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Purification, Characterization, and cDNA Cloning of a Myofibril-Bound Serine Proteinase from the Skeletal Muscle of Crucian Carp (*Carassius auratus*)[†]

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A myofibril-bound serine proteinase (MBSP) was highly purified from the skeletal muscle of crucian carp (*Carasius auratus*) by acidic treatment of myofibril solution and chromatographies on Q-Sepharose and benzamidine-Sepharose 6B. MBSP revealed a main protein band of approximately 28 kDa on SDS-polyacrylamide gel electrophoresis (PAGE) and was particularly inhibited by serine proteinase inhibitors. Substrate-specificity analysis revealed that the enzyme specifically cleaved at the carboxyl side of arginine and lysine residues, suggesting the characteristics of a trypsin-type serine proteinase. MBSP gene was cloned on the basis of the N-terminal sequence and the conserved active site peptide of serine proteinases together with 5′-rapid amplification of cDNA ends (5′-RACE) and 3′-RACE. The coding region gave an amino acid sequence of 242 residues including the initiation methionine and a signal peptide of 20 residues. Amino acid residues of His^{60}, Asp^{106}, and Ser^{196} consisting of the catalytic triad of serine proteinases were conserved in the sequence. Crucian carp MBSP shared relatively high identities with other serine proteinases, especially in well-conserved regions.

KEYWORDS: Crucian carp; myofibril; serine proteinase; cDNA cloning

INTRODUCTION

In the degradation of myofibrillar proteins in vivo, both lysosomal and nonlysosomal pathways are responsible (*1*−*6*). For the nonlysosomal pathway, three protein degradation systems are involved, including the ubiquitin-proteasome system (*2*), the calpain-μ and -m system (*3*), and a serine proteinase system (*4*−*6*). Concerning the serine proteinase system, the involvement of a myofibril-bound serine proteinase (MBSP) is noteworthy. MBSP was initially identified by Toyohara et al. (*7*) and was later purified to homogeneity from carp (*Cyprinus carpio*) skeletal muscle by Osatomi et al. (*8*). More recently, proteinases with characteristics similar to MBSP were also purified from lizard fish (*9*) and mouse (*10*), and these enzymes were characterized as trypsin-type serine proteinases. On the other hand, a myofibril-bound serine proteinase with chymotrypsin characteristic was isolated from hamster skeletal muscle, and its primary structure was determined (*11*). All these data suggested the ubiquitous existence of MBSP-like proteinase in the muscle of different animal species. Though the precise function of MBSP in vivo still remains unknown, much evidence revealed that such enzymes degrade myofibrillar proteins such as myosin heavy chain, α-actinin, actin, and tropomyosin effectively at neutral pH (*8*, *10*), indicating their possibility in the involvement in myofibrillar protein metabolism.

In our previous work, we identified the existence of a myofibril-bound serine proteinase (MBSP) in the skeletal muscle of crucian carp which is supposed to be the responsible proteinase for the thermal gel degradation of fish jelly products as MBSP degrades myofibrillar proteins especially at the temperature around 55 °C under low alkaline conditions (*12*). Although MBSPs from common carp (*8*, *13*) or lizard fish (*9*) were all purified to homogeneity, little information about their primary structures is available.

Therefore, in the present study, we tried to purify and characterize the biochemical properties of a myofibril-bound serine proteinase from the skeletal muscle of crucian carp and determine the primary structure of the proteinase by cDNA cloning.

MATERIALS AND METHODS

Fish. Crucian carp (*Carasius auratus*) was purchased alive from a local market in Jimei, Xiamen, China. The fish was subdued in iced water for 10 min and was sacrificed instantly. Skeletal muscle was collected and immediately used for experiment.

Chemicals. Q-Sepharose and benzamidine-Sepharose 6B were purchased from Amersham Bioscieces. *t*-Butyloxy-carbonyl-Phe-Ser-Arg-4-methyl-coumaryl-7-amide (Boc-Phe-Ser-Arg-MCA) and other

[†] The sequence of crucian carp MBSP has been registered in GenBank with accession no. DQ872434.

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synthetic fluorogenic peptide substrates (MCA-substrates) were obtained from Peptide Institute. Lima bean trypsin inhibitor (LBTI) was from Worthington biochemical corporation, phenylmethanesulfonyl fluoride (PMSF) and benzamidine were products from Sigma-Aldrich, l-3-carboxy-trans-2,3-epoxy-propionyl-L-leucine-4-guanidinobutylamide (E-64) was a product of Amresco, pepstatin and Pefabloc SC were purchased from Roche, and protein standard for SDS-polyacrylamide gel electrophoresis (PAGE) was from Bio-Rad. All other chemicals used were of the highest reagent grade from commercial sources.

Protein Analysis. Protein in column eluates was determined by measuring the absorbance at the wavelength of 280 nm. The concentration of proteins after each purification step was determined by the method of Lowry et al. (*14*) using bovine serum albumin as standard. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (*15*), and the gel was silver stained.

Assay of Enzyme Activity. Routinely, the proteolytic activity of crucian carp MBSP was measured using Boc-Phe-Ser-Arg-MCA as a substrate. The reaction was initiated by adding 25 μL of enzyme solution to the incubation mixture containing 875 μL of 20 mM Tris-HCl (pH 8.0) and 100 μL of 10 μM substrate in a total volume of 1 mL and was incubated at 55 °C for 20 min. The reaction was terminated by adding 1.5 mL of stopping reagent (methyl alcohol:*n*-butyl alcohol: distilled water = 35:30:35, v/v), and the fluorescence intensity of the liberated 7-amino-4-methylcoumarin (AMC) was measured by a spectrofluorometer at excitation wavelength of 380 nm and emission wavelength of 450 nm. One unit of proteolytic activity was defined as the amount that liberated 1 nmol of AMC per min under the assay conditions. Proteolytic activity assays in chromatographies were performed in duplicate and variation between duplicate samples was always <5%. The mean values were used.

Temperature dependence of MBSP activity was assayed at 25−80 °C using 20 mM Tris-HCl buffer (pH 8.0). pH dependence of enzymatic activity was assayed at 55 °C in reaction mixtures at pH 6.0−10.0 with 20 mM of different buffers (pH 6.0−7.5, phosphate buffer; pH 7.5−8.5, Tris-HCl buffer; pH 8.5−10, glycine-NaOH buffer). All analyses were carried out in triplicate, and the results are shown as average values ± standard deviation (95% confidence levels).

Purification of the Myofibril-Bound Serine Proteinase. All purification steps were carried out at 0∼4 °C. Purification procedure was carried out as described (*8*) with some modifications. Briefly, crucian carp skeletal muscle (300 g) was minced and homogenized using a Polytron (PT-2100, Kinematica, Switzerland) with 4-fold of 20 mM phosphate buffer (pH 7.5). After centrifugation at 10 000*g* for 20 min, the supernatant was discarded and the pellet was resuspended in the above buffer followed by homogenization for 1 min and centrifugation. The washing, homogenization, and centrifugation were repeated four more times to remove sarcoplasmic proteins effectively. The final resulting precipitate was further homogenized with 4-fold of 20 mM phosphate buffer (pH 6.4) containing 0.5 M KCl and 5 mM $Na_4P_2O_7$. After standing for 2 h, the homogenate was filtered through a nylon net to remove fibrous matter, and the resulting solution was adjusted to pH 4.0 with 3 N HCl, followed by centrifugation at 12 000*g* for 20 min. The pellet was discarded while the supernatant, which was regarded as crude MBSP, was adjusted to pH 8.0 using 1 N NaOH and was dialyzed against 20 mM Tris-HCl buffer (pH 8.0) and subsequently was applied to a column of Q-Sepharose (2.5 × 16 cm) equilibrated with the dialysis buffer. After washing the column with starting buffer to the absorbance at 280 nm reaching baseline, a linear gradient elution of NaCl from 0 to 0.5 M was performed in the same buffer at a flow rate of 1 mL/min. Active fractions were pooled and dialyzed against 50 mM Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl and immediately were applied to a column of benzamidine-Sepharose 6B (1 × 4 cm). Bound proteins were eluted using 10 mM HCl and the eluted fractions were collected in tubes containing 50−100 μL of 1.0 M Tris-HCl buffer (pH 8.5) for immediate neutralization. Active fractions from the final stage were used for enzymatic property analysis.

Determination of the N-Terminal Amino Acid Sequence. Highly purified crucian carp MBSP from benzamidine-Sepharose 6B chromatography was applied to SDS-PAGE using a 15% gel and was electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. After a brief staining with Coommasie brilliant blue R-250, the protein band corresponding to 28 kDa was cut out and submitted to amino acid sequencing using a protein sequencer (Applied Biosystems, model 492).

RNA Preparation and cDNA Synthesis. Total RNA was prepared from skeletal muscle using TRIzol reagent (Invitrogen, Carlsbad, CA). First-stranded cDNA was synthesized with SuperScript III Reverse Transcriptase (Invitrogen) and an Oligo$(dT)_{18}$ primer according to the manufacture's instruction.

Reverse Transcription−Polymerase Chain Reaction (RT−PCR). On the basis of the N-terminal amino acid sequence, two degenerate oligonucleotide primers were designed as sense primers for PCR and nested-PCR: 5′-AT(T/C/A)AT(T/C/A)GG(T/C/A/G)GG(T/C/A/G)TA-(T/C)GA(A/G)-3′ and 5′-CA(A/G)CC(T/C/A/G)TGGCA(A/G)GC-(T/C/A/G)TT(T/C)CT(T/C/A/G). The antisense degenerate primer 5′-GG(T/C/A/G)CC(T/C/A/G)CC(T/C/A/G)GA(A/G)TC(T/C/A/G)CC-3′ was designed on the basis of the well-conserved active site sequence of "GDSGGP" in most serine proteinases. Using these primers and the cDNA synthesized, a fragment of MBSP gene with the size of about 500 bp was amplified by PCR in a thermal cycler, GeneAmp 9700 (Applied Biosystems, United States). The PCR program was performed as following: 3 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 48 °C, 1 min at 72 °C, and final extension of 7 min at 72 °C. The PCR product was purified from agarose gel and was cloned into pGEM-T Easy vector (Promega) followed by DNA sequence analysis.

5′ and 3′-Rapid Amplification of cDNA Ends (5′ and 3′-RACE). From the sequence information described above, gene-specific primers were designed for 3′-RACE. The first-strand cDNA for 3′RACE was synthesized from skeletal muscle total RNA using an Oligo (dT)-adaptor primer, 5′-GGCCACGCGTCGACTAGTAC$(T)_{17}$-3′. The cDNA was first amplified between forward primer 1 (5′-GTGAAGCCAATC-CCTCTGCCA-3′) and the adaptor primer 5′-GGCCACGCGTCGAC-TAGTAC-3′ and then performed a nested PCR using forward primer 2 (5′-AGTGCTTGGTTTCTGGATGG-3′) and the adaptor primer. Parameters for 3′-RACE were the same as described above except the annealing temperature (52 °C). The PCR product (600 bp) was purified, cloned, and sequenced.

On the basis of the information of DNA fragments amplified using degenerate primers and 3′RACE, gene-specific primers for 5′RACE were designed. The first-stranded cDNA for 5′RACE was synthesized using a specific primer 5′-CTACAGGGCCACCTGAATCC-3′ and added poly (A) into the 3′-terminus by terminal deoxynucleotidyl transferase (Invitrogen). Sense primer (5′-GGCCACGCGTCGACTAG-TAC$(T)_{17}$-3′) and antisense primer (5′-CACCTGAATCCCCCTG-GCAT-3′) were used for first PCR amplification. Sense primer (5′-GGCCACGCGTCGACTAGTAC-3′) and antisense primer (5′-GGGTGGCAGGTTGTTTCAGCTTG-3′) were used for nested-PCR amplification. The PCR program used was the same as mentioned above except the annealing temperature (50 °C). The PCR product (400 bp) was purified, cloned, and sequenced.

DNA sequencings were analyzed at the Invitrogen Biotechnological Co. Ltd (Shanghai, China) with the DNA sequencer ABI PRISM 3730 (CA). The sequencing was performed three times and the sequences were confirmed. The full-length sequence of crucian carp MBSP was obtained by overlapping the 500 bp fragment, the 3′RACE fragment (600 bp), and the 5′RACE fragment (400 bp).

The nucleotide sequence data are available in the GenBank database under the accession number DQ872434 for the crucian carp MBSP cDNA.

RESULTS

Purification of Crucian Carp MBSP. To obtain high homogeneity of myofibril-bound serine proteinase (MBSP) without possible contamination of sarcoplasmic serine proteinases, myofibril from skeletal muscle was carefully prepared and extensively washed with low ionic strength buffer until serine proteinase activity in the soluble fraction could not be detected. To enhance the efficiency of MBSP extraction, several salts

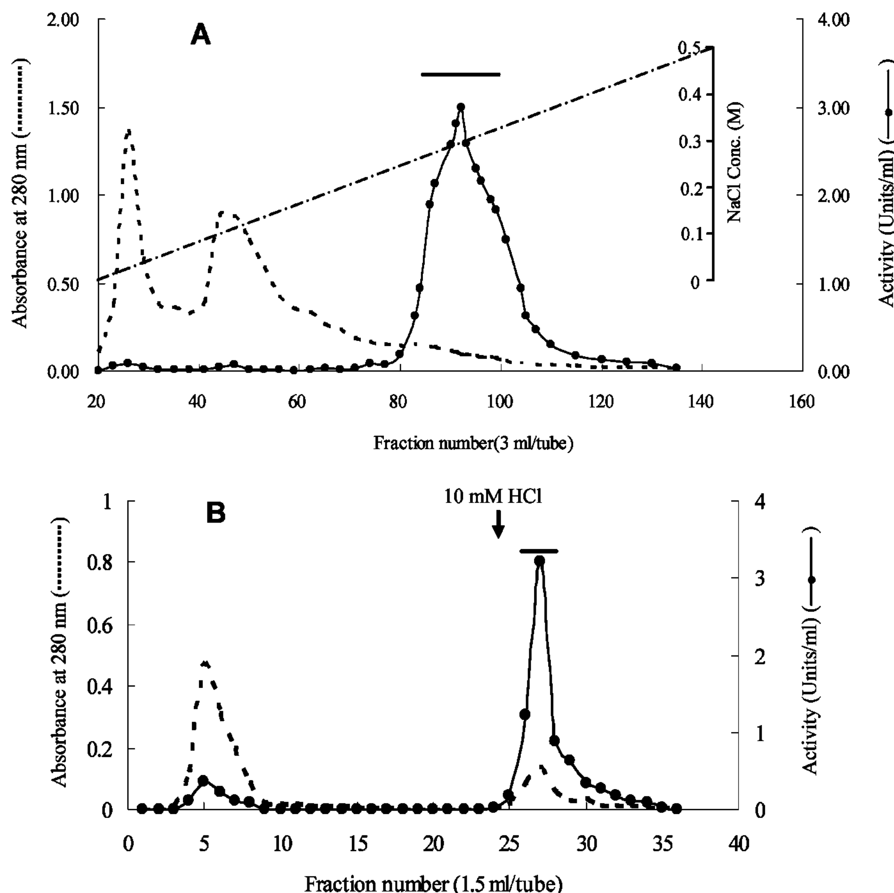

Figure 1. Chromatographic purification of crucian carp MBSP. (**A**) Q-Sepharose chromatography. (**B**) benzamidine-Sepharose 6B chromatography. Active fractions under the bar were pooled.

Table 1. Purification Result of MBSP from the Skeletal Muscle of Crucian Carp

step	total protein (mg)	total activity (U)	specific activity (U/mg)	purity (-fold)	yield (%)
crude enzyme	404.7	237.6	0.6	1	100
Q-Sepharose	5.3	67.5	12.7	21.2	28.4
benzamidine-Sepharose 6B	1.2	18.7	15.6	26	7.9

and detergents including Brij-35 and Triton X-100 were tested to optimize the extraction conditions. The enzyme was most effectively dissociated from myofibril with an acidic treatment at pH 4.0, and the process was accompanied by the obvious denaturation of most myofibrillar proteins. The chromatographic profile on Q-Sepharose showed that a single enzymatic active peak was eluted from a linear gradient of 0−0.5 M NaCl in 20 mM Tris-HCl buffer (pH 8.0) (**Figure 1A**) while most contaminating proteins were removed. After the benzamidine-Sepharose 6B affinity column, a sharp enzymatic active peak was obtained (**Figure 1B**). Active fractions from this peak were pooled for SDS-PAGE and enzymatic property analyses.

Table 1 summarizes the purification results of MBSP from crucian carp skeletal muscle. Starting from 300 g of crucian carp muscle, about 1.2 mg of MBSP was obtained. The overall recovery was 7.9% with a 26-fold increase in specific activity. The molecular mass of MBSP was approximately 28 kDa on SDS-PAGE (**Figure 2A**), which agreed well with the gel filtration result using Sephacryl S-200 column (**Figure 2B**), suggesting the enzyme is a monomer.

Effect of Inhibitors. The effect of various proteinase inhibitors on the activity of MBSP in hydrolyzing Boc-Phe-Ser-Arg-MCA is summarized in **Table 2**. Serine proteinase inhibitors including benzamidine, Pefabloc SC, and PMSF as well as LBTI showed high degree of inhibition. Inhibitors for asparatic (pepstatin) and cysteine (E-64) proteinases did not show much effect. No inhibitory effect of EDTA, an inhibitor for metalloproteinases, was identified. This result indicated that MBSP belongs to the serine proteinase family.

Substrate Specificity. To characterize the substrate specificity of the enzyme, various fluorogenic MCA-substrates were incubated with purified MBSP, and the amounts of AMC released from the substrate were measured. Crucian carp MBSP hydrolyzed Boc-Gln-Arg-Arg-MCA most rapidly among the substrates tested (**Table 3**). Other substrates containing arginine or lysine residues were also cleaved to some degree. However, arginine residue was favored over lysine residue. MBSP revealed low activity against Z-Phe-Arg-MCA and Z-Arg-Arg-MCA, which are substrates for cathepsin L and B. No cleavage to Arg-MCA, a substrate for aminopeptidase, and to Suc-Leu-Leu-Val-Tyr-MCA, a substrate for chymotrypsin, was identified. Combining its sensitivity to chemicals and substrate specificity, it could be concluded that crucian carp MBSP is a trypsin-type serine proteinase.

Optimum Temperature and pH. Temperature dependence of MBSP activity was assayed at 25−80 °C using 20 mM Tris-HCl buffer (pH 8.0). pH dependence of enzymatic activity was assayed at 55 °C in reaction mixtures at pH 6.0−10.0 with 20 mM of different buffers (pH 6.0−7.5, phosphate buffer; pH 7.5−8.5, Tris-HCl buffer; pH 8.5−10, glycine-NaOH buffer). As shown in **Figure 3A**, the enzyme revealed optimum temperature

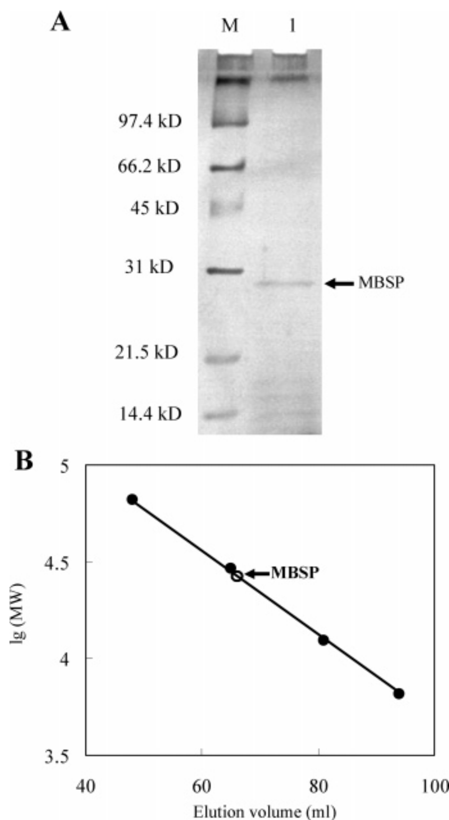

Figure 2. SDS-polyacrylamide gel electrophoresis of the purified MBSP (**A**) and its native molecular mass on Sephacryl S-200 gel filtration (**B**). (**A**) The purified MBSP was subjected to electrophoresis on a 12% gel followed by silver staining. Lane M, molecular marker; lane 1, purified MBSP. (**B**) The native molecular mass of MBSP was determined by gel filtration on Sephacryl S-200. The protein standards (closed circles) were bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa), and aprotinin (6.5 kDa). Arrowhead indicates the elution position (open circle) of crucian carp MBSP.

Table 2. Effects of Various Proteinase Inhibitors on MBSP Activity[a]

inhibitor	concentration (mM)	relative activity (%)
none		100
benzamidine	5	0
Pefabloc SC	1	2
PMSF	5	4
LBTI	0.1	7
E-64	0.28	105
pepstatin	0.15	97
EDTA	5	102

[a] Purified crucian carp MBSP was preincubated with each reagent in 20 mM Tris-HCl buffer (pH 8.0) at room temperature for 20 min. Remaining activity was assayed at pH 8.0, 55 °C, using Boc-Phe-Ser-Arg-MCA as substrate. Proteolytic activity assays were performed in duplicate and variation between duplicate samples was always <5%. The mean values were used.

of 55 °C using Boc-Phe-Ser-Arg-MCA as substrate. The pH optimum was 8.0 (**Figure 3B**). At pH 6.5, only 28.9% enzymatic activity remained while at pH 9.5, only 13.4% activity was detected indicating that the functional pH of crucian carp MBSP is in a narrow range.

Thermal Stability. The thermostability of purified MBSP was examined by incubating the enzyme at various temperatures in 20 mM Tris-HCl buffer (pH 8.0) at 25−90 °C for 30 min, and the remaining activity was determined. As shown in **Figure 4**, the enzyme was quite stable up to 70 °C as it retained 55% of its initial activity after incubation at that temperature for 30 min, further confirming its potential myofibrillar protein degradation ability in food processing, especially in the case of fish cake production where heating treatment is always performed (*12*, *16*).

Table 3. Substrate Specificity of MBSP on Synthetic Fluorogenic Substrates[a]

substrates (10 μM)	relative activity (%)
Boc-Phe-Ser-Arg-MCA	100
Boc-Gln-Arg-Arg-MCA	132
Boc-Val-Pro-Arg-MCA	123
Boc-Leu-Lys-Arg-MCA	40
Boc-Val-Leu-Lys-MCA	28
Boc-Glu-Lys-Lys-MCA	19
Boc-Leu-Arg-Arg-MCA	9
Z-Phe-Arg-MCA	0
Arg-MCA	0
Suc-Leu-Leu-Val-Tyr-MCA	0

[a] Proteolytic activity was assayed at pH 8.0, 55 °C, in duplicate and variation between duplicate samples was always <5%. The mean values were used.

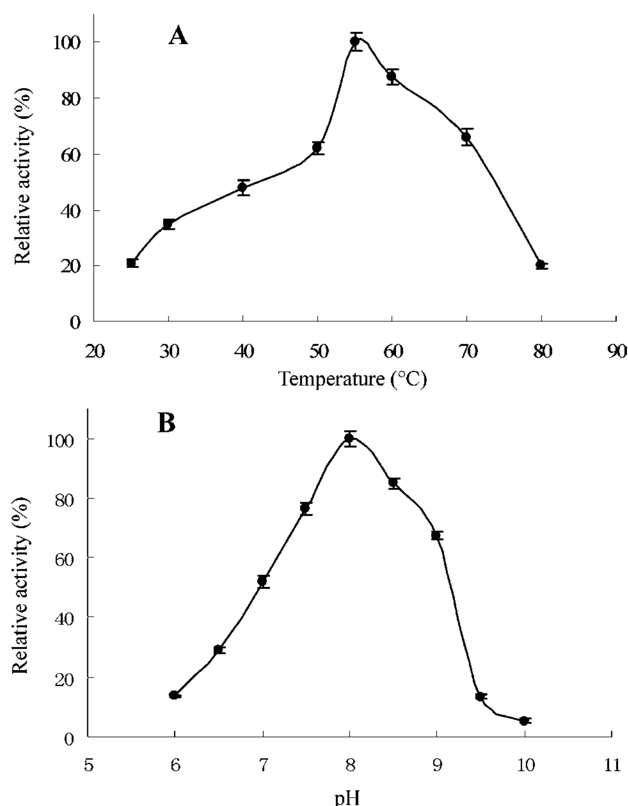

Figure 3. Optimum temperature (**A**) and pH (**B**) of MBSP. The enzyme activity was determined using Boc-Phe-Ser-Arg-MCA as substrate. The error bars represent replicate measurements.

N-Terminal Amino Acid Sequence of Purified MBSP. To analyze the N-terminal sequence, purified MBSP on SDS-PAGE was blotted onto a PVDF membrane and was subjected to protein sequencing. A sequence of 27 amino acid residues was identified as IIGGYEXRPHSQPWQAFLTDNRISXGG, where "X" represents an unidentified amino acid residue or a cysteine residue. The N-terminal fragment shared 77.8% homology to common carp MBSP (*13*) and was used to design degenerate sense primers for PCR and nested-PCR.

cDNA Cloning of Crucian Carp MBSP. The degenerate primers used in PCR generated a cDNA fragment of about 500 bp. On the basis of the sequence information of this fragment, gene-specific primers were subsequently designed and used in

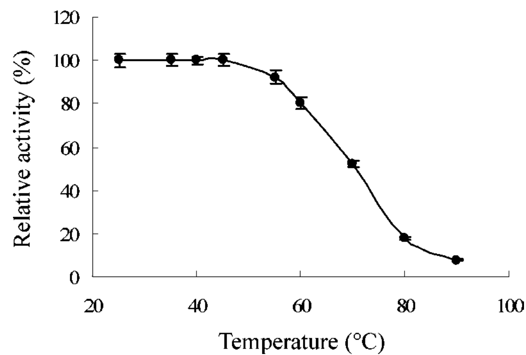

Figure 4. Thermostability of crucian carp MBSP. MBSP solution in 20 mM Tris-HCl buffer (pH 8.0) was preincubated at various temperatures for 30 min. After preincubation, the enzyme solution was immediately cooled in ice water and the remaining activity was determined. The error bars represent replicate measurements.

3′RACE and 5′RACE. Determination of crucian carp MBSP was performed by comparing the overlapped sequence fragments and the full-length sequence, and deduced amino acid sequence is shown in **Figure 5**.

Crucian carp MBSP was 1050 bp in length including the start codon ATG at positions 34−36, the stop codon TAA at positions 760−762, and a poly A tail. The open reading frame of crucian carp MBSP is 726 bp and the predicted protein consists of 242 amino acid residues. Combined with the information of the N-terminal amino acid sequence of native protein, it is obvious that MBSP was processed to the mature form of 222 amino acid residues by cleaving a signal peptide of 20 amino acid residues at Glu[20]-Ile[21]. Though the calculated molecular mass of the active MBSP was 25 kDa, which is relatively smaller than the value of 28 kDa estimated by SDS-PAGE (**Figure 2**), the N-terminal sequence (27 amino acid residues) of the deduced mature MBSP is in complete accordance with that as determined by protein sequencing, strongly suggesting our present data is correct. A homology search in GenBank databases revealed that mature crucian carp MBSP shares relatively high homology to other serine proteinases especially in the conserved regions (**Figure 6**). It has 54.5% identity to chum salmon trypsin (*17*);

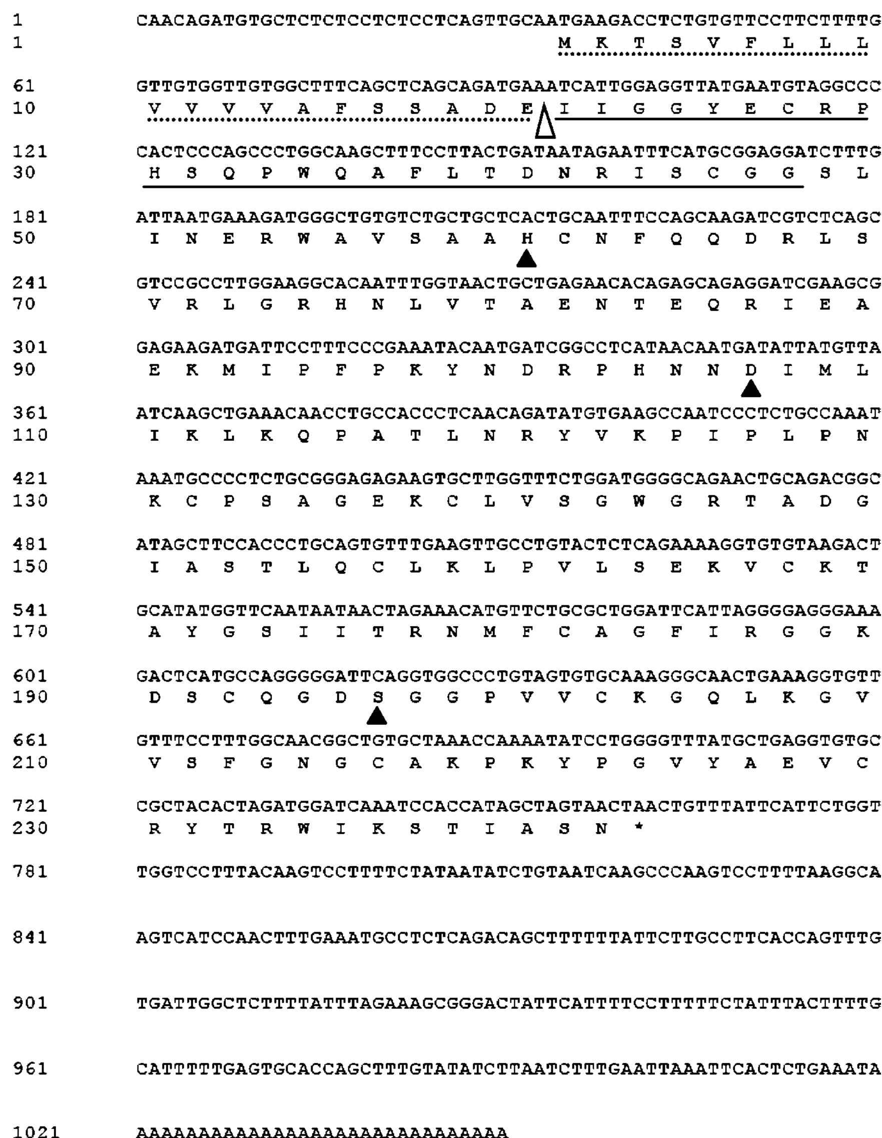

Figure 5. Nucleotide and deduced amino acid sequences of crucian carp MBSP. Residue numbers for both nucleotide and amino acid are indicated in the left of each row. A cleavage site (open triangle) for a signal peptide is shown. The signal peptide sequence is dot-lined and a line indicates the N-terminal amino acid sequence of mature MBSP. The His[60], Asp[106], and Ser[196] that form the triad active site of serine proteinase are marked as closed triangles.

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crucian carp MBSP     IIGGYECRPHSQPWQAFLTDN.....RISCGGSLINERNA    35
chum salmon trypsin   IVGGYECKAYSQPHQVSLNSG.....YHFCGGSLVNENWV    35
cod trypsin-I         IVGGYECTKHSQAHQVSLNSG.....YHFCGGSLVSKDWV    35
porcine trypsin       IVGGYTCAANSIPYQVSLNSG.....SHFCGGSLINSQWV    35
rat trypsin-IA        IVGGYTCPEHSVPYQVSLNSG.....YHFCGGSLINDQWV    35
hamster mekratin      IIGGVESKPHSRPYMAHLEIVTERGFTASCGGFLITPEFV    40
human KLK14           IIGGHTCTRSSQPWQAALLAGPRR..RFLCGGALLSGQWV    38
                      * **      *      *           *** *

crucian carp MBSP     VSAAHCNFQQDRLSVRLGRHNLVTAENTEQRIEAEKMIPF    75
chum salmon trypsin   VSAAHC..YKSRVEVRLGEHNIKVTEGSEQFISSSRVIRH    73
cod trypsin-I         VSAAHC..YKSVLRVRLGEHHIRVNEGTEQYISSSSVIRH    73
porcine trypsin       VSAAHC..YKSRIQVRLGEHNIDVLEGNEQFINAAKIITH    73
rat trypsin-IA        VSAAHC..YKSRIQVRLGEHNINVLEGDEQFINAAKIIKH    73
hamster mekratin      MTAAHCK..GKEITVTLGAHDVSKAESTQQKIKVKKQIAH    78
human KLK14           ITAAHCG..RPILQVALGKHNLRRWEATQQVLRVVRQVTH    76
                        ****        * ** *     *   *

crucian carp MBSP     PKYNDRPHNNDIMLIKLKQPATLNRYVKPIPLP..NKCPS   113
chum salmon trypsin   PNYSSYNIDNDIMLIKLSKPATLNTYVQPVALP..SSCAP   111
cod trypsin-I         PNYSSYNINNDIMLIKLTKPATLNQYVHAVALP..TECAA   111
porcine trypsin       PNFNGNTLDNDIMLIKLSSPATLNSRVATVSLP..RSCAA   111
rat trypsin-IA        PNYSSWTLNNDIMLIKLSSPVKLNARVAPVALP..SACAP   111
hamster mekratin      PNYSFYSKLHDIMFLKLQKKAKEDPSVDTIPLPSPSDFLK   118
human KLK14           PNYNSRTHDNDLMLLQLQQPARIGRAVRPIEVT..QACAS   114
                      *         * *   *         *

crucian carp MBSP     AGEKCLVSGWGRTADG...IASTLQCLKLPVLSEKVCKTA   150
chum salmon trypsin   AGTMCTVSGWGNTMSS.TADKNKLQCLNIPILSYSDCNNS   150
cod trypsin-I         DATMCTVSGWGNTMSS.VADGDKLQCLSLPILSHADCANS   150
porcine trypsin       AGTECLISGWGNTKSSGSSYPSLLQCLKAPVLSDSSCKSS   151
rat trypsin-IA        AGTQCLISGWGNTLSNGVNNPDLLQCVDAPVLSQADCEAA   151
hamster mekratin      PGKMCRAAGWGRTGVT.EPTSERLREVKLRIMDKGACKN.   156
human KLK14           PGTSCRVSGWGTISSPIARYPASLQCVNINISPDEVCQKA   154
                          *   ***            *            *

crucian carp MBSP     YGSIITRNMFCAGFIRGGKDSCQGDSGGPVVCKGQLKGVV   190
chum salmon trypsin   YPGMITNAMFCAGYLEGGKDSCQGDSGGPVVCNGELQGVV   190
cod trypsin-I         YPGMITQSMFCAGYLEGGKDSCQGDSGGPVVCNGVLQGVV   190
porcine trypsin       YPGQITGNMICVGFLEGGKDSCQGDSGGPVVCNGQLQGIV   191
rat trypsin-IA        YPGEITSSMICVGFLEGGKDSCQGDSGGPVVCNGQLQGIV   191
hamster mekratin      YYHYHYDFQVCVGSPRRVRSAYKGDSGGPLLCAGVAHGIV   196
human KLK14           YPRTITPGMVCAGVPQGGKDSCQGDSGGPLVCRGQLQGLV   194
                      *         * *          ******  * *   * *

crucian carp MBSP     SFGNG.CAKPKYPGVYAEVCRYTRWIKSTIASN          222
chum salmon trypsin   SWGYG.CAEPGNPGVYAKVCIFNDWLTSTMATY          222
cod trypsin-I         SWGYG.CAERDHPGVYAKVCVLSGWVRDTMANY          222
porcine trypsin       SWGYG.CAQKNKPGVYTKVCNYVNWIQQTIAAN          223
rat trypsin-IA        SWGYG.CALPDNPGVYTKVCNFVGWIQDTIAAN          223
hamster mekratin      SYGRG...DAKPPAVFTRISSYVPWINIVIKASS         226
human KLK14           SWGMERCALPGYPGVYTNLCKYRSWIEETMRDK          227
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Figure 6. Alignment of primary structures of mature serine proteinases. The mature sequence of crucian carp MBSP was compared with that of chum salmon trypsin (*17*), cod trypsin-I (*18*), porcine trypsin (*19*), rat trypsin (*17*), hamster mekratin (*11*), and human KLK14 (*20*). Mekaratin is a chymotrypsin-type serine proteinase from the skeletal muscle of hamster while KLK14 is a trypsin-type serine proteinase from human kallikrein family. Identical amino acid residues are asterisked.

52.2% to cod trypsin-I (*18*); 55.1% to porcine trypsin (*19*); 54.2% to rat trypsin-IA (*17*); 36.2% to hamster mekratin (*11*), a chymotrypsin-type serine proteinase from the skeletal muscle of hamster; and 47.2% to KLK14 (*20*), a trypsin-type serine proteinase from human. The catalytic triad (His^{60}, Asp^{106}, and Ser^{196}) of serine proteinases is well conserved in crucian carp MBSP.

DISCUSSION

In the present study, by acidic treatment of myofibril, ion-exchange chromatography, and affinity chromatography, we have successfully isolated crucian carp MBSP from skeletal muscle. Approximately 1.2 mg MBSP was obtained from 300 g skeletal muscle, with a yield of 7.9%. Such a recovery is the highest compared with MBSPs from common carp (*8*) and lizard fish (*9*). Two reasons were proposed. The first is the content of MBSP in the muscle of crucian carp is higher than that in common carp and lizard fish, while the second is that the present purification method is more effective than previous methods used (*8*, *9*). The molecular mass of the enzyme was estimated as approximately 28 kDa both on SDS-PAGE (**Figure 2A**) and gel filtration on Sephacryl S-200 column (**Figure 2B**). This value agrees well with that of common carp MBSP (*8*) while it is different from that of marine lizard fish MBSP which was identified as a homodimer consisting of two identical subunits (28 kDa) (*9*). This phenomenon may be explained as crucian carp and common carp are all freshwater fish and shared high species homology during genetic evolution while lizard fish is marine fish and its genetic relationship to crucian carp and common carp is much farther.

Serine proteinase inhibitors such as Pefabloc SC, benzamidine, STI, and PMSF effectively inhibited MBSP activity, while inhibitors toward asparatic proteinase (pepstatin), cysteine proteinase (E-64), and metalloproteinase (EDTA) did not show much effect. Substrate-specificity assay toward MCA-substrates revealed that only those with arginine and lysine residues at P_1 were hydrolyzed while substrates for chymotrypsin-type serine proteinase (Suc-Leu-Leu-Val-Tyr-MCA) and for cathepsin B, L (Z-Phe-Arg-MCA) and for aminopeptidase (Arg-MCA) were not affected. This substrate specificity is quite similar to that of common carp MBSP (*8*, *13*) but is something different from that of lizard fish MBSP which specifically cleaves at the carboxyl side of arginine residue (*9*). Combining inhibitor susceptibility with substrate specificity, it is obvious that crucian carp MBSP is also a trypsin-type serine proteinase.

The full-length sequence of crucian carp MBSP consists of 242 amino acid residues. A fragment of 20 residues including the initiation methionine was regarded as signal peptide as this fragment is absent in the N-terminal sequence of purified MBSP. Comparing the primary sequence of MBSP with other serine proteinases, mature MBSP shared relatively high identities to chum salmon trypsin (*17*), cod trypsin-I (*18*), rat trypsin-IA (*17*), porcine trypsin (*19*), hamster mekratin (*11*), and KLK14 from human skeletal muscle (*20*). Although the primary sequence of common carp MBSP has not been determined yet, crucian carp MBSP shared 77.8% identity with common carp MBSP in the N-terminal 27 amino acid residues (*13*). This homology is the highest among serine proteinases compared, suggesting the close relationship between crucian carp and common carp. As shown in **Figure 5**, the catalytically important residues in serine proteinases, that is, His^{60}, Asp^{106}, and Ser^{196}, were well conserved. There are 12 Cys residues at positions 27, 45, 61, 131, 138, 156, 167, 181, 192, 202, 216, and 229 that may form six intramolecular disulfide bonds necessary for full activity. Combined with the N-terminal amino acid sequence data, it is obvious that zymogen activation of MBSP occurs between Glu^{20} and Ile^{21}. No potential glycosylation motif in the sequence, however, could be identified, suggesting crucian carp MBSP is not a glycoprotein. Thus, the molecular mass difference of MBSP as estimated by SDS-PAGE (28 kDa) and calculated from nucleotide sequence (25 kDa) may ascribe to the mobility of the enzyme on the gel.

Serine proteinases play important roles in the processing of polypeptide precursors and many biological processes (*21*). Recently, more and more transmembrane serine proteinases have been purified and characterized and their sequence has been determined (*22*). The identification of serine proteinases in the skeletal muscle of hamster (*11*) and mouse (*10*, *23*) has revealed the importance of such enzymes in the metabolism of muscle. However, different from MBSP, hamster serine proteinase mekratin is chymotrypsin-like and the molecular weight of mouse serine proteinase (120 kDa) is much higher than crucian carp MBSP. Although human kallikreins (KLK) composing at least 15 members are trypsin-like serine proteinases and are expressed in prostate and skeletal muscle under normal conditions (*20*), sequence homology analysis revealed their difference

(**Figure 6**). Crucian carp MBSP is also different from a more recently identified transmembrane serine protease TMPRSS6, which is mainly expressed strongly in thyroid and weakly in trachea and is quite possibly a glycosylated protein (*24*).

Although the existence of enzymes similar to crucian carp MBSP has been identified in the skeletal muscle of common carp (*8*), lizard fish (*9*), and mouse (*5*, *10*), little information is available in revealing their primary structures. Our present data gave the first report of the full-length sequence of MBSP. Combining the enzymatic characteristics and the sequence alignment, it is obvious that crucian carp MBSP is a unique trypsin-type serine proteinase, which is expressed in normal skeletal muscle. Further study to reveal the role of MBSP in the involvement in the nonlysosomal pathway of intracellular protein degradation is clearly of importance. The physiological function as well as its variation of expression level at different culturing seasons also needs to be clarified.

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