increase of incubation time when hydrolyzed with proteinase K, thermolysin and the mixture of these enzymes. Myofibrillar protein showed higher rate of hydrolysis when treated with thermolysin than of proteinase K. The myofibrillar structure is a complex system with many protein–protein interactions (Morzel et al. 2006) and even protein–lipid interactions (Chelh et al. 2007) which can affect its susceptibility to proteolysis (Morzel et al. 2006).

Solubility of a protein is primarily dependent on the distribution of hydrophobic and hydrophilic amino acids on the surface of a protein and on the thermodynamics of the protein–water interactions (Kristinsson and Rasco 2000). Solubility profile of sarcoplasmic and myofibrillar protein hydrolysates were significantly different. By increasing the time of hydrolysis, the soluble protein content of sarcoplasmic and myofibrillar protein hydrolysates was increased from 32.2 to 410.2 and from 85.4 to 570.6 mg/g, respectively (Table 1). The solubility of sarcoplasmic protein after 30 min incubation with proteinase K, thermolysin and the mixture of these enzymes decrease whereas myofibrillar protein solubility was still increasing after 2 h incubation with proteases. A 100% protein solubility is never achieved which is related partly to peptide–peptide interactions and partly to the presence of compact structures in the protein that cannot be hydrolyzed. Increased solubility of intact fish myofibrillar protein with partially enzymatic hydrolysis, is well-documented which is due to the smaller size of peptides and newly exposed amino and carboxyl groups that enable more interactions with water and increase hydrophilicity of the hydrolysates (Kristinsson and Rasco 2000). The peptide content of sarcoplasmic and myofibrillar protein hydrolysates increased by the increase in time of incubation. The peptide content of sarcoplasmic and myofibrillar protein hydrolysates were increased from 16.9 to 196.9 and from 82.2 to 274.7 mg/g, respectively.

Amino acid composition of sarcoplasmic and myofibrillar protein hydrolysates Fig. 1 shows a typical chromatograph

Fig. 1 A HPLC chromatograph of separation of amino acids of *C. striatus* myofibrillar protein fraction with no enzyme treatment using HCl digestion. AABA: DL- α -aminobutyric acid

of HPLC separation of amino acids of *C. striatus* myofibrillar protein fraction with no enzyme treatment using HCl digestion. Compositions of 18 amino acids of sarcoplasmic and myofibrillar proteins and their hydrolysates using proteases are summarized in Table 2. The major amino acids were glutamic acid (16.6%) in MT fraction followed by aspartic acid (11.2%) in sarcoplasmic protein fraction with no enzyme treatment and lysine (10.4% of total amino acids) in MT fraction. Comparison of amino acids of some freshwater fish meat proteins revealed that *C. striatus* had higher content of amino acids among other freshwater fishes (Ghassem et al. 2009; Zuraini et al. 2006) with the major amino acids of glutamic acid, aspartic acid followed by lysine (Ghassem et al. 2009). Different amino acid compositions of sarcoplasmic and myofibrillar proteins hydrolysates were observed. Sarcoplasmic protein exhibited higher content of essential amino acids (His, Thr, Val, Leu, Phe) than myofibrillar protein when there was no enzyme treatment (Table 2).

As results indicate, SP showed higher content of essential amino acids of Trp, Lys, Met and Thr than ST and STP whereas, MT fraction showed higher amount of essential amino acids of His, Lys, Met and Thr than MP fraction. Content of other essential amino acids such as Leu and His of sarcoplasmic and Leu, Ile and Val of myofibrillar protein fractions decreased when hydrolyzed with either thermolysin or proteinase K. However, an increase in the amount of amino acids of Val, Ile, Phe of sarcoplasmic protein and of Trp of myofibrillar protein was observed when the mixture of enzymes were applied (Table 2).

Table 2 Amino acid composition of sarcoplasmic and myofibrillar proteins of *C. stratus* and their hydrolysates (%)

AA%	Sarcoplasmic protein	SP ^a	ST	STP	Myofibrillar protein	MP ^b	MT	MTP
Asp	11.1±1.01	8.0±2.12	8.6±1.01	8.1±0.30	8.6±0.11	9.7±0.44	9.1±0.31	9.4±0.15
Ser	5.1±1.12	4.7±2.21	4.5±2.22	4.3±0.41	4.9±1.01	4.3±0.25	4.3±0.45	4.2±0.24
Glu	10.3±1.31	8.4±1.92	8.1±1.53	8.1±1.04	13.2±2.13	16.4±0.30	16.6±0.55	15.6±0.51
Gly	8.6±0.32	8.6±0.10	9.2±1.01	7.6±2.22	6.2±1.37	4.7±0.55	4.9±0.55	4.7±0.55
His ^c	3.2±0.45	3.7±0.19	3.5±0.43	3.1±0.31	2.3±0.33	2.1±0.46	2.4±0.23	2.1±0.42
Arg	4.9±1.85	6.4±0.28	7.3±0.25	7.1±1.45	5.4±0.45	6.9±0.21	6.6±0.12	6.7±0.12
Thr ^c	4.4±1.11	4.6±0.10	4.3±0.38	4.5±0.32	4.1±0.20	4.6±1.10	4.8±0.15	4.7±0.45
Ala	7.0±0.20	7.8±0.10	7.4±2.11	7.2±0.22	7.1±1.01	6.2±0.11	6.1±2.12	6.0±0.22
Pro	3.8±0.13	3.6±1.55	4.1±2.20	2.8±0.42	3.8±1.53	3.7±0.26	3.8±0.95	3.5±0.75
Tyr	1.8±1.14	3.8±2.12	2.0±1.00	4.2±1.16	2.1±1.26	3.3±0.33	3.6±2.12	3.8±1.00
Val ^c	5.4±1.05	5.2±0.42	5.2±0.14	7.1±0.32	4.9±0.30	4.7±2.12	4.8±2.26	4.6±2.01
Met ^c	2.11±0.21	2.5±0.21	2.3±0.54	2.1±2.35	3.0±0.54	3.1±2.75	3.2±2.33	3.1±2.13
Lys ^c	9.3±0.21	9.9±0.64	9.5±1.20	7.7±1.33	9.9±1.77	9.3±0.81	10.4±1.35	10.1±1.34
Ile ^c	4.7±2.13	4.0±1.01	4.4±1.29	7.6±0.45	4.9±0.91	4.5±0.60	4.5±0.12	4.4±0.65
Leu ^c	8.1±0.86	7.7±0.90	7.7±0.12	7.6±0.22	8.0±0.73	7.8±0.23	7.9±1.05	7.7±1.22
Phe ^c	6.2±0.48	6.1±0.21	6.0±0.42	6.7±0.10	4.7±0.15	3.4±0.22	3.4±1.01	3.4±1.01
Cys	0.2±1.29	1.0±0.30	0.9±0.31	0.6±0.42	1.5±1.01	0.3±1.80	0.4±0.20	0.5±0.52
Trp ^c	2.9±1.14	4.2±1.61	2.8±0.20	2.7±0.30	4.5±1.00	2.8±0.21	3.7±0.43	5.2±0.51

^a SP, sarcoplasmic hydrolysed with proteinase K; ST, sarcoplasmic hydrolysed with thermolysin, STP, sarcoplasmic hydrolysed with mixture of thermolysin and proteinase K.

^b MP, Myofibrillar protein hydrolysed with proteinase K; MT, Myofibrillar protein hydrolysed with thermolysin; MTP, Myofibrillar protein hydrolysed with mixture of thermolysin and proteinase K.

^c Essential amino acids, Data are expressed as mean ± SD of three separate determinations.

Proteolytic activity of thermolysin indicated that primary specificity of this protease was against hydrophobic amino acid residues such as α -leucine, α -isoleucine, α -phenylalanine, whose amino groups donate the carboxyl group, make this protease a useful catalyst for preparation of blocked dipeptide and tripeptide derivatives (Wayne and Fruton 1983). However, proteinase K is a stable broad-spectrum serine protease, commonly used for its broad specificity (Kraus and Femfert 1976). Proteinase K degrades many

proteins even in the native state with the predominant site of cleavage of peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids with blocked α -amino groups (Kraus and Femfert 1976).

SDS-PAGE analysis The SDS-PAGE method described by Laemmli cannot separate polypeptides with masses below about 15 kDa. The most generally used technique is the one developed by Schagger and von Jagow (1987) with the

Fig. 2 SDS-PAGE pattern of sarcoplasmic (a) and myofibrillar (b) proteins and of their hydrolysates obtained after 0.5 h and 2 h incubation, respectively. In both SDS-PAGE profiles, lane 1 represents protein marker; lane 2, control (unhydrolyzed samples); lane 3, proteinase K treated samples; lane 4 thermolysin treated samples and lane 5 shows samples treated with the mixture of both enzymes

Fig. 3 Michaelis-Menten curve of *C. striatus* protein fractions using o-phthalaldehyde (OPA) spectrophotometric assay. Initial data were obtained after 30 min incubation for myofibrillar (a) and 10 min incubation for sarcoplasmic protein (b) at 37 °C by adding 50 µg of thermolysin and proteinase K at various concentration of sarcoplasmic and myofibrillar proteins (0.2–10 mg/mL). Data are average of three different experiments

use of high concentration gels and the incorporation of materials to resolve the polymers of low molecular weight. Representative SDS-PAGE electrophoregrams of *C. striatus* sarcoplasmic and myofibrillar protein hydrolysates in a discontinuous tris-tricine gel system containing SDS are shown in Fig. 2 (a,b). As shown in lane 2, unhydrolyzed sarcoplasmic protein fraction showed both high and low molecular weight proteins, whereas hydrolysis pattern of sarcoplasmic-enzyme treated samples were different (Fig. 2a). After 0.5 h incubation with proteases, no high molecular protein fraction was detected on SDS-PAGE profile. Hydrolysis of sarcoplasmic protein with thermolysin and mixture of enzymes exhibited mainly low molecular bands ranged from 18.3 to 10.2 kDa, while proteinase K hydrolysate samples resulted in several peptide fragments, with sizes ranging from 38 to 10.2 kDa. This result is compatible with that of Table 1 which 0.5 h incubation of sarcoplasmic protein with proteinase K showed higher DH than thermolysin treated samples.

SDS-PAGE electrophoregram of myofibrillar enzyme treated samples were significantly different from that of sarcoplasmic hydrolysates (Fig. 2b). Hydrolysis patterns of thermolysin-treated myofibrillar samples were almost similar to that of proteinase K with the presence of protein portions of molecular weights ranging from 81 to 51.2 kDa for proteinase K and 85 to 48.5 kDa for thermolysin treated samples and with one single peptide

fragment of 30.8 kDa molecular weight which remained intact after 2 h incubation (Lanes 3 and 4, Fig. 2b). When treated with mixture of enzymes, not many differences were observed on SDS-PAGE pattern. Hydrolysis pattern of myofibrillar protein sample of *C. striatus* indicated excessive hydrolysis with thermolysin, proteinase K and the mixture of enzymes after 2 h by DH of over 80% (Table 1).

Kinetic study of proteases To predict suitability of sarcoplasmic and myofibrillar protein and proteolytic activity of thermolysin and proteinase K, kinetic parameters of proteases were measured using OPA assay. Initial velocity (V_0) of each reaction was measured at different substrate concentrations (0.2–10 mg/mL) during 10 min for sarcoplasmic and 30 min for myofibrillar protein. Values of V_0 were then plotted versus substrate concentration to create a Michaelis-Menton kinetic model for myofibrillar and sarcoplasmic protein fractions (Fig. 3 (a,b)). The value of V_0 when enzyme is fully saturated with substrate is called maximum velocity (V_{max}). Michaelis constant (K_m) shows the affinity of enzyme towards the substrate and is corresponds to half of V_{max} . These kinetic parameters of an enzymatic reaction provide information about specificity and mechanism of reaction. Higher K_m means enzyme needs higher concentration of substrate with lower affinity toward substrate in an enzymatic reaction (Shargel et al. 2005; Taylor and Diers-Caviness 2003). To measure kinetic parameters of hydrolysis

Table 3 Kinetic parameters of hydrolysis of sarcoplasmic and myofibrillar proteins by thermolysin and proteinase K

	Thermolysin			Proteinase K		
	K_m^a (mg/mL)	K_{cat}^b (1/s)	K_{cat}/K_m^c (mL/mg s)	K_m (mg/mL)	K_{cat} (1/s)	K_{cat}/K_m (mL/mg s)
Sarcoplasmic protein	2.79 ± 0.01	3.10×10^2	1.11×10^2	0.96 ± 0.02	1.9×10^2	1.98×10^2
Myofibrillar protein	19.68 ± 0.03	13.11×10^2	0.66×10^2	3.46 ± 0.05	1.87×10^2	0.54×10^2

^a K_m : Michaelis constant

^b K_{cat} : Catalytic constant

^c K_{cat}/K_m ratio: Catalytic efficiency ratio

of sarcoplasmic and myofibrillar protein, values of V_0 and substrate concentration of both enzymes (Salami et al. 2008) were plotted reciprocally (Lineweaver-Burk plot). In this study both enzymes when acting on peptide bonds of sarcoplasmic and myofibrillar protein showed Michaelis-Menton behavior ($R > 0.97$) (Fig. 3). Kinetic parameters of thermolysin and proteinase K were significantly different. Both enzymes indicate different K_m during hydrolysis of sarcoplasmic and myofibrillar proteins (Table 3) which both had lower K_m for sarcoplasmic than myofibrillar protein. When sarcoplasmic and myofibrillar proteins were used as substrates separately, thermolysin had higher K_m than proteinase K, suggesting that proteinase K had higher affinity towards both substrates and bound to substrates more tightly than thermolysin (Table 3).

Catalytic constant (K_{cat}) value is the number of substrate molecule turnover per enzyme molecule per second. It incorporates the rate constants for all the reactions in a multiple enzymatic system and gives a direct measure of catalytic production under optimum conditions (Eisenthal et al. 2007). As results in Table 3 show, in both cases of sarcoplasmic and myofibrillar protein hydrolysis, thermolysin exhibited higher K_{cat} than proteinase K. The rate of myofibrillar turnover was higher than sarcoplasmic protein when hydrolyzed by thermolysin, whereas proteinase K showed higher K_{cat} when sarcoplasmic protein used as substrate (Table 3). These results are in agreement with those of DH and peptide content showed in Table 1, where sarcoplasmic and myofibrillar protein contained high amount of peptides when hydrolyzed by proteinase K and thermolysin, respectively.

Catalytic efficiency ratio (K_{cat}/K_m), is a more useful tool for comparison of enzymes, as it provides more information about reaction products per unit of time. It shows direct comparison of the effectiveness of enzyme towards different substrates (Bisswanger 2004; Salami et al. 2008). Based on kinetic parameter results (Table 3), thermolysin and proteinase K revealed higher K_{cat}/K_m ratio when sarcoplasmic protein was used as substrate than myofibrillar protein. However proteinase K had higher K_{cat}/K_m ratio than thermolysin in case of hydrolyzing sarcoplasmic protein, while using myofibrillar protein as substrate, the efficiency of thermolysin was higher than proteinase K (Table 3). Although lower K_m of both enzymes for sarcoplasmic protein suggests that this protein is a better substrate than myofibrillar protein, with respect to cleavage specificity of each enzyme, substrates turnover rate and enzyme efficiency, it was suggested that sarcoplasmic and myofibrillar protein are suitable substrates for proteinase K and thermolysin hydrolytic reaction, respectively.

Conclusion

Investigation of *C. striatus* sarcoplasmic and myofibrillar protein characteristics revealed different proteolytic and kinetic activity when hydrolyzed with thermolysin and proteinase K. Results of this study indicated faster digestion of sarcoplasmic than myofibrillar protein in the same enzymatic condition, however, the rate of turnover and enzyme efficiency suggested that sarcoplasmic protein is suitable substrate for proteinase K hydrolytic reaction whereas thermolysin has higher affinity for myofibrillar protein samples. The extent of hydrolysis depends on specific cleavage of each enzyme, available target sites on peptide bonds of each substrate and accessibility of target sites for each enzyme. Dipeptide and tripeptide derivatives produced from *C. striatus* protein extract hydrolysates would be investigated with respect to potential ACE inhibitory activity in future research.

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