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Further Characterization of a Sarcoplasmic Serine Proteinase from the Skeletal Muscle of White Croaker (*Argyrosomus argentatus*)

Min-Jie Cao[1]*, Kenji Hara[2], Ling Weng[1], Nong Zhang[3], and Wen-Jin Su[1]

[1]*College of Biological Engineering, Jimei University, Jimei, Xiamen, 361021, China; fax: +86-592-6180470; E-mail: mjcao@jmu.edu.cn*
[2]*Faculty of Fisheries, Nagasaki University, Nagasaki, 852-8521, Japan*
[3]*Fisheries Research Institute of Fujian Province, Xiamen, 361012, China*

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Abstract—A trypsin-type serine proteinase (WSP) was purified previously from the sarcoplasmic fraction of skeletal muscle of white croaker (*Argyrosomus argentatus*) by Yanagihara et al. ((1991) *Nippon Suisan Gakaishi*, **57**, 133-142). However, further research on WSP was not carried out. In the present study, we determined the N-terminal amino acid sequence of this enzyme (27 amino acid residues), which revealed relatively high identity in the conserved region to other trypsin-type serine proteinases. Degradation action of WSP on neuropeptides is also reported in this manuscript. The results show that WSP only cleaves at the carboxyl side of Arg or Lys residue of the peptides, especially between dibasic amino acid residues such as Arg–Arg and Arg–Lys.

Key words: amino acid sequence, white croaker, serine proteinase, dibasic, identity, peptide

Many biologically functional peptides and proteins are produced by specific endoproteolytic processing of their precursors at monobasic or dibasic residues, and most of the processing enzymes are supposed to have highly specific trypsin-like activities [1, 2]. Among such processing enzymes the yeast Kex2 (also known as kexin (EC 3.4.21.61)) family proteases are most extensively studied. The Kex2 family proteases are Ca^{2+}-dependent, subtilisin-like serine proteases which include PC2 (PC for prohormone or proprotein convertase), PC1/3, PC4, PACE4, PC5/6, LPC/PC7/PC8/SPC7, furin (EC 3.4.21.85), etc. [3, 4]. Among them, furin (*fur* for the proto-oncogene *c-fes/fps* upstream region) was the first proprotein convertase to be identified. Further studies on cellular localization have demonstrated that within cells, PC2, PC1/3, and PC5/6 are mainly located in secretory granules, while furin and LPC/PC7/PC8/SPC7 are located in *trans*-Golgi network (TGN) [3]. Investigation of substrate specificity of the Kex2 proteases revealed that these enzymes specifically process protein or peptide precursors into their mature forms by cleaving mainly at the carboxyl side of dibasic pairs such as Lys–Arg or Arg–Arg. In contrast, protein or peptide precursors could also be processed into active forms by cleavage after monobasic sites or between the dibasic pairs by trypsin-like proteases other than those of the Kex2 family [5]. Several papers have described the purification and characterization of these serine proteases from different animals, such as bull [6], rat [7], and pig [8].

In contrast to the rapid research progress of serine proteases from mammalian tissues and yeast cells, only a few research works have been done on purification of serine proteases from fish [9-12]. Much less work has been performed on the study of primary structure and cleavage specificity of serine proteinases from fish [12, 13].

In the present study, we analyzed the N-terminal amino acid sequence of trypsin-type serine proteinase (WSP) and investigated its cleavage specificity towards neuropeptides containing dibasic and monobasic residues.

MATERIALS AND METHODS

Fish. White croaker (*Argyrosomus argentatus*) with average body weight of 300 g was freshly obtained from

* To whom correspondence should be addressed.

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Nagasaki fish market, Nagasaki, Japan, and immediately transported in ice to the laboratory of the Faculty of Fisheries, Nagasaki University. The fish meat was collected and stored at −35°C for further experimental use.

Chemicals. *t*-Butyloxycarbonyl-Val-Leu-Lys-4-methyl-coumaryl-7-amide (Boc-Val-Leu-Lys-MCA), neurotensin, α-neoendorphin, bovine adrenal medulla dodecapeptide (BAM-12P), dynorphin A 1-13, methionyl-lysyl-bradykinin, and lysyl-bradykinin were purchased from Peptide Institute (Osaka, Japan). Oxidized insulin B chain was a product of Serva (Germany). DEAE-Sephacel, Sephadex G-150, Q-Sepharose, and benzamidine-Sepharose 6B were products from Pharmacia (Sweden). Acetonitrile (HPLC grade) was obtained from Nacalai Tesque (Japan). Chemicals for WSP purification were used as described by Yanagihara et al. [9].

WSP purification and activity assay. The purification and activity assay of WSP were carried out as described by Yanagihara et al. [9]. Briefly, white croaker muscle (1 kg) was minced with 2-fold of ice-cold distilled water and homogenized by a polytron (PT-2120; Kinematica, Switzerland). The mixture was centrifuged at 10,000*g*, 4°C, for 20 min. The supernatant was fractionated by ammonium sulfate at 50-75% saturation. After centrifugation, the precipitate was dissolved in 5 mM KH_2PO_4-$Na_2B_4O_7$ buffer, pH 7.5, and dialyzed against the same buffer. The dialyzed solution was applied to a DEAE-Sephacel column, and the active fractions were eluted by a linear gradient of 0-0.5 M NaCl. Fractions enzymatically active towards the substrate Boc-Val-Leu-Lys-MCA were pooled, concentrated by ultrafiltration, and loaded on a Sephadex G-150 column. After chromatography, active fractions were pooled, dialyzed, and applied to Q-Sepharose. Purified WSP was finally obtained by benzamidine-Sepharose 6B affinity column chromatography. Enzyme activity was assayed in duplicate using 700 μl of 50 mM borate buffer (pH 8.0), 100 μl of 10 μM Boc-Val-Leu-Lys-MCA, and 200 μl enzyme solution at 60°C for 10 min and stopped by adding 1.5 ml of stopping reagent (methanol–butanol–water, 35 : 30 : 35 v/v). The enzyme activity unit (U) was defined as the amount of enzymatic protein required to release 1 nmol of AMC (7-amino-4-methyl-coumarin) per min.

Determination of the N-terminal amino acid sequence. WSP preparation purified using benzamidine-Sepharose 6B chromatography (approximately 100 pmol) was loaded on a ProSpin™ (Applied Biosystems, USA) PVDF membrane cartridge, and then the membrane was washed with distilled water to remove NaCl in the sample and directly submitted to sequence analysis using a model 492 protein sequencer (Applied Biosystems) equipped with an on-line phenylthiohydantoin amino acid 120 A analyzer.

Degradation of peptides and analysis of the resulting peptide fragments. A 50-μl sample of purified WSP (0.5 U) was incubated with each peptide (10 nmol) at 37°C for 2 h (except oxidized insulin B chain, which was incubated for 24 h) in 0.1 ml of 0.1 M Tris-HCl buffer, pH 8.0. The reaction mixture was immediately subjected to reverse-phase high performance liquid chromatography (RP-HPLC) on a μBONDPAK C_{18} column (3.9 × 300 mm; Waters, USA). Elution was carried out with a linear gradient of acetonitrile (0-70%) containing 0.1% trifluoroacetic acid for 50 min at a flow rate of 1.0 ml/min by monitoring the absorbance at 215 nm. The hydrolyzed peptide products obtained from RP-HPLC were identified by collecting corresponding fractions and analyzing their sequences using the model 492 protein sequencer or by a model L-8500 amino acid analyzer (Hitachi, Japan).

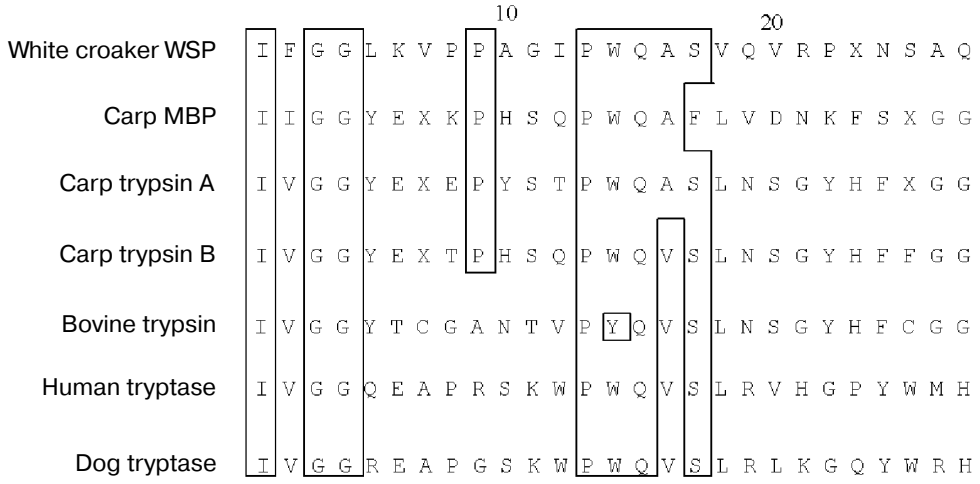

Fig. 1. Alignment of the N-terminal amino acid sequence of WSP with those of carp MBP, carp trypsin A, carp trypsin B, bovine trypsin, human tryptase, and dog tryptase. Identical residues are boxed.

RESULTS AND DISCUSSION

The N-terminal amino acid sequence of white croaker WSP was determined to the 27th residue and compared with those of various serine proteinases from different sources. WSP showed homology of 30% to a myofibril-bound serine proteinase from common carp [13], 33% to carp trypsin A [14], 30% to carp trypsin B [14], 22% to bovine trypsin [15], 26% to human tryptase [16], and 26% to dog tryptase [17] (Fig. 1). This homology is relatively low. However, in the conserved region 1 of most trypsin-type serine proteinases, the homology is significantly higher, reaching 35-53%. Searching the protein data bases of SwissPro and GenBank, no identical sequence to WSP could be found, suggesting it is a new member of the trypsin family. This partial primary structural data shown here gave us important information for primer design for future work concerning molecular cloning of the enzyme.

After incubation of neurotensin with purified WSP at 37°C for 2 h, RP-HPLC of the reaction product revealed three peaks (Fig. 2a). Based on amino acid sequence analysis and comparison of retention time on HPLC with the original peptide, the identities of these peptides were confirmed, with peak 1 as Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg; peak 2: Arg-Pro-Tyr-Ile-Leu, and peak 3, original peptide, suggesting that WSP exclusively cleaved the Arg–Arg dibasic bond of this substrate. No cleavage occurred at the carboxyl site of the second residue of the arginine pair. This phenomenon may be ascribed to the existence of Pro both at P2 and P2′, which caused the "proline-directed arginyl cleavage" as described [18]. A similar result was also identified in our previous work using carp myofibril-bound serine proteinase (MBP) [13]. To further characterize the substrate specificity of the enzyme, various peptides were incubated with it respectively and the resultant mixtures analyzed by RP-HPLC. Figure 2 shows the RP-HPLC results of WSP cleavage of three representative peptides—neurotensin (a), BAM-12P (b), and lysyl-bradykinin (c).

Figure 3 summarizes the result of WSP cleavage of different neuropeptides. Except lysyl-bradykinin, all peptides were attacked by WSP. Though neurotensin, BAM-12P, and dynorphin A1-13 contained both dibasic and monobasic amino acid residues, it was found that WSP preferably cleaved the substrates at the sites between dibasic pairs such as Arg–Arg, Lys–Arg, and Arg–Lys with 2 h of incubation, indicating that the cleavage efficiency of WSP is higher to all the peptides containing dibasic residues. However, prolonged incubation (24 h) also caused degradation at the carboxyl sites of monobasic amino acids such as in the case of oxidized insulin B chain as determined by amino acid analysis.

Interestingly, though lysyl-bradykinin also contains a Lys–Arg pair at the N-terminus of the peptide, this pair was not split by WSP, in contrast to methionyl-lysyl-

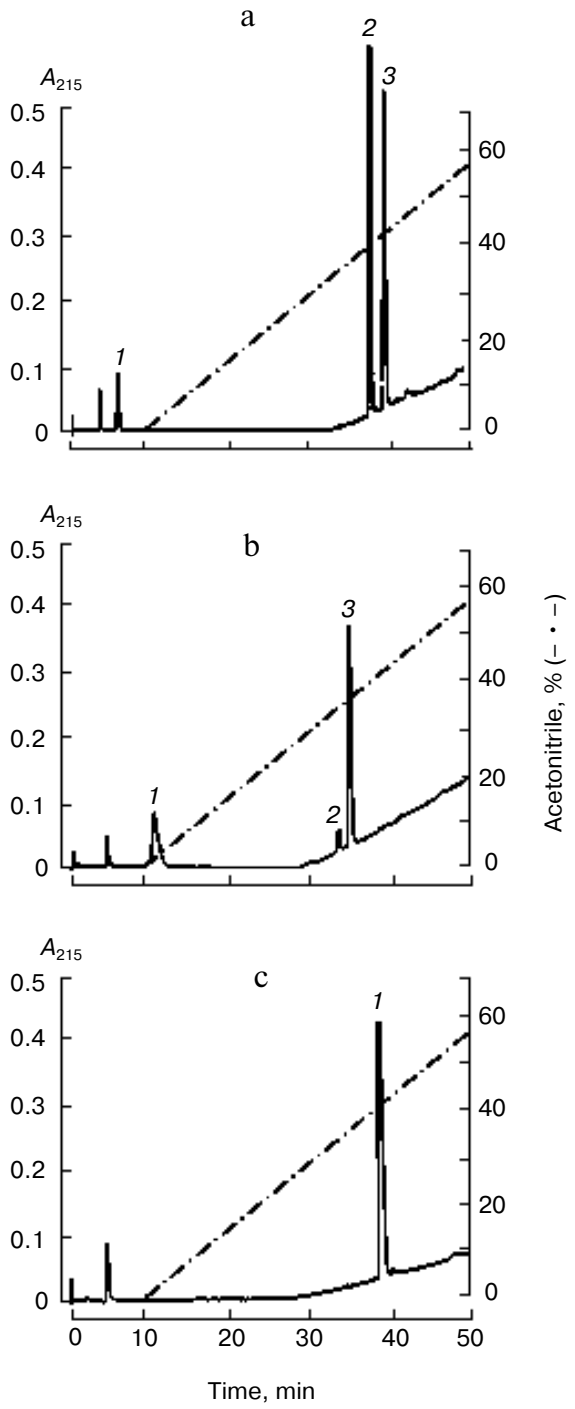

Fig. 2. RP-HPLC profiles of three respective peptides—neurotensin (a), BAM-12P (b), and lysyl-bradykinin (c)—reacting with WSP. Peptides (10 nmol) were incubated with 0.5 U of purified WSP at 37°C for 2 h and the reaction mixtures were submitted to reverse-phase HPLC, respectively. Based on the protein sequence analyses and comparison of retention time with the original peptides, the respective peaks were identified. a) Neurotensin: peak *1*) Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg; peak *2*) Arg-Pro-Tyr-Ile-Leu; peak *3*) original peptide. b) BAM-12P: peak *1*) Arg-Val-Gly-Arg-Pro-Glu; peak *2*) Tyr-Gly-Gly-Phe-Met-Arg, peak *3*) original peptide. c) Lysyl-bradykinin, no cleavage was identified.

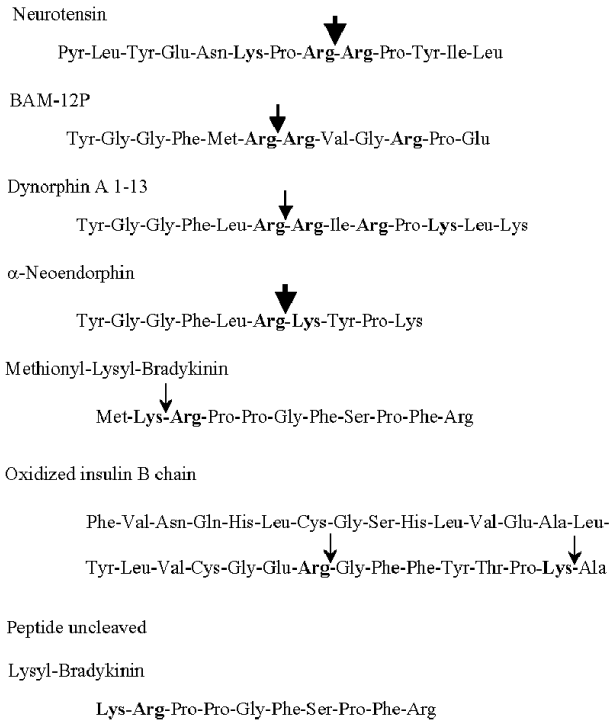

Fig. 3. Cleavage sites of peptides by serine proteinase WSP from white croaker. The cleavage extent was calculated based on the percentage of disappearance of the original peptide. Heavy, medium, and thin arrows indicate major (original peptide disappearance percentage >40%), medium (original peptide disappearance percentage = 20-40%), and small (original peptide disappearance percentage <20%) cleavage sites, respectively. Pyr, pyroglutamyl.

bradykinin, suggesting that the primary structure of the peptide is quite important for the cleavage by WSP. A similar result was also obtained using carp MBP [13]. However, different from carp MBP, unique cleavage at the carboxyl site of glycine of peptides lysyl-bradykinin and methionyl-lysyl-bradykinin was not identified after WSP treatment, suggesting minor differences in substrate specificity of these enzymes.

At present, the physiological function of WSP remains ambiguous. As a sarcoplasmic serine proteinase, its degradation of myofibrillar proteins such as myosin heavy chain and actin was dramatic [9]. Though the existence of myofibril-bound serine proteinase (MBSP) was also identified in white croaker [19], the extremely low content of MBSP in fish muscle suggests the possibility that it may cooperate with WSP in the non-lysosomal pathway and plays an essential role in the turnover of myofibrillar proteins *in vivo*.

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