Identification of a *modori*-inducing proteinase in the threadfin bream: Molecular cloning, tissue distribution and proteinase leakage from viscera during ice storage

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Abstract

Previously we purified and characterized a sarcoplasmic serine proteinase (SSP) from the belly muscle of the threadfin bream as a *modori*-inducing proteinase. In our attempt to clarify the structure and physiological functions of SSP, we successfully cloned the full-length cDNA of SSP (ORF 726 bp). The deduced amino acid sequence of SSP (241 residues) was highly homologous to fish trypsinogen. The distribution of SSP mRNA and the proteinase activity in the tissue indicated that SSP was mainly synthesized and existed in the digestive system under physiological conditions. After ice storage of the threadfin bream without gutting, a high SSP activity was detected only in the belly muscle because of SSP leaked from the viscera. Therefore, it is desirable to use edible proteinase inhibitor to inactivate the leaked SSP during production of *surimi*-based products or to take effective measures to prevent the proteinase leakage during post-harvest storage.

Keywords

Serine proteinase; Molecular cloning; Tissue distribution; Proteinase leakage; *Modori* phenomenon

1. Introduction

Surimi-based products prepared from fish muscle proteins, are widely produced and consumed around the world. During the heating process when preparing such products, myofibrillar protein degradation takes place and weakens the *surimi* gel. This phenomenon is called *modori*. The *modori* phenomenon is mainly attributed to endogenous proteinases that possess the ability to degrade myofibrillar proteins. These proteinases are thermally activated at temperatures of 50-70℃, and then the highly activated proteinases degrade the myofibrillar proteins during heating process and induce the *modori* phenomenon (Jiang, 2000).

Thus far, various types of muscle endogenous proteinases involved in myofibrillar protein degradation have been purified and characterized. Many researchers have revealed that the *modori* phenomenon is induced by muscle endogenous proteinases, especially serine and cysteine proteinases (Yoshida, Ohta, Kuwahara, Cao, Hara, & Osatomi, 2015; Hu, Ji, Jiang, Zhang, Chen, & Ye, 2015; Wongwichian, Chaijan, Panpipat, Klomklao, & Benjakul, 2016; Suwansakornkul, Itoh, Hara, & Obatake, 1993; Singh & Benjakul, 2018). However, most researchers have focused on the muscle endogenous proteinases, while visceral proteinases have received little attention. Recently, it has been reported that proteinases attributed to the *modori* phenomenon in white croaker may not be muscle endogenous proteinases but visceral proteinases that contaminated the muscles during fish dissection (Ueki, Wan, & Watabe, 2016a, 2016b; Ueki, Matsuoka, Wan, & Watabe, 2018). These findings suggest that visceral proteinases are also involved in the *modori* phenomenon of *surimi*-based products.

In general, visceral proteinases are present in the internal organs or digestive system. When the fish dies, however, the visceral enzymes may migrate to other tissues during storage and result in proteinase leakage. The leakage of proteinases from viscera into fish muscle has been found in some special fish species, including capelin, anchovy and herring (Gildberg, 1978; Martinez & Gildberg, 1988; Felberg, Slizyte, Mozuraityte, Dahle, Olsen, & Martinez, 2009). In these fish, the visceral proteinases rapidly leak into the belly muscle during post-mortem and cause serious autolysis that end up in the disruption of the abdominal wall, which was referred

64 to as belly bursting (Veliyulin, Felberg, Digre, & Martinez, 2007). As an example, the visceral
65 proteinases were demonstrated to have leaked into the belly muscle of the herring within 24 h
66 during ice storage and caused serious autolysis of the belly muscle (Felberg et al., 2009). These
67 findings suggest that once visceral proteinases leak into the muscle tissue, it may cause serious
68 autolysis of the belly muscle and result in a decrease in the quality of the fish for consumption
69 as food. For the *surimi* industry, the leakage of visceral proteinases into the muscle may induce
70 the *modori* phenomenon of *surimi*-based products.
71 Threadfin bream, one of the most important commercial fish species, is considered to be the
72 best raw material for *surimi*-based products. However, the problem of *modori* phenomenon is
73 occasionally observed in industrial production resulting in huge economic losses. In a previous
74 study (Liu et al., 2019), we purified a sarcoplasmic serine proteinase (SSP) from the belly
75 muscle of the threadfin bream as a *modori*-inducing proteinase (with an optimum temperature
76 of 50°C), and it was found to be a homodimer by SDS-PAGE and have a highly homologous
77 N-terminal amino acid sequence with fish trypsin. Moreover, SSP showed a high activity in the
78 belly muscle, but had almost no activity in the dorsal muscle (Liu et al., 2020). Based on the
79 SDS-PAGE, homology analysis, and the uneven distribution in muscle parts, SSP was
80 suspected to be a novel trypsin that may have leaked from the digestive system during storage.
81 In fact, the uneven distribution of proteinase activity in different muscle parts (dorsal and
82 belly muscles) is not physiological distribution, and this may be attributed to proteinase
83 contamination during dissection or proteinase leakage during storage. In white croaker, another
84 important raw material for *surimi*-based products, a higher proteolytic activity was observed in
85 the belly muscle and was reported to be responsible for the *modori* phenomenon. Moreover, the
86 higher proteolytic activity in the belly muscle was considered to be caused by contamination of
87 visceral proteinases during fish dissection rather than muscle endogenous proteinases (Ueki,
88 Matsuoka, Wan, & Watabe, 2018). On the other hand, we found that a high SSP activity was
89 detected in the belly muscle of the threadfin bream but not in the dorsal muscle, even though
90 the fish was carefully dissected to avoid contamination of proteinases from the viscera during
91 dissection (Liu et al., 2020). Therefore, we hypothesized that visceral proteinases leaked to the

belly muscle during post-harvest storage and were involved in the *modori* phenomenon.

In this study, we aimed to clarify the leakage of SSP from the viscera into fish muscles during post-harvest storage and its effect on the *modori* phenomenon. We cloned the full-length cDNA of SSP and investigated the distribution of SSP mRNA and the proteinase activity in the tissue. We also investigated the leakage of SSP during post-harvest storage and its effect on the gel properties of the *surimi* gel. To our knowledge, this is the first report of a leaked visceral proteinase, rather than muscle endogenous proteinase, as the cause of the *modori* phenomenon in the threadfin bream. This study also gives new insight on improving the quality of *surimi*-based products.

2. Materials and methods

2.1. Fish

Threadfin breams (*Nemipterus virgatus*) were commercially caught in the Hirado Sea in Nagasaki, Japan, and the fish were put into a holding tank with ice. Fish were kept in ice and transported to the laboratory through Nagasaki fish market within 24 h. Fish were then carefully dissected and filleted to prevent muscle tissue from being contaminated by visceral enzymes. The tissues used for RNA extraction were treated by five volumes of RNAlater (Thermo Fisher Scientific, San Jose, CA, USA) overnight to stabilize the RNA in the tissue and stored at −80℃ until use. The tissues used for activity detection were washed with cold distilled water and stored at −30℃ until use.

2.2. Total RNA isolation

Total RNA was isolated from the tissues using ISOGEN II (Nippongene, Tokyo, Japan) according to the manufacturer's protocol.

2.3. cDNA cloning

The cDNA synthesis, RT-PCR and RACE were performed according to the method of

Yoshida et al. (2013) with a slight modification. For RT-PCR, degenerated primers (sense primer: 5'-ATHGTNGGNGGNTAYGARTGYC-3' and antisense primer: 5'-ACAGGACCACCWGARTCNCC-3') were designed based on the N-terminal amino acid sequence of purified SSP in our laboratory (Liu et al., 2019) and the highly conserved active site of trypsin-like serine proteinases, respectively. For 3'-RACE, an SSP specific primer (sense primer: 5'-GCAAGCCCGCCACCCTCAACC-3') was designed based on the SSP cDNA sequence determined by RT-PCR, and the anchor primer (APS-BR: 5'-GGCCACGCGTCGACTAGTAC-3') was used as an antisense primer. For 5'-RACE, an oligo-dT primer (CDS-BR: 5'-GGCCACGCGTCGACTAGTAC$(T)_{16}$-3') and a specific primer (antisense primer: 5'-GGACTTGTAGCAGTGAGCAGC-3') designed based on the SSP cDNA sequence determined by RT-PCR were used for cDNA amplification.

2.4. DNA sequencing

DNA sequencing was performed by the dideoxy chain termination method using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with 3130 Genetic Analyzer (Applied Biosystems).

2.5. Sequence and phylogenetic tree analysis

Protein BLAST was performed at the GenBank website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and sequence alignment (https://www.ebi.ac.uk/Tools/msa/clustalo/) was performed among SSP, trypsinogens, myofibril-bound serine proteinase (MBSP) and hyaluronan binding protein 2 (HABP2) from other fish. With the amino acid sequences of SSP, trypsinogens, MBSPs and HABP2, a phylogenetic tree was generated using Neighbor Joining method by MEGA7 (https://megasoftware.net/). It is worth noting that MBSP is widely present in fish muscle and is responsible for the degradation of myofibrillar proteins during *surimi* gel production and causes the *modori* phenomenon (Cao & Osatomi, 2016).

2.6. Tissue distribution of SSP mRNA

To investigate the distribution of SSP mRNA in the tissue, total RNA was extracted from various tissues (hepatopancreas, pyloric caeca, intestinal tract, gonad, heart, dorsal muscle, and belly muscle) of the threadfin bream. And then, 500 ng of total RNA was subjected to reverse transcription using oligo-dT primer CDS-BR and M-MLV reverse transcriptase (Promega). The synthesized cDNAs of the various tissues were used as templates for quantitative real-time PCR (qRT-PCR) with SSP specific primers (sense primer: 5'-GAACTGGGTTGTGTCTGCTGCTC-3' and antisense primer: 5'-GAGGTGATGAACTGCTCGTTAC-3') and Power SYBR Green PCR Master Mix (Applied Biosystems) in a 7300 Real Time PCR System (Applied Biosystems). The qRT-PCR was performed as follows: 2 min at 50℃, 10 min at 95℃, 40 cycles of 15 s at 95℃, 1 min at 60℃. As a reference gene, 18S ribosomal RNA gene was amplified using specific primers (sense primer: 5'-ATTCCGATAACGAACGAGAC-3' and antisense primer: 5'-CCACTTGTCCCTCTAAGAAG-3') that designed based on the 18S ribosomal RNA gene sequences of the banded sergeant (GenBank: KX015768.1) and the spotbanded scat (GenBank: KJ774763.1).

2.7. Preparation of the crude enzyme

The tissue was minced and homogenized with 3-folds (w/v) of Buffer A (50 mM Tris-HCl, pH 7.5, containing 5 mM $CaCl_2$) and centrifuged at 12,800 ×g for 20 min. The supernatant was used as the crude enzyme.

2.8. Gelatin zymography

Gelatin zymography is a sensitive method for determining the activity of gelatinolytic proteinases (Snoek-van Beurden & Von den Hoff, 2005). Trypsin-like serine proteinases, such as SSP, shows gelatinolytic activity. In our previous study, SSP can be clearly detected at 50 kDa on gelatin zymography even in the crude extract (Liu et al., 2020). Hence, the SSP activity assay was carried out using this method. Gelatin zymography was performed using 10%

polyacrylamide gel containing 0.1% gelatin as described by Yoshida et al. (2009). After electrophoresis, the gel was incubated in Buffer A for 2 h at 50℃ and then stained with CBB R-250.

2.9. Tissue distribution of SSP activity

To investigate the distribution of SSP activity in the tissue, the crude enzyme was extracted from various tissues (same with the distribution of SSP mRNA in the tissue) of the threadfin bream, and the activity of the crude enzyme was determined using gelatin zymography. The crude enzyme was diluted for 100 times and 1 μL of the dilution was applied to gelatin zymography. The experiment was carried out with five independent individuals.

2.10. Experiment of SSP leakage

To investigate the leakage of SSP from viscera into the fish muscle during ice storage, the threadfin breams with or without gutting were stored in ice for 72 h. Then, SSP activity in the dorsal and belly muscles was determined using gelatin zymography. The pyloric caeca of the fresh threadfin bream were used as a positive control. Crude enzymes prepared from muscle and pyloric caeca were diluted for 10 and 100 times, respectively. The experiment was carried out with five independent individuals.

2.11. Preparation and evaluation of surimi gel

Fish muscle was minced using a food processor and washed with cold 0.1% NaCl solution (4℃) at a ratio of 1:1.5 (w/v). The mixture was stirred gently for 270 sec, and the mince was dewatered using filter paper until the moisture reached 81%. The washed mince was then mixed thoroughly with 5% sorbitol, 4% sucrose and 0.25% polyphosphate. The mixture was kept at −30℃ and referred to as frozen *surimi*.

To prepare *surimi* gel, the frozen *surimi* was thawed at 4℃ overnight and ground with 3% sodium chloride for 3 min at 4℃ using a food processor. The *surimi* was then packed into a polyvinylidene chloride bag (diameter: 35 mm) and incubated at 30℃ for 1 h, and 90℃ for 30

192 min. After heating, the gels were immediately cooled in iced water for 30 min and left at room
193 temperature for about 1 h until the time their gel strengths were measured.

194 The *surimi* gels were sliced into 25 mm-thick pieces and their gel strength and strain were
195 measured by a CR-500DX rheometer (Sun Scientific Co., Tokyo, Japan) equipped with a
196 spherical plunger (diameter: 5 mm) with a rising rate of the sample table at 6 cm/min. The
197 strength and strain of *surimi* gels were calculated from the mean of eight measurements.

198 *2.12. Statistics*

199 All analyses were performed using the GraphPad5 software program (GraphPad Prism,
200 Software Inc., San Diego, CA, USA) by one-way ANOVA with a Tukey's multiple comparison
201 test.

202 **3. Results and discussion**

203 *3.1. cDNA cloning of SSP*

204 In order to clarify the structure and physiological functions of SSP, we cloned the full-length
205 cDNA of SSP from the hepatopancreas of the threadfin bream (Fig. 1). The sequence consisted
206 of a 5'-non-coding region of 24 bp, an open reading frame of 726 bp and a 3'-non-coding region
207 of 109 bp. The open reading frame encoded a putative protein of 241 amino acid residues,
208 which contained a signal peptide of 15 amino acid residues (Met^{1}-Ala^{15}), an activation peptide
209 of 5 amino acid residues (Phe^{16}-Lys^{20}) and a mature form protein of 221 amino acid residues.
210 The first 22 N-terminal amino acid residues of the mature form protein were identical to that of
211 the purified SSP (Liu et al., 2019), indicating that the cloned cDNA was the gene encoding SSP.
212 The theoretical molecular weight of the mature form SSP was calculated to be 24.3 kDa.
213 Actually, the native SSP was a homodimer linked by disulfide bond(s), and the molecular
214 weight of its monomer was determined to be 22.5 kDa (Liu et al., 2019), which was slightly
215 lower than the theoretical molecular weight. The difference in the molecular weight of the SSP
216 may be ascribed to the mobility of the protein on the gel or autolysis (Zhou et al., 2012).

3.2. Sequence analysis

Comparison of the amino acid sequences of SSP and serine proteinases from other fish was shown in Fig. 2. SSP was highly homologous to trypsinogens from other fish with a homology of 75-87%. The catalytic triad (His^{60}, Asp^{104}, Ser^{195}) of trypsinogens was completely conserved in SSP. The 12 Cys residues (at positions of 27, 45, 61, 129, 136, 155, 166, 180, 191, 201, 215, 228) of trypsinogens were also completely conserved in SSP, and may form intramolecular or intermolecular disulfide bonds to stabilize the structure of the molecule and to form homodimer.

3.3. Phylogenetic tree analysis

Phylogenetic tree analysis was performed with the amino acid sequences of SSP and serine proteinases from other fish using Neighbor Joining method (Fig. 3). As a result, the serine proteinases were classified into three major groups: trypsinogen, MBSP and HABP2. SSP was in the same clade of trypsinogen, but not the muscle endogenous proteinases MBSP or HABP2. Phylogenetic tree analysis also showed that SSP was closer to trypsin rather than to the muscle endogenous serine proteinases (MBSP and HABP2) from other fish (Fig. 3). From these results, SSP is likely to be a novel trypsin that exists as a homodimer. Trypsins have been purified from many tissues of fish, such as hepatopancreas (Zhou et al., 2012; Cao, Osatomi, Suzuki, Hara, Tachibana, & Ishihara, 2000; Balti, Barkia, Bougatef, Ktari, & Nasri, 2009), intestinal tract (Jellouli, Bougatef, Daassi, Balti, Barkia, & Nasri, 2009; Klomklao, Kishimura, Nonami, & Benjakul, 2009; Unajak et al., 2012), pyloric caeca (Khantaphant & Benjakul, 2010; Marcuschi et al., 2010), and spleen (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2006; Klomklao, Benjakul, Visessanguan, Kishimura, Simpson, & Saeki, 2006; Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2007; Poonsin et al., 2019). It is well known that trypsin is a digestive enzyme and is responsible for the hydrolysis of dietary proteins in the digestive system. Additionally, it has been reported that splenic trypsin may play some roles in the immune system (Koshikawa et al., 1998). However, there was no related report on trypsin present in fish muscle.

3.4. Tissue distribution of mRNA expression and proteinase activity of SSP

In order to elucidate the distribution of SSP in the tissues, its mRNA expression and proteinase activity in various tissues were investigated using qRT-PCR and gelatin zymography, respectively. As shown in Fig. 4A, SSP was expressed in the hepatopancreas, pyloric caeca, intestinal tract and spleen, and the highest expression was detected in the pyloric caeca. As shown in Fig. 4B, the SSP activity was detected in the hepatopancreas, pyloric caeca, intestinal tract, spleen and gonads, and the activity in the pyloric caeca and intestinal tract was much higher than in other tissues. In contrast, there was no mRNA expression or proteinase activity detected in the dorsal and belly muscles. From these results, it was suggested that SSP was mainly synthesized in any tissue of the digestive system and secreted to the digestive tract as a zymogen (Pro-SSP), thereafter the Pro-SSP was converted to a mature form by its autolytic activity in the pyloric caeca and intestinal tract. As we assumed, there was no SSP mRNA expression or activity detection in the muscles, and this indicated that SSP was not a muscle endogenous proteinase and might be a novel trypsin. Moreover, SSP was expressed and present in the spleen, indicating that SSP may play some roles in the immune system. In the gonads, there was no SSP mRNA expression even though proteinase activity was detected. This may be due to the fact that gonads are close to the intestinal tract where the activated SSP is rich and easily leaks during post-harvest.

3.5. SSP leakage during ice storage

SSP did not exist in the muscle under physiological conditions, and the only way it was detected in the muscle was considered to be from leaking from the viscera. Therefore, an experiment was designed to investigate the leakage of SSP from viscera into the fish muscle. Threadfin breams with or without gutting were stored in ice for 72 h, and the SSP activity in the dorsal and belly muscles was determined using gelatin zymography. As shown in Fig. 5, there was no SSP activity detected in both muscles for the fresh fish and the ice stored fish with gutting. On the other hand, SSP activity in the dorsal and belly muscles of the fish without gutting increased after ice storage for 72 h, and the activity in the belly muscle was much higher

than in the dorsal muscle. These results indicate that SSP leaked from viscera into the muscles of the threadfin bream during ice storage. Considering the difference of SSP activity between dorsal and belly muscles, SSP initially leaked into the belly muscle during ice storage and spread into the dorsal muscle as a result of prolonged storage time.

3.6. Effects of SSP leakage on the properties of surimi gel

SSP was purified and characterized as a *modori*-inducing proteinase, and the gel strength of the threadfin bream *surimi* was significantly reduced by the addition of purified SSP (Liu et al., 2019). MBSP, which was known as a *modori*-inducing proteinase, was hardly detected in the muscles of the threadfin bream (Liu et al., 2020). Therefore, the leakage of SSP could probably be the main factor for the *modori* phenomenon. However, it was not clear whether the leaked SSP can induce the *modori* phenomenon or whether the leaked SSP can be removed by washing process. To clarify the effects of SSP leakage on the *modori* phenomenon, we used ice stored (72 h) threadfin bream to prepare the washed and unwashed *surimi*, and investigated their gel properties. As a result, the gel strength of the washed *surimi* prepared from the belly muscle was significantly lower than that of the dorsal muscle (Fig. 6). In general, most of the undesirable substances (such as lipid, blood, and soluble proteinases) in the muscles can be removed during *surimi* production, while the leaked SSP cannot be effectively removed by washing process (data not shown). Therefore, the leakage of SSP was considered to be the main factor causing the *modori* phenomenon of the threadfin bream. In addition, the gel strengths of unwashed *surimi* both prepared from dorsal and belly muscle were much lower than washed *surimi*, this may be also caused by the leaked SSP in the threadfin bream muscle.

According to the report of Oujifard, Benjakul, Ahmad, & Seyfabadi (2012), the gel strength of commercial frozen *surimi* (grade A) of the threadfin bream is about 600 g, while the gel strength of the *surimi* prepared from the dorsal muscle of the ice stored (72 h) threadfin bream is about 400 g. The differences in gel strength may be caused by the freshness of raw materials or processing conditions. In addition to *surimi* gel, another commercially available protein gel is gelatin gel (cold-set gel), which is made of denatured collagen, and its gel strength is

297 generally weaker than *surimi* gel (Sinthusamran, Benjakul, Swedlund, & Hemar, 2017; Zhang,
298 He, & Simpson, 2017). Although the gel forming mechanisms between the two kind of gels are
299 different, considering SSP also possesses gelatin hydrolyzing activity, it may also play a
300 negative role on gel properties during gelatin gel production.

301 ## 4. Conclusion

302 We successfully cloned the full-length cDNA of SSP from the hepatopancreas of the
303 threadfin bream. The deduced amino acid sequence of SSP was highly homologous to fish
304 trypsinogen. SSP was mainly synthesized and existed in the digestive system under
305 physiological conditions. SSP could have leaked into the belly muscle during post-harvest ice
306 storage and may be involved in the *modori* phenomenon of the threadfin bream as an exogenous
307 proteinase. Therefore, it is desirable to use edible proteinase inhibitors to inactivate the leaked
308 SSP in order to improve the quality of *surimi*-based products of the threadfin bream. Moreover,
309 it is necessary to take appropriate measures to prevent the leakage of visceral proteinases of
310 fish during post-harvest storage.

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313 ## Conflict of interest

314 The authors declare that they do not have any conflicts of interest.

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425 snakehead (*Channa argus*). *Comparative Biochemistry and Physiology, 161*B, 247-254.

426 **Figure captions**

427 **Fig. 1** Nucleotide and deduced amino acid sequence of SSP from hepatopancreas of the
428 threadfin bream. The N-terminal amino acid sequence of purified SSP is shaded in black and
429 showed by white letters. Initial codon (ATG) and stop codon (TAA) are shown in boldface.
430 The signal peptide sequence is underlined and the activation peptide sequence is dot-lined. The
431 active site residues of serine proteinase are indicated in boxes.

432 **Fig. 2** Alignment of the amino acid sequences of SSP, trypsinogen, MBSP and HABP2. SSP
433 (threadfin bream, this study); Try, trypsinogen (red seabream, BAL14139.1; gilt-head bream,
434 ABE68639.1; Japanese anchovy, BAB40330.1; Japanese dace, BAG55217.2; grass carp,
435 ACS71745.1); MBSP (common carp, Cao & Osatomi, 2016; silver carp, ACE62930.1; goldfish,
436 ABI31732.1); HABP2 (red seabream, BAN66753.1; gilt-head bream, AJW65884.1). Identical
437 amino acid residues with SSP of the threadfin bream are shaded in gray. The active site residues
438 of serine proteinases are shaded in black and showed by white letters. The cysteine residues of
439 serine proteinases are indicated in boxes.

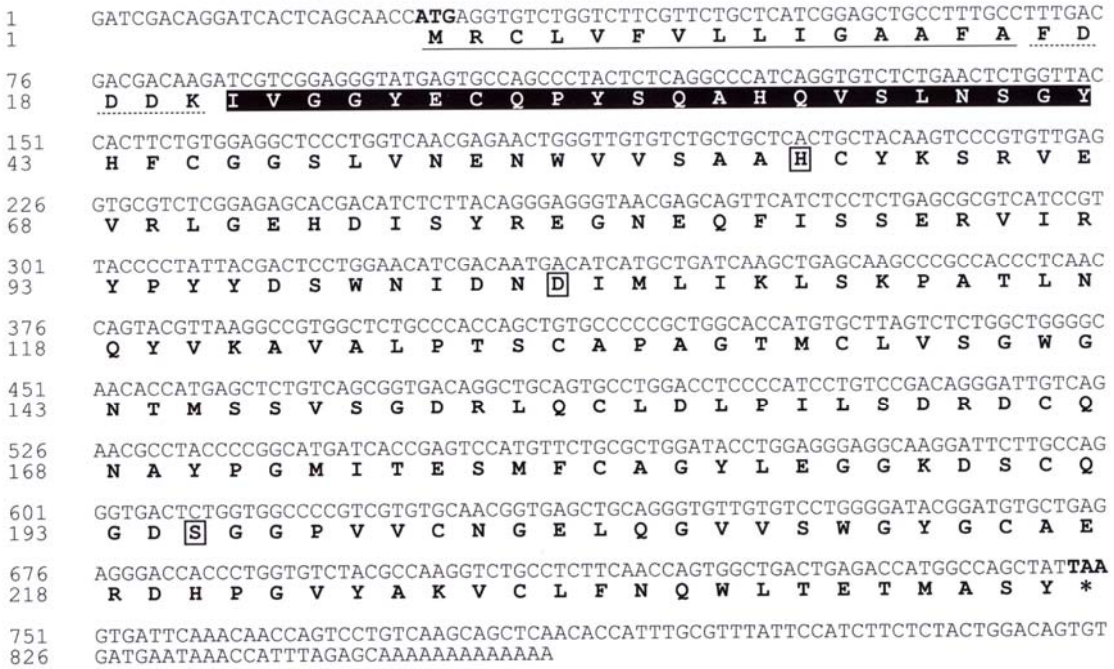
440 **Fig. 3** Phylogenetic tree of amino acid sequence of SSP and serine proteinases from other fish
441 by Neighbor Joining method. The branch lengths are drawn according to the genetic distance.

442 **Fig. 4** (A) Distribution of SSP mRNA in the tissue relative to 18S rRNA in the threadfin bream
443 determined using qRT-PCR (n=2~5). (B) Distribution of SSP activity in the tissue of the
444 threadfin bream determined using gelatin zymography. M, protein standards; Hp,
445 hepatopancreas; P, pyloric caeca; I, intestinal tract; S, spleen; G, gonads; H, heart; D, dorsal
446 muscle; B, belly muscle. Different letters on the bars indicate significant differences.

447 **Fig. 5** Leakage of SSP from viscera into the dorsal (A) and belly (B) muscles of the threadfin

448 bream during ice storage (72 h). The activity of SSP was determined using gelatin zymography.
449 M, protein standards; 1, the muscle of fresh threadfin bream; 2, the muscle of ice stored
450 threadfin bream with gutting; 3, the muscle of ice stored threadfin bream without gutting; 4, the
451 pyloric caeca of fresh threadfin bream.
452 **Fig. 6** Effects of leakage of visceral proteinases on the gel strength (A) and gel strain (B) of
453 frozen *surimi* prepared from the dorsal and belly muscles of the threadfin bream. Different
454 letters on the bars indicate significant differences.

Fig. 1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1 M R C L V F V L L I G A A F A F D
76 GACGACAAGATCGTCGGAGGGTATGAGTGCCAGCCCTACTCTCAGGCCCATCAGGTGTCTCTGAACTCTGGTTAC
18 D D K I V G G Y E C Q P Y S Q A H Q V S L N S G Y
151 CACTTCTGTGGAGGCTCCCTGGTCAACGAGAACTGGGTTGTGTCTGCTGCTCACTGCTACAAGTCCCGTGTTGAG
43 H F C G G S L V N E N W V V S A A H C Y K S R V E
226 GTGCGTCTCGGAGAGCACGACATCTCTTACAGGGAGGGTAACGAGCAGTTCATCTCCTCTGAGCGCGTCATCCGT
68 V R L G E H D I S Y R E G N E Q F I S S E R V I R
301 TACCCCTATTACGACTCCTGGAACATCGACAATGACATCATGCTGATCAAGCTGAGCAAGCCCGCCACCCTCAAC
93 Y P Y Y D S W N I D N D I M L I K L S K P A T L N
376 CAGTACGTTAAGGCCGTGGCTCTGCCCACCAGCTGTGCCCCCGCTGGCACCATGTGCTTAGTCTCTGGCTGGGGC
118 Q Y V K A V A L P T S C A P A G T M C L V S G W G
451 AACACCATGAGCTCTGTCAGCGGTGACAGGCTGCAGTGCCTGGACCTCCCCATCCTGTCCGACAGGGATTGTCAG
143 N T M S S V S G D R L Q C L D L P I L S D R D C Q
526 AACGCCTACCCCGGCATGATCACCGAGTCCATGTTCTGCGCTGGATACCTGGAGGGAGGCAAGGATTCTTGCCAG
168 N A Y P G M I T E S M F C A G Y L E G G K D S C Q
601 GGTGACTCTGGTGGCCCCGTCGTGTGCAACGGTGAGCTGCAGGGTGTTGTGTCCTGGGGATACGGATGTGCTGAG
193 G D S G G P V V C N G E L Q G V V S W G Y G C A E
676 AGGGACCACCCTGGTGTCTACGCCAAGGTCTGCCTCTTCAACCAGTGGCTGACTGAGACCATGGCCAGCTATTAA
218 R D H P G V Y A K V C L F N Q W L T E T M A S Y *
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Fig. 2

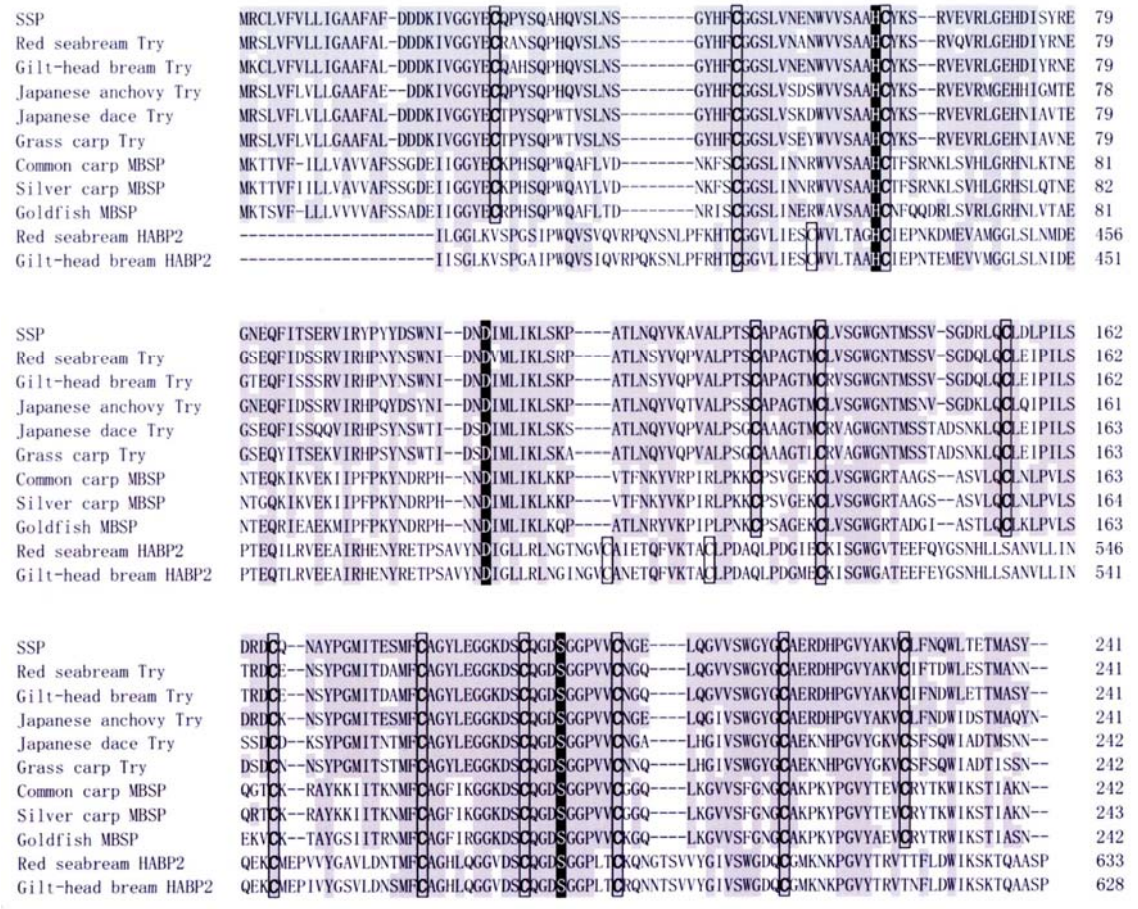
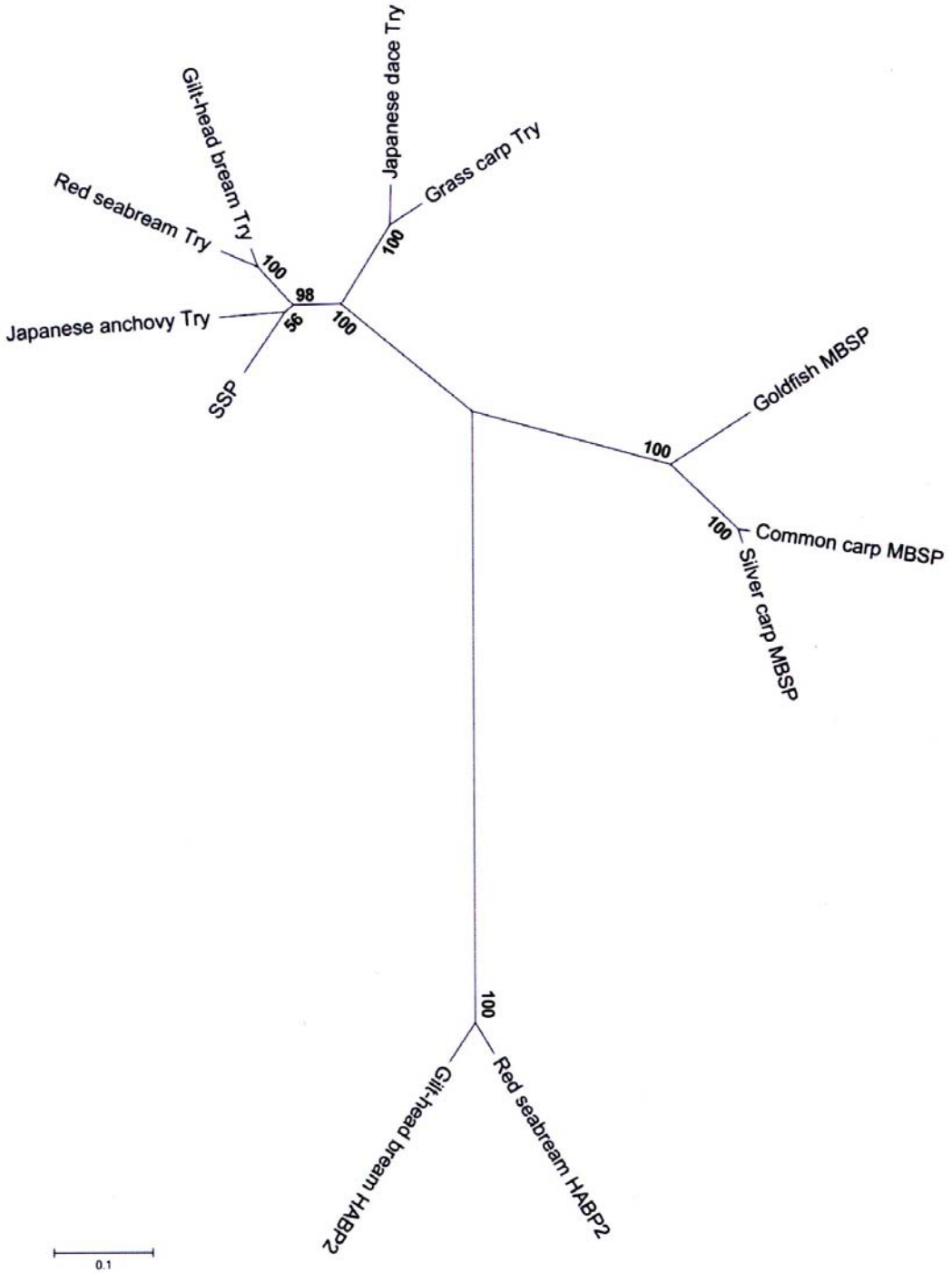

SSP
Red seabream Try
Gilt-head bream Try
Japanese anchovy Try
Japanese dace Try
Grass carp Try
Common carp MBSP
Silver carp MBSP
Goldfish MBSP
Red seabream HABP2
Gilt-head bream HABP2

Fig. 3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Grass carp Try
Gilt-head bream Try
Red seabream Try
Japanese anchovy Try
SSP
Goldfish MBSP
Common carp MBSP
Silver carp MBSP
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98
100
55
100
100
100
0.1

Fig. 4

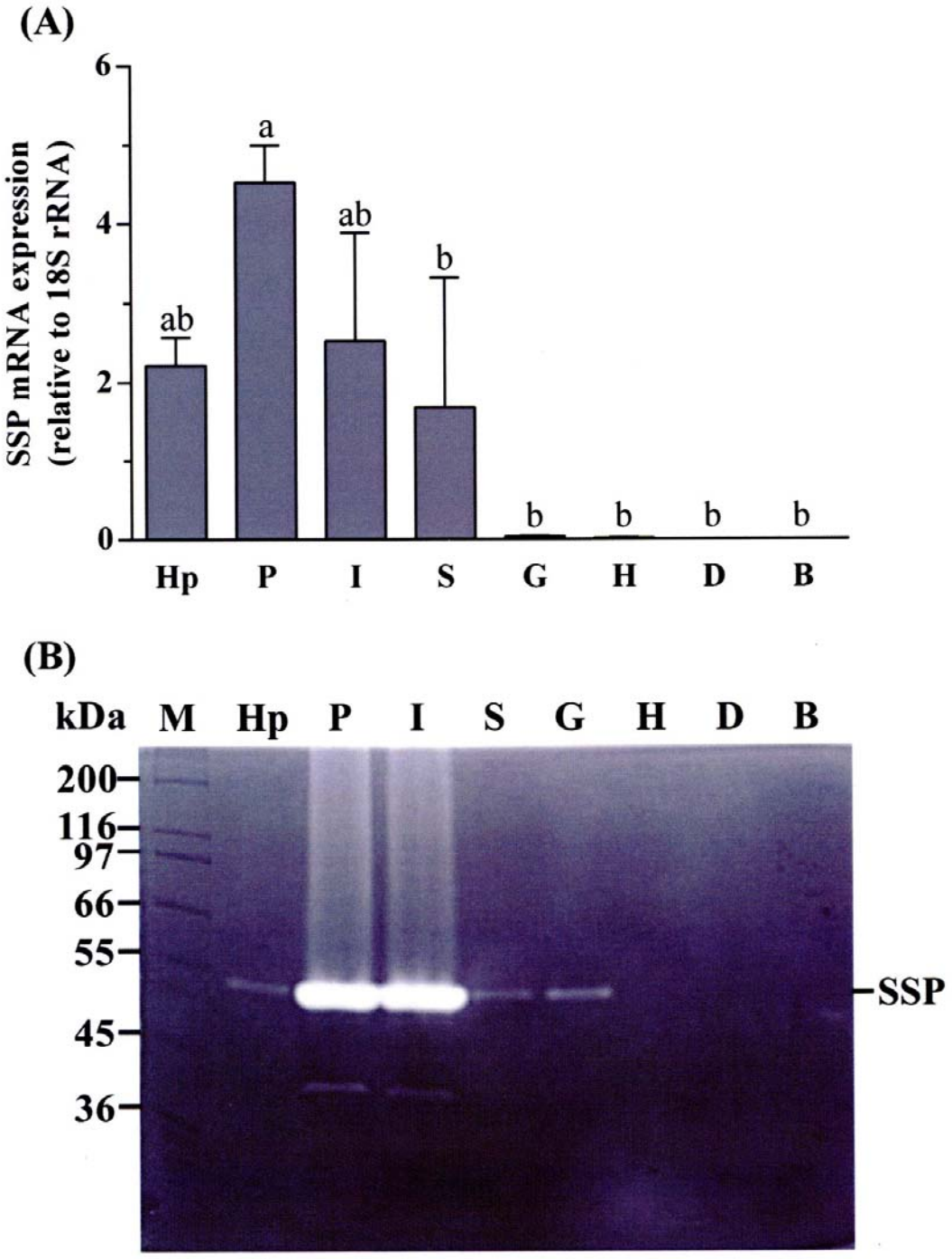

(A)
SSP mRNA expression
(relative to 18S rRNA)
6
4
2
0
a
ab
ab
b
b
b
b
b
Hp
P
I
S
G
H
D
B
(B)
kDa
M
Hp
P
I
S
G
H
D
B
200
116
97
66
55
45
36
SSP

Fig. 5

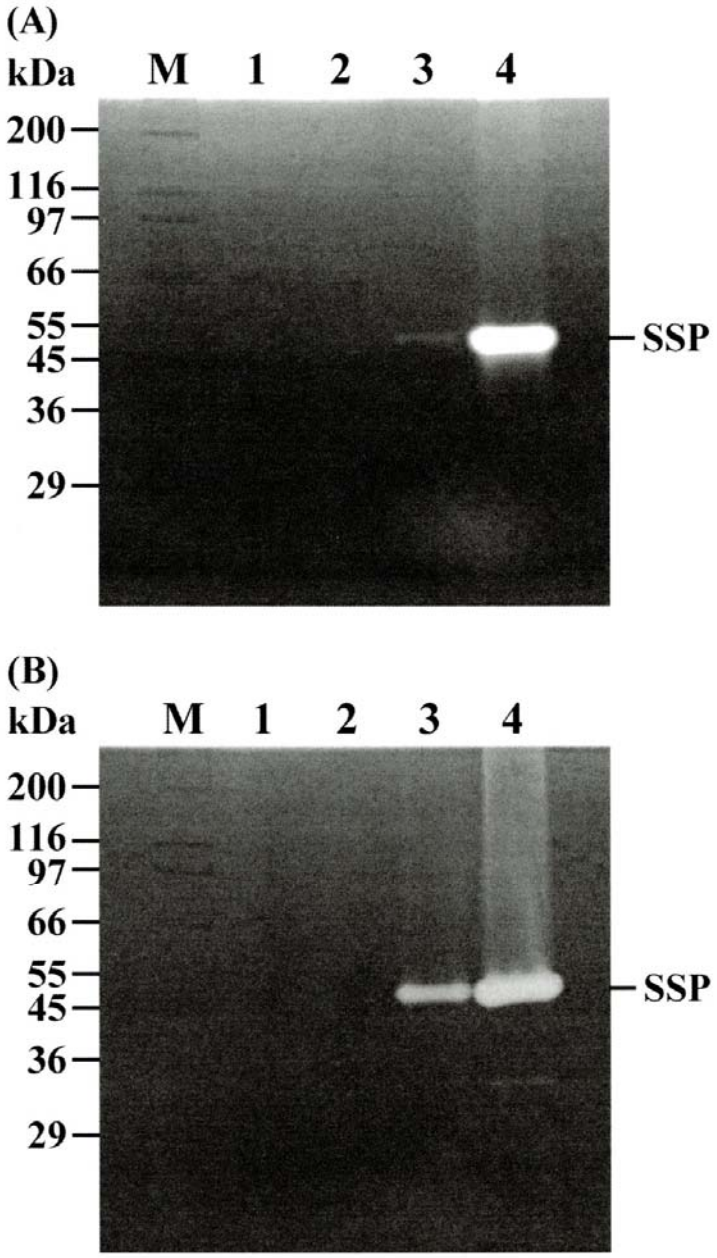
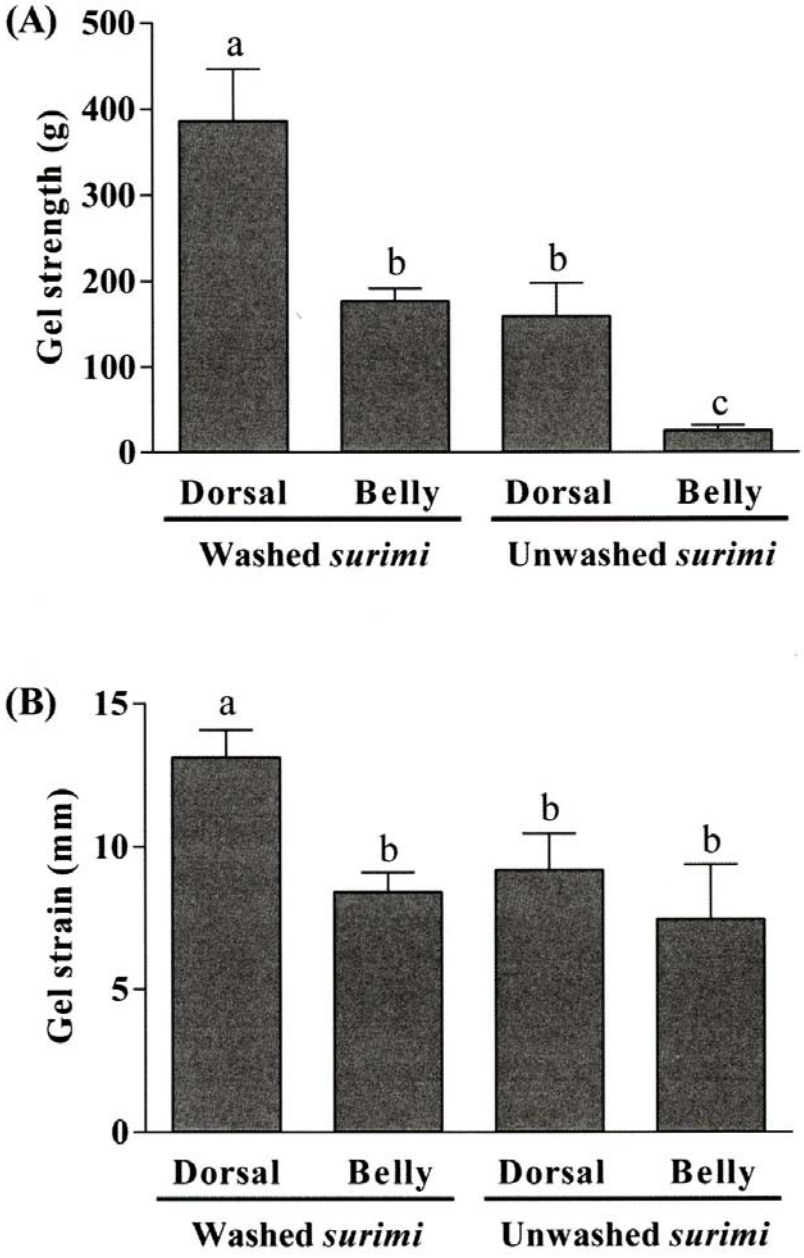

(A)
kDa
M
1
2
3
4
200
116
97
66
55
45
36
29
SSP
(B)
kDa
M
1
2
3
4
200
116
97
66
55
45
36
29
SSP

Fig. 6


(A)
Gel strength (g)
500
400
300
200
100
0
a
b
b
c
Dorsal
Belly
Dorsal
Belly
Washed surimi
Unwashed surimi
(B)
Gel strain (mm)
15
10
5
0
a
b
b
b
Dorsal
Belly
Dorsal
Belly
Washed surimi
Unwashed surimi