J. Biochem. 123, 650-658 (1998)

Purification and Characterization of Myonase from X-Chromosome Linked Muscular Dystrophic Mouse Skeletal Muscle

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Received for publication, October 22, 1997

A chymotrypsin-like proteinase, designated myonase, was successfully purified to homogeneity from X-chromosome linked muscular dystrophic mouse skeletal muscle by affinity chromatography on agarose conjugated with lima bean trypsin inhibitor as ligand. The molecular mass of the purified myonase was determined to be 26 kDa by SDS-PAGE and to be 25,187 Da by mass spectrometry. The native enzyme is a single chain molecule and a monomeric protein without sugar side-chains. The nucleotide sequence of myonase mRNA is similar to mouse mast cell proteinase 4 (MMCP-4) cDNA. This is the first report of a native enzyme whose amino acid sequence closely corresponds to MMCP-4 cDNA. Myonase has chymotrypsin-like activities and hydrolyzes the amide bonds of synthetic substrates having Tyr and Phe residues at the P_1 position. Myonase is most active at pH 9 and at high concentration of salts. Myonase preferentially hydrolyzes the Tyr4-Ile5 bond of angiotensin I and the Phe20-Ala21 bond of amyloid β-protein, and it is less active towards the Phe8-His9 bond of angiotensin I and the Phe4-Ala5 and Tyr10-Glu11 bonds of amyloid β-protein. Myonase is completely inhibited by such serine proteinase inhibitors as chymostatin, diisopropylfluorophosphate and phenylmethylsulfonyl fluoride, but not by *p*-tosyl-L-phenylalanine chloromethyl ketone, *p*-tosyl-L-lysine chloromethyl ketone, pepstatin, E-64, EDTA, and *o*-phenanthroline. It is also inhibited by lima bean trypsin inhibitor, soy bean trypsin inhibitor, and human plasma α_1-antichymotrysin. These properties match those of chymase, but unlike chymase, myonase does not interact with heparin in the regulation of its activity. Myonase was immunohistochemically localized in myocytes, but not in mast cells.

Key words: MDX-mouse muscle, myonase, mRNA, purification, serine proteinase.

Muscular dystrophy is characterized by myofibrillar proteolysis followed by cell necrosis. The skeletal muscle in MDX-mouse, which is X-chromosome linked muscular dystrophic mouse (C57BL/10ScSn-mdx), is degenerated, but degeneration is followed by complete regeneration in the process of muscular dystrophy (*1*). Proteinases related to degradation of myofibrilla in muscular dystrophy are known to be cathepsins, especially cathepsins B and L (*2, 3*), which are derived from phagocytes, mainly macrophages, that invade dystrophic muscle. The role of calcium-dependent proteinases (calpains) has also been extensively studied in relation to the pathogenesis of muscular dystrophy (*e.g., 4, 5*). In addition, the transitory calpain, p94, is found to be rapidly turned over by autolysis in skeletal muscle (*6*). On the other hand, we have found that MDX-mouse skeletal muscle contains large amounts of a chymotrypsin-like proteinase that specifically degrades a muscle structural protein designated C-protein (*7*).

Recently it was reported that mast cells appeared in the skeletal muscle of the genetic muscular dystrophy in human, dog, and mouse (*8*). Mast cells contain serine proteinases, so called chymases, which are similar in cleavage specificity to pancreatic chymotrypsin (*9*). Mast cell heterogeneity in rodents is well-known to be associated with the differential expression of a number of serine proteinases in granules. DNAs and RNAs of four mast cell-specific serine proteinases have been characterized in the rat as RMCP-1, RMCP-2, RMCP-3, and RMCT (*10-15*). Rat mucosal mast cells (MMC) express the chymotryptic serine proteinase RMCP-2 and no detectable levels of the chymotryptic proteinase RMCP-1, which is only found in connective tissue mast cells (CTMC) (*13, 16*). On the other hand, seven mast cell-specific serine proteinases (MMCP-1 to MMCP-7) (*17-25*) and mast cell-specific carboxypeptidase A (MMC-CPA) (*26*) have been characterized in the mouse mast cells. MMCP-1 and MMCP-2 are preferentially expressed in mouse MMC, whereas MMCP-4,

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Abbreviations: AMC, 7-amido-4-methylcoumarin; CTMC, connective tissue mast cells; MDX-mouse, X-chromosome linked muscular dystrophic mouse (C57BL/10ScSn-mdx); MMC, mucosal mast cells; MMCP, mouse mast cell proteinase; RACE, rapid amplification of cDNA ends; RMCP, rat mast cell proteinase. Enzymes: horseradish peroxidase [EC 1.11.1.7]; cathepsin B [EC 3.4.22.1]; cathepsin L [EC 3.4.22.15]; chymase [EC 3.4.21.39].

MMCP-5, MMCP-6, and MMC-CPA are preferentially expressed in serosal mast cells (CTMC). Bone marrow-derived mast cells express MMCP-1, MMCP-2, MMCP-4, MMCP-5, MMCP-6, MMCP-7, and MMC-CPA (*20, 25-30*) and rat skeletal muscles express RMCP-1 (*31*).

We previously believed that the chymotrypsin-like proteinase in the MDX-mouse skeletal muscle specifically degraded the fast type C-protein, but further study revealed that it degraded other structural proteins such as myosin heavy chain, especially proteins with high molecular mass, although the fast type C-protein was the preferred substrate. Because of its presence in myofibrilla, the proteinase was named myonase. We have purified and characterized myonase in the MDX-mouse skeletal muscle and analyzed its mRNA to study how it differs from chymase.

MATERIALS AND METHODS

Materials—Human plasma α_1-antichymotrysin was from Calbiochem. Other proteinase inhibitors and lima bean trypsin inhibitor-conjugated agarose (LBTI-agarose) were purchased from Sigma. Porcine mucosal heparan sulfate, porcine mucosal de-*N*-sulfated heparin, low molecular mass heparins (3 and 6 kDa) from porcine intestinal mucosa were from Sigma. Anti-myonase IgG from rabbit and monoclonal anti-myonase specific peptide IgG were produced by Iatron Lab. Anti-rat mast cell chymase (RMCP-1) IgG from rabbit was a generous gift from Dr. Kido, Tokushima Univ. The monoclonal anti-human mast cell chymase IgG was purchased from Chemicon International. All other reagents were at least analytical grade.

Extraction of Myonase—Skeletal muscle (120 g wet weight) dissected from MDX-mice was homogenized with 0.6 M NaCl/10 mM Tris-HCl, pH 7.8, in a cartridge mill chamber. The precipitate obtained by centrifuging at $10,000 \times g$ for 20 min was washed three times by repeating both homogenization with the above buffer and centrifugation. The final suspension of the precipitate was passed through a nylon cloth to remove fibrous materials and centrifuged. Myonase could not be extracted with 0.6 M NaCl or KCl/10 mM Tris-HCl, pH 7.8, but extraction was successful with 2 M NaCl or KCl/10 mM Tris, pH 10.3. Thus, myonase was extracted from the precipitate by suspending it in 100 ml of 2 M NaCl/10 mM Tris, pH 10.3, and centrifuging at $356,000 \times g$ for 20 min. This process was repeated once more. The extracts (200 ml) containing solubilized myonase were pooled and passed through a pulp-sheet on a Buchner funnel by gentle suction to eliminate fat-like materials.

Affinity Chromatography—The proteinase solution was passed through two straightly connected columns (each 16 i.d. $\times$4 mm) of lima bean trypsin inhibitor (LBTI)-agarose which were equilibrated with 2 M NaCl/10 mM Tris-HCl, pH 9. After washing the columns with 2 M NaCl/10 mM Tris-HCl, pH 9, myonase was eluted with 2 M NaCl/10 mM HCl. The eluted solution was collected manually in 2-ml fractions in tubes containing 0.2 ml of 1 M sodium acetate, pH 4.3. The proteinase activity of each fraction was assayed. Active peaks were pooled and dialyzed against 1 mM sodium acetate, pH 4.3, overnight. All procedures were performed at 4°C. The dialyzate was kept at −80°C until use. A part of the dialyzate was dried on a Speed-Vac for the analyses by SDS-PAGE, N-terminus sequence, and mass spectrometry.

SDS-PAGE Analysis and Western Blotting—Discontinuous SDS-PAGE of the dissolved protein in 2% SDS/5% β-mercaptoethanol/62.5 mM Tris-HCl, pH 6.8/10% glycerol/0.002% bromphenol blue (SDS-treatment mixture) was carried out as described by Laemmli (1970) (32) on 12% acrylamide mini-slab gels (separation gel; 7$\times$8 cm). The gel was stained by the picrate-Coomassie Brilliant Blue R250 method (*33*). Another gel was electroblotted onto a nitrocellulose membrane (Nitro Plus™ 2000, Micron Separation) in a solution of 48 mM Tris/39 mM glycine/1.3 mM SDS/20% methanol. The blotted protein was treated with antiserum against myonase and detected using horseradish peroxidase-conjugated anti-(rabbit or mouse IgG+IgL), as described by Towbin *et al.* (*34*).

Assay of Myonase Activity—Enzyme activity was measured by hydrolysis of the synthetic peptide Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (AMC). The reaction mixture containing proteinase, 50 mM Tris-glycine, pH 9.0, 2 M NaCl, and 10 μM Suc-Leu-Leu-Val-Tyr-AMC in a final volume of 100 μl was incubated at 30°C for 20 min. The proteinase reaction was stopped by adding 1.4 ml of 2 M NaCl/0.1 M sodium acetate, pH 4.3. The production of free AMC was monitored at 380 nm (excitation) and 460 nm (emission) using a fluorescence spectrophotometer model F-2000 (Hitachi). One unit of proteinase activity was defined as 1 nmol of AMC production per min.

Protein Content Determination—Protein content was determined by the Bradford method (*35*) using reagents purchased from Bio-Rad. BSA was used as reference.

Active-Site Titration—Active-site titration of myonase was carried out by using [1,3-^{3}H]diisopropyl fluorophosphate (310.8 GBq/mmol, DuPont) (*36*). Attempts to titrate the operation molarity of myonase were undertaken with *p*-nitrophenyl *p'*-guanidinobenzoate (Sigma) (*37*) and 4-methylumbelliferyl *p*-guanidinobenzoate (Sigma) (*38*).

Substrate Kinetics—Kinetic constants (K_m and V_{max}) were determined from the hydrolysis rates at six separate substrate concentrations by using Lineweaver-Burk plots. Correlation coefficients were typically greater than 0.99 and never lower than 0.96.

Degradation of Biogenic Peptides and Analysis of the Resulting Fragments—Angiotensin I (2.57 nmol, Nova-biochem) and amyloid β-protein (fragment 1-40) (6.93 nmol, Sigma) were hydrolyzed with myonase at the enzyme-substrate ratio (E/S) of 1/12,240 and 1/82,000, respectively, in buffer consisting of 50 mM NH_4HCO_3-acetate, pH 9.0, at 30°C for 1 h. After drying *in vacuo* in a Speed-Vac (vacuum-centrifuge), the digests were analyzed by mass spectrometry. Additionally, the digests of angiotensin I were separated by reverse-phase HPLC. Individual peaks monitored at 215 nm were manually collected and characterized by sequencing and mass analysis. The digests of amyloid β-protein were separated by electrophoresis on 16.6% acrylamide slab gel according to Schögger and von Jagow (1987) (*39*). After electrophoresis, the gel was electroblotted onto a microporous polyvinylidene fluoride membrane (Immobilon™-PSQ, Millipore) in a solution of 10 mM 3-cyclohexylamino-1-propanesulfonic acid (Sigma), pH 11.0/10% methanol. After transfer, the membrane was stained with 0.2% Coomassie Brilliant Blue R-250 in 45% methanol/10% acetate. Stained protein

bands were excised and used for sequencing.

Digestion of Myonase and Isolation of Peptides—Myonase (70 pmol) was digested with endoproteinase Glu-c (Sigma), Lys-c (Sigma), or thermolysin (Boehringer Mannheim). The resulting digests were separated by reverse-phase HPLC. Peaks in each case monitored at 215 nm were collected manually, dried *in vacuo* in a Speed-Vac and sequenced.

Amino Acid Sequence Analysis—N-terminal sequence analysis of myonase was performed on a gas-phase protein sequencer, model PPSQ-10 (Shimadzu) using Edman degradation.

Mass Spectrometry—Mass analysis was performed using a matrix-assisted laser desorption/ionization time of flight mass spectrometer, model Kompact-MALDI I (Shimadzu-Kratos) equipped with a nitrogen laser (337 nm). In each analysis, 1 μl of peptide/protein solution in 0.1% aqueous trifluoroacetic acid/30% acetonitrile was mixed with 1 μl of sinapinic acid (10 mg/ml) in the above solvent mixture as matrix. Spectra were calibrated externally with angiotensin I, insulin, and BSA.

Deglycosylation—Purified myonase (2.5 μg) was denatured by heating in a boiling-water bath for 10 min and then cooled to room temperature. The denatured sample was incubated with 10 μl of deglycosylation buffer (150 mM phosphate, pH 7.0/50 mM EDTA/1% 2-mercaptoethanol) and 1 μl of peptide-*N*-glycosidase F (0.2 unit, Boehringer Mannheim) or endo-α-acetylgalactosaminidase (0.5 milliunit, Boehringer Mannheim) at 37°C overnight. Then a further 1 μl of glycosidase or galactosaminidase was added and incubation was continued for 24 h. As a control an aliquot of myonase was treated by the same procedure in the absence of glycosidase and galactosaminidase. The reaction was terminated by addition of an equal volume of 2-fold concentrated SDS-treatment mixture and then submitted to SDS-PAGE.

mRNA Isolation—Extraction of total RNAs from skeletal muscle (0.4 g wet weight) and selection of their poly(A)$^+$ RNA were performed with messenger RNA isolation kit from Stratagene.

Rapid Amplification of cDNA Ends (RACE) and Sequencing—For 3′RACE, poly(A)$^+$ RNA (0.13 μg) extracted from mouse skeletal muscle was reverse-transcribed with the 3′RACE abridged anchor primer (5′-GGG CCA CGC GTC GAC TAG TAC TTT TTT TTT TTT TTT TT-3′) by Super Script II reverse transcriptase (Life Technologies). The resulting first strand cDNA was amplified by PCR (25 cycles) with the gene-specific primer P1(+) (5′-ATT ATT GGT GGT GTT GAG TCT AGA-3′, which sequence was deduced from the N-terminal amino-acid sequence of myonase) and the abridged universal amplification primer (AUAP) (5′-GGC CAC GCG TCG ACT AGT AC-3′) by using 3′RACE system kit from Life Technologies according to the manufacturer's manual. The product was amplified by the second PCR with primer P2(+) (5′-GGG TTC ACA GCT ACC TGT GGT GGG-3′, which sequence was deduced from the N-terminal amino-acid sequence of myonase) and AUAP. After electrophoresis, the PCR product of the expected size was cut from the 1% agarose gel and purified with a Prep-A-Gene DNA purification kit (Bio-Rad). For 5′RACE, the poly(A)$^+$ RNA was reverse-transcribed with the primer P1(−) (5′-GGC TGA GGA CAG GCC GGG GG-3′, which was expected from the sequence analysis by 3′RACE) by use of Super Script II reverse transcriptase with a 5′RACE system kit from Life Technologies according to the manufacturer's manual. The synthesized cDNA was isolated by using a Sephaglas Band Prep kit from Pharmacia Biotech according to the manufacturer's manual. The isolated cDNA was tailed with dCTP by terminal deoxynucleotidyl transferase (Life Technologies) according to the manufacturer's manual. The first-strand cDNA tailed with oligo dC was amplified by PCR with the primers P2(−) (5′-GGT GAC AGG ATG GAC ACA TGC T-3′, which sequence was deduced from the sequence analysis by 3′RACE) and 5′RACE abridged anchor primer (5′-GGC CAC GCG TCG ACT AGT ACG GGG GGG GGG-3′) by using a 5′RACE system kit from Life Technologies according to the manufacturer's manual. The product was amplified further by the second PCR with the primer P3(−) (5′-TTA ATC CAG GGC ACA TAT GA-3′, which sequence was deduced from the sequence analysis by 3′RACE) and AUAP. After electrophoresis, the PCR product of the expected size was cut from the 1% agarose gel and purified with a Prep-A-Gene purification kit.

The nucleotide sequence was determined by use of an Applied Biosystem model 373 A DNA Sequencer using an ABI PRISM dye terminator cycle sequencing core kit.

Immunohistochemical Procedure—A frozen section (10 μm thick) of femoral muscle from MDX-mouse (44-day-old) on a silane-coated slide was fixed with chilled acetone and pretreated with 0.3% H_2O_2 to remove the endogenous peroxidase. The section was incubated with 1% BSA in PBS to block non-specific binding of antiserum. The slide was washed three times for 3 min each with 0.01 M sodium phosphate/0.85% NaCl, pH 7.2 (PBS). Rabbit anti-myonase IgG (17.8 μg/ml) was applied to each slide. After incubation in a moist chamber overnight, antiserum was rinsed off three times for 3 min each with PBS. A second antiserum, goat anti-rabbit IgG conjugated with horse radish peroxidase (Bio-Rad) was applied. After rinsing, the section was stained with 3,3′-diaminobenzidine tetra-hydrochloride dehydrate (DAB) as usual.

RESULTS

Purification of Myonase—Myonase was stable during extraction, but further purification by gel filtration or ion exchange column chromatography was not possible due to drastic loss of recovery even in the presence of BSA (1 mg/ml). However, affinity chromatography with LBTI-agarose succeeded in purifying myonase by using a short column bed and rapid elution.

Purity and Molecular Mass of Myonase—Starting from 120 g of skeletal muscle, 24 μg of purified myonase was obtained in 22% yield of enzyme-activity with 126,000-fold purification from the homogenate of muscle tissue. Myonase was judged to be homogeneous by SDS-PAGE, N-terminus sequencing and mass spectrometry. Figure 1 shows SDS-PAGE and mass spectrum of the purified myonase. The molecular mass of myonase was estimated to be 26 kDa by SDS-PAGE (Fig. 1A), which was consistent with the 25,187 Da found by mass spectrometry (Fig. 1B). This molecular mass is similar to that of chymase from human (*40–43*), mouse (*17*), rat (*11*), cow (*44*), and hamster (*45*). The N-terminal amino acid sequence (46 residues) of myonase was determined to be IIGGVESRPH

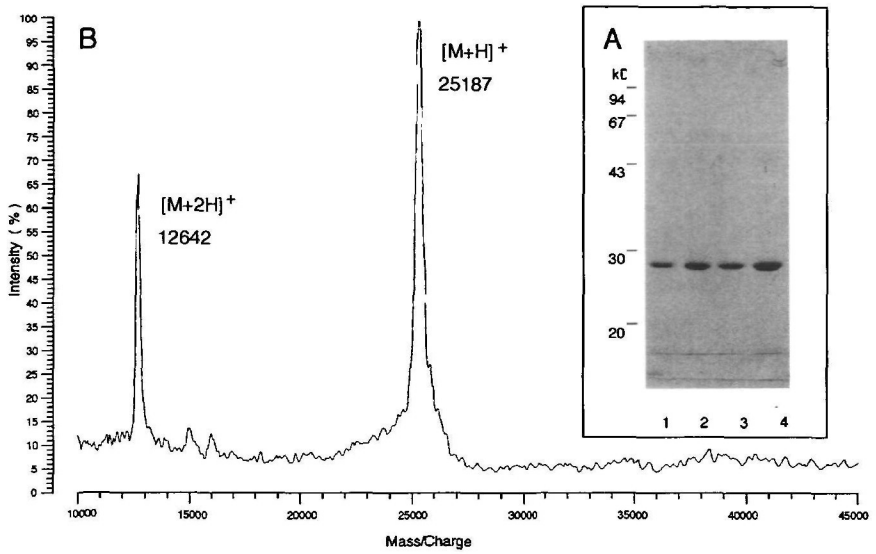

Fig. 1. **SDS-PAGE and mass spectrum of purified myonase.** A: The purified myonase (0.37, 0.60, 0.85, and 1.40 μg in lane 1, 2, 3, and 4, respectively) was analyzed on a 12% acrylamide gel under reducing conditions. Molecular mass standards used were α-lactalbumin (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), BSA (67 kDa), and phosphorylase *b* (94 kDa). The proteins were visualized by Coomassie Blue R250 staining. B: The purified myonase in 0.1% trifluoroacetic acid/30% acetonitrile was analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry using sinapinic acid as matrix. $[M+H]^+$ and $[M+2H]^+$ were 25,187 and 12,642, respectively.

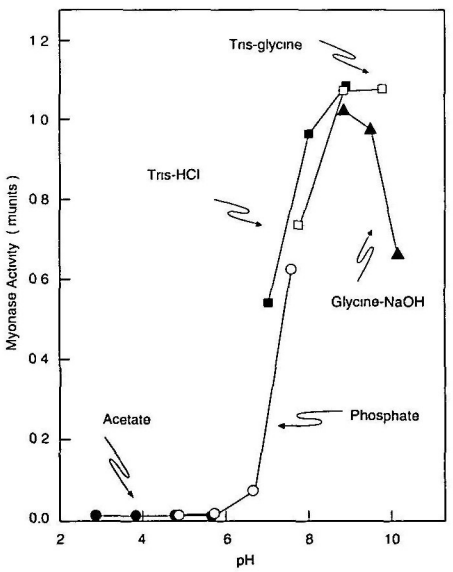

Fig. 2. **Optimum pH of myonase activity.** Myonase activity was estimated by using the synthetic substrate Suc-Leu-Leu-Val-Tyr-AMC as described in "MATERIALS AND METHODS" and with the following buffers: 50 mM sodium acetate (●), 50 mM sodium phosphate (○), 50 mM Tris-HCl (■), 50 mM Tris-glycine (□), and 50 mM glycine-NaOH (▲). The enzyme reaction was done at an ionic strength of 2.05 M and a temperature of 30°C.

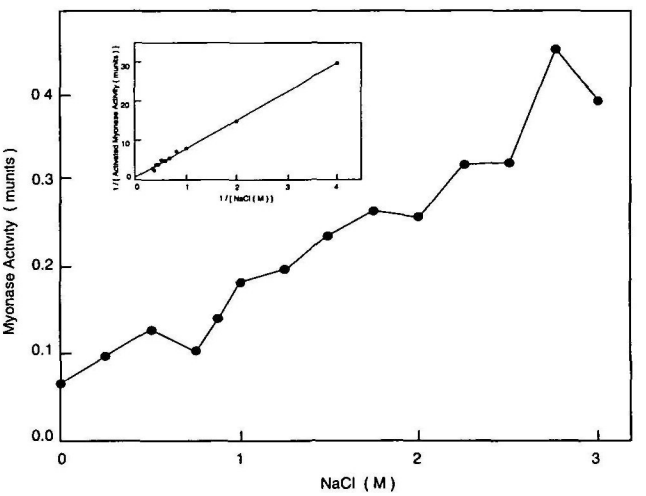

Fig. 3. **Myonase activity required a high salt concentration.** Myonase activity was estimated using the synthetic substrate Suc-Leu-Leu-Val-Tyr-AMC as described in "MATERIALS AND METHODS" but at various concentrations of salt. Inset shows a double reciprocal plot of the activated myonase activity ([activity at various NaCl concentrations] − [activity at 0 M NaCl]) against NaCl concentration.

SRPYMAHLEI TTERGFTATC GGFLITRQFVLTAAHC by amino acid sequence analysis.

Dependence of Enzyme-Activity on pH and Salt Concentration—The pH dependence of myonase was studied with the synthetic substrate Suc-Leu-Leu-Val-Tyr-AMC at an ionic strength of 2.05 M and a temperature of 30°C. It shows a bell-shaped profile under non-saturated substrate concentrations with a pH optimum around pH 9.0 (Fig. 2). The enzyme activity required a high concentration of salt (Fig. 3). For half of the maximum activity, 11.6 M NaCl or KCl was calculated from the double reciprocal plots (Fig. 3, inset).

Enzyme Stability—The purified myonase was unstable at room temperature or −20°C, but stable for at least one year at −80°C. Half of the initial activity was lost within 10 min at 30°C in either the presence or the absence of NaCl (2 M). BSA stabilizes this enzyme, but causes inhibition at a concentration of more than 0.5 mg/ml. The stabilizing effect of BSA could be seen at a concentration of less than 0.05 mg/ml. Heparin did not stabilize myonase. Earlier reports suggested that rat mast cell chymase was stimulated by heparin (*46-49*). However, myonase activity was neither stimulated nor inhibited by heparin sulfate (1 ng/ml to 1 mg/ml), de-*N*-sulfated heparin (1 ng/ml to 1 mg/ml), 6 kDa heparin (1 ng/ml to 1 mg/ml), or 3 kDa heparin (1 ng/ml to 1 mg/ml) at either high (2.05 M) or low (0.05 M) ionic strength.

Specificity to Fluorogenic Peptide Substrates—Several

fluorogenic peptides with different amino acid sequences were investigated as potential substrates for myonase. It was demonstrated that peptides having a hydrophobic amino acid such as Tyr or Phe at P_1 were greatly favored as substrates of myonase (Table I). In general, peptides having a hydrophilic amino acid at P_1 were not favored substrates.

Hydrolytic Sites of Biogenic Peptides—Hydrolyzed fragments of angiotensin I were detected at 552 and 786 Da by mass spectrometry. Amino acid sequencing confirmed that these fragments were Asp1-Tyr4 and Ile5-Leu10. Thus, angiotensin I was specifically hydrolyzed at Tyr4-Ile5. But myonase was less active for the Phe8-His9 bond in angiotensin I. This specific site is the same as RMCP-1 (*9*) and angiotensin II-forming hamster chymase (*45*). Hydrolyzed fragments of amyloid β-protein were detected at 2,460 and 1,886 Da by mass spectrometry. The amino acid sequence of these fragments indicated that their correspondence to Asp1-Phe20 and Ala21-Val40. This was confirmed by comparison with the synthetic peptide fragments, Asp1-Phe20 and Ala21-Val40. Thus, amyloid β-protein was specifically hydrolyzed at the Phe20-Ala21 bond, but less at bonds such as Phe4-Arg5 and Try10-Glu11.

Inhibition Studies Indicating That Myonase Is a Serine Proteinase—The activity of myonase was further studied with various proteinase inhibitors to reveal its function. As shown in Fig. 4, a significant inhibitory effect was observed with the classical serine proteinase inhibitors: chymostatin (IC_{50} 0.25 μM), diisopropyl fluorophosphate (IC_{50} 130 μM), and phenylmethylsulfonyl fluoride (IC_{50} 66 μM), while a minimal effect was observed with pepstatin and E-64, inhibitors of aspartyl and cysteine proteinases, respectively. No inhibition was observed by EDTA or *o*-phenanthroline, classical inhibitors of metalloproteinases. These results indicate that myonase is a serine proteinase. Additional experiments showed that *p*-tosyl-L-phenylalanine chloromethyl ketone and *p*-tosyl-L-lysine chloromethyl ketone do not inhibit myonase. The activity of myonase was inhibited by lima bean trypsin inhibitor [IC_{50} 1.8 μg/ml (0.9 μM)], soy bean trypsin inhibitor [IC_{50} 0.8 μg/ml (0.4 μM)], and human plasma α_1-antichymotrysin [IC_{50} 2.86 μg/ml (0.42 μM)] (Fig. 4), but not by chicken egg white ovomucoid trypsin inhibitor or bovine pancreatic trypsin inhibitor. Chicken egg white ovoinhibitor inhibited the myonase activity similarly to rat mast cell chymase (*10*). Recently it was reported that heparin prevented the inhibition by α_1-antichymotrysin of rat mast cell chymase (*49*). But the inhibition of myonase (0.42 pmol/ml) activity with α_1-antichymotrysin (84 pmol/ml) was not prevented by heparin sulfate (1 ng/ml to 1mg/ml), de-*N*-sulfated heparin (1 ng/ml to 1 mg/ml), 6 kDa heparin (1 ng/ml to 1 mg/ml), or 3 kDa heparin (1 ng/ml to 1 mg/ml) at either high (2.05 M) or low (0.05 M) ionic strength.

Immunoreactivity of Myonase—The proteinase activity of myonase was completely inhibited by anti-myonase IgG from rabbit, but not by anti-rat mast cell chymase IgG from rabbit, and monoclonal anti-human mast cell chymase IgG. Furthermore, myonase was detected by anti-myonase IgG from rabbit and monoclonal anti-myonase specific

TABLE I. **Kinetic constants for hydrolysis of peptide substrates by myonase.** The reactions were carried out as described in "MATERIALS AND METHODS." The synthetic substrates carbobenzoxy-L-phe-L-arg-AMC (Z-Phe-Arg-AMC), *t*-butyloxycarbonyl-L-glu-L-ala-L-arg-AMC (Boc-Gln-Ala-Arg-AMC), *t*-butyloxycarbonyl-L-glu-L-lys-L-lys-AMC (Boc-Glu-Lys-Lys-AMC), *N*-methoxysuccinyl-L-ala-L-ala-L-pro-L-val-AMC (Suc(OMe)-Ala-Ala-Pro-Val-AMC), a-*N*-succinyl-L-ala-L-ala-L-ala-AMC (Suc-Ala-Ala-Ala-AMC), a-*N*-succinyl-L-ala-L-ala-L-pro-L-phe-AMC (Suc-Ala-Ala-Pro-Phe-AMC), a-*N*-succinyl-L-ala-L-pro-L-ala-AMC (Suc-Ala-Pro-Ala-AMC), a-*N*-succinyl-L-arg-L-pro-L-phe-L-his-L-leu-L-leu-L-val-L-tyr-AMC (Suc-Arg-Pro-Phe-His-Leu-Leu-Val-Tyr-AMC), a-*N*-succinyl-gly-L-pro-AMC (Suc-Gly-Pro-AMC), a-*N*-succinyl-gly-L-pro-L-leu-gly-L-pro-AMC (Suc-Gly-Pro-Leu-Gly-Pro-AMC), a-*N*-succinyl-L-isoleu-L-isoleu-L-isoleu-L-try-AMC (Suc-Ile-Ile-Try-AMC), a-*N*-succinyl-L-leu-L-leu-L-val-L-tyr-AMC (Suc-Leu-Leu-Val-Tyr-AMC), benzoyl-L-arg-AMC (Bz-Arg-AMC), L-leu-AMC (Leu-AMC), L-met-AMC (Met-AMC), L-pyroglx-AMC (Pyr-AMC) and L-phe-AMC (Phe-AMC) were purchased from Peptide Institute (Osaka), and L-Tyr-AMC (Tyr-AMC) was from Bachem.

	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\cdot\mu M^{-1}$)
Suc-Leu-Leu-Val-Tyr-AMC	20	39.6	1.980
Suc-Ala-Ala-Pro-Phe-AMC	25	11.9	0.476
Suc-Arg-Pro-Phe-His-Leu-Leu-Val-Tyr-AMC	2.2	2.4	1.090
Boc-Gln-Ala-Arg-AMC	19	2.4	0.126
Suc-Ile-Ile-Try-AMC	13	1.0	0.077
Boc-Glu-Lys-Lys-AMC	59	0.3	0.005
Suc(OMe)-Ala-Ala-Pro-Val-AMC	40	0.3	0.008
Z-Phe-Arg-AMC	No hydrolysis		
Suc-Ala-Pro-Ala-AMC	No hydrolysis		
Suc-Ala-Ala-Ala-AMC	No hydrolysis		
Suc-Gly-Pro-AMC	No hydrolysis		
Suc-Gly-Pro-Leu-Gly-Pro-AMC	No hydrolysis		
Tyr-AMC	No hydrolysis		
Phe-AMC	No hydrolysis		
Bz-Arg-AMC	No hydrolysis		
Met-AMC	No hydrolysis		
Pyr-AMC	No hydrolysis		
Leu-AMC	No hydrolysis		

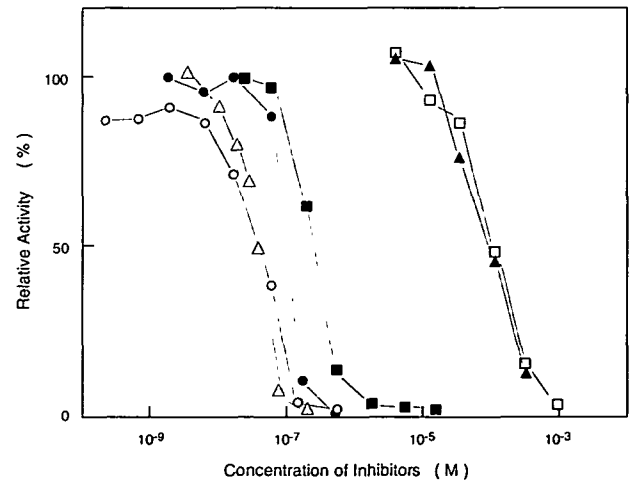

Fig. 4. **Inhibition of myonase by proteinase inhibitors.** Reaction mixtures containing myonase, inhibitor, and salt were incubated for 10 min on ice just before starting the proteinase reaction. Proteinase reaction was started by adding Tris-glycine buffer, pH 9.0, and Suc-Leu-Leu-Val-Tyr-AMC at 30°C as described in "MATERIALS AND METHODS." The inhibitors are lima bean trypsin inhibitor (●), soy bean trypsin inhibitor (○), chymostatin (■), diisoprophyl fluorophosphate (□), antichymotrysin (△), and phenylmethylsulfonyl fluoride (▲).

peptide IgG on Western blotting and slightly detected by anti-rat mast cell chymase IgG, but not detected by monoclonal anti-human mast cell chymase IgG, as shown in Fig. 5.

Primary Structure of Myonase—To learn the structural basis, the primary structure of myonase was determined by sequencing with automated Edman degradation. The N-terminal amino acid sequence (46 residues) of myonase was as described above. Purified myonase was digested with endoproteinase Lys-c, Glu-c, and thermolysin. Thirteen peptides of the resulting digests were purified by HPLC and sequenced to reveal a sequence of 67 amino acids. Comparison of these sequences with those in current data bases showed that they exactly matched the sequence derived from the previously described MMCP-4 cDNA (*23*) (Fig. 6). Both tryptic and CNBr digests gave no matrix-assisted laser desorption/ionization time of flight mass spectra, hence further purification of these digests was not carried out. Myonase appeared to be resistant to these conditions or to be fully degraded. Myonase mRNA was isolated in order to determine the full amino acid sequence of myonase based on the deduced nucleotide sequence of the N-terminal amino acid sequence. The full nucleotide sequence of myonase mRNA was similar to MMCP-4 cDNA (*23*) (Fig. 6), except that nucleic acids at positions 164, 319, 462, 523, 571, 622, 709, and 719 were changed from A to T, from A to G, from C to T, from T to C, from G to A, from C to T, from C to T, and from G to A, respectively. The corresponding amino acids at positions 41, 92, 140, 160, 176, 193, 222,

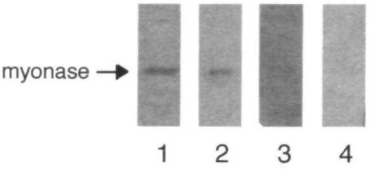

Fig. 5. **Immunoreactivity of myonase.** Purified myonase (0.3 μg in each lane) was separated on a 12% acrylamide gel under reducing conditions. After Western blotting, the blotted protein was detected by anti-myonase IgG from rabbit (17.8 μg/ml, lane 1), monoclonal anti-myonase specific peptide IgG (17.8 μg/ml, lane 2), anti-rat mast cell chymase IgG from rabbit (17.8 μg/ml, lane 3), and monoclonal anti-human mast cell chymase IgG (17.8 μg/ml, lane 4).

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                                                CCCT TATGGCACTT CTCTTGCCTT CTGGGGCTGG AGCTGAGGAG      44

                ATT ATT GGT GGT GTT GAG TCT AGA CCA CAT TCT CGC CCT TAC ATG GCC CAT CTG GAG ATC     103
  1             Ile Ile Gly Gly Val Glu Ser Arg Pro His Ser Arg Pro Tyr Met Ala His Leu Glu Ile
                ACC ACT GAG AGA GGG TTC ACA GCT ACC TGT GGT GGG TTT CTC ATA ACC CGC CAA TTT GTG     163
 21             Thr Thr Glu Arg Gly Phe Thr Ala Thr Cys Gly Gly Phe Leu Ile Thr Arg Gln Phe Val
                TTG ACT GCT GCA CAC TGT AGT GGA AGA GAA ATC ACT GTC ACC CTT GGA GCT CAT GAT GTG     223
 41             Leu Thr Ala Ala His Cys Ser Gly Arg Glu Ile Thr Val Thr Leu Gly Ala His Asp Val
                AGC AAG ACA GAA TCC ACA CAG CAG AAG ATA AAA GTA GAA AAA CAA ATC GTT CAC CCA AAG     283
 61             Ser Lys Thr Glu Ser Thr Gln Gln Lys Ile Lys Val Glu Lys Gln Ile Val His Pro Lys
                TAC AAC TTC TAT TCC AAT CTC CAT GAC ATC ATG TTG CTG AAG CTT CAA AAG AAA GCC AAA     343
 81             Tyr Asn Phe Tyr Ser Asn Leu His Asp Ile Met Leu Leu Lys Leu Gln Lys Lys Ala Lys
                GAG ACT CCC TCT GTG AAT GTA ATT CCT CTG CCT CGT CCT TCT GAC TTT ATC AAG CCG GGG     403
101             Glu Thr Pro Ser Val Asn Val Ile Pro Leu Pro Arg Pro Ser Asp Phe Ile Lys Pro Gly
                AAG ATG TGC CGG GCA GCT GGC TGG GGG CGA ACT GGA GTG ACA GAA CCT ACC TCA GAT ATA     463
121             Lys Met Cys Arg Ala Ala Gly Trp Gly Arg Thr Gly Val Thr Glu Pro Thr Ser Asp Ile
                CTG AGG GAG GTG AAA CTG AGA ATC ATG GAT AAA GAG GCC TGT AAA AAC TAT TGG CAT TAC     523
141             Leu Arg Glu Val Lys Leu Arg Ile Met Asp Lys Glu Ala Cys Lys Asn Tyr Trp His Tyr
                GAC TAT AAC CTC CAG GTC TGC GTG GGC AGT CCC AGA AAG AAA AGA TCA GCA TAC AAG GGA     583
161             Asp Tyr Asn Leu Gln Val Cys Val Gly Ser Pro Arg Lys Lys Arg Ser Ala Tyr Lys Gly
                GAC TCT GGA GGA CCT CTA CTG TGT GCT GGG GTG GCC CAT GGT ATT GTA TCT TAT GGA CGC     643
181             Asp Ser Gly Gly Pro Leu Leu Cys Ala Gly Val Ala His Gly Ile Val Ser Tyr Gly Arg
                GGA GAT GCA AAG CCC CCT GCA GTC TTC ACC CGA ATC TCC TCA TAT GTG CCC TGG ATT AAC     703
201             Gly Asp Ala Lys Pro Pro Ala Val Phe Thr Arg Ile Ser Ser Tyr Val Pro Trp Ile Asn
                AGA GTT ATA AAG GGC AAG  TAGTGAAAAG CCTGACCTGC GTGCATCAGA GTCTTCAAGC CAGAGCTCTT     771
221             Arg Val Ile Lys Gly Lys

                CTGATAACCC TTGGGTTCAA CAAAGCATGT GTCCATCCTG TCACCTGCCT GCCCCCGGCC TGTCCTCAGC      841
                CTGCCCCCAG CCTGCCCCCA AGATGATCTG AAAGATGAAT TCTGTGATGA TGGACTGTTC CCTGTAATGC      911
                ACCTCAGTAA AGCCCTAACC TCCAGCAAAA AAAAAAAAA                                         950
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Fig. 6. **Nucleotide sequence of myonase mRNA and primary structure of myonase.** Nucleotide sequence of myonase mRNA was determined using both 3′RACE and 5′RACE as described in "MATERIALS AND METHODS." Some nucleotide residues (marked by *) were different from the previous report of MMCP-4 cDNA (*23*). The N-terminal amino acid sequence (⊢—) of myonase was determined by use of a gas-phase protein sequencer, model PPSQ-10 (Shimadzu) using the Edman degradation. Amino acid sequences of myonase fragments produced by endoproteinase Glu-c (-----), Lys-c (·····) and thermolysin (----) were coincident with the deduced amino acid sequence from the nucleotide sequence of myonase mRNA.

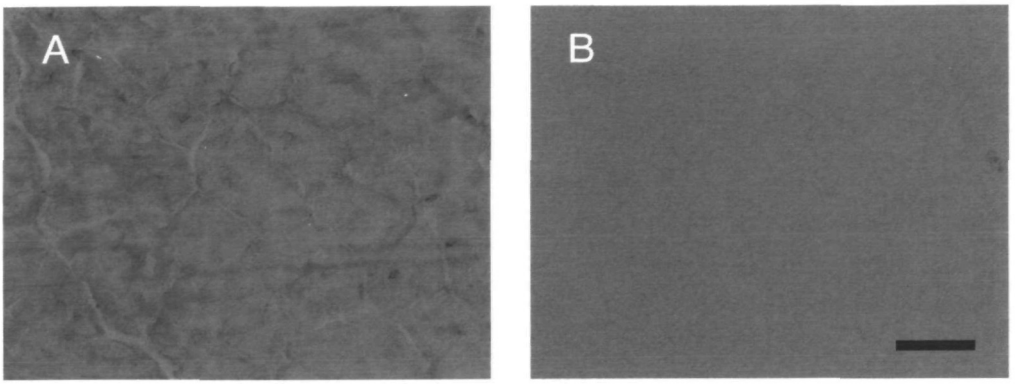

Fig. 7. **Immunohistochemical localization of myonase in mouse femoral muscle.** Femoral muscle was dissected from 44-day-old male MDX-mouse. A: Staining with anti-myonase IgG from rabbit. B: Staining with control IgG from rabbit. Original magnifications ×400. Bar=50 μm.

and 226 were Leu, Leu, Ile, Tyr, Ser, His, Val, and Lys, respectively.

Is Myonase a Glycoprotein?—To investigate whether post-translational glycosylation of myonase occurred, the enzyme was incubated with peptide-*N*-glycosidase F to remove *N*-linked carbohydrate, or endo-α-acetylgalactosaminidase to remove *O*-linked carbohydrate. No change in the apparent molecular mass as determined by SDS-PAGE was detected even after prolonged deglycosylation treatment with *O*-glycosidase and *N*-glycosidase. The molecular mass of myonase by mass spectrometry was identical to that calculated from the amino-acid sequence deduced from the nucleotide sequence of myonase mRNA. Thus, myonase does not contain any carbohydrate moiety.

Immunohistochemical Localization of Myonase—As shown in Fig. 7, myonase was located solely in myocytes of femoral muscle, not in mast cells. The usual methodological control used in typical immunohistochemical studies did not stain (Fig. 7B). Muscle fibers in 44-day-old mouse were mixed regenerating fibers and degenerating fibers. Immunoreactivity to myonase was present in regenerating fibers, but not in degenerating fibers.

DISCUSSION

A serine proteinase from MDX-mouse skeletal muscle has been isolated and purified. In its molecular mass, optimal pH of proteinase activity and susceptibility to inhibitors including lima bean trypsin inhibitor, soy bean trypsin inhibitor, chymostatin, diisopropyl fluorophosphate, and phenylmethylsulfonyl fluoride, the isolated enzyme was similar to the rat mucosal and peritoneal mast cell-derived proteinases, RMCP-1 and -2 (*11*) and the mouse mast cell proteinase (*17*), but it differed in immunoreactivity. The activity of myonase was also inhibited by ovoinhibitor, but not by *p*-tosyl-L-phenylalanine chloromethyl ketone and ovomucoid. These results show that myonase belongs to the chymase family.

Myonase preferentially cleaves the Tyr4-His5 bond of angiotensin I, in which it is similar to RMCP-1 (*9*) and angiotensin II-forming hamster chymase (*45*) but different from human heart chymase (*40, 41*), which hydrolyzes the Phe8-His9 bond to produce angiotensin II. It is unclear which moiety in the proteinase of the chymase family is responsible for these preferential cleavage positions of the peptide bond. Amyloid β-protein was specifically hydrolyzed at the Phe20-Ala21 bond to give the fragments Asp1-Phe20 and Ala21-Val40. This is the first report of this cleavage in amyloid β-protein using the purified proteinase, but the physiological significance of these fragments remains to be assessed. Recently it was reported that neuroblastoma cells from mouse could produce the fragment Asp1-Phe20 from amyloid β-protein (*50*). This cell line may have a proteinase like myonase.

Myonase mRNA is identical with MMCP-4 cDNA from mouse mast cells. But the native enzyme corresponding to this cDNA has not been reported. Myonase is the first protein to be isolated for which the primary structure is similar to MMCP-4.

Chymase contained in mast cells is conjugated with a proteoglycan such as heparin (*51*). The proteoglycan regulates the intracellular localization and the enzymatic activity of the chymase (*46-49, 51*). The similarity in the primary structure to chymase (*52*) suggested that myonase would also be regulated by a proteoglycan. Myonase, however, did not show any interaction with heparin in the regulation of its activity. The lack of regulation by heparin might be due to the absence of sugar in the myonase molecule. These considerations suggest that myonase is regulated by a mechanism different from that of chymase.

Calpain is believed to be related to the pathogenesis of muscular dystrophy due to the defect of dystrophin (*4, 5*). A defect of dystrophin results in the deregulation of a calcium channel, allowing unregulated entry of calcium into the cell. The increase in $[Ca^{2+}]_{in}$ activates calpain, which is diffused the cell plasma, to autolyze partially. The autoproteolytically modified calpain is relocated in the membranes through the interaction with phospholipids (*53*), and this relocated calpain cleaves the membrane skeletal proteins, causing cell necrosis. This sequential process is the most likely route for pathogenesis of muscular dystrophy.

Myonase has chymase-like activity and appears during the pathological process of muscular dystrophy. Thus, it may regulate the degeneration-regeneration of muscle rather than the pathogenesis of muscular dystrophy. Dystrophin-defective muscular dystrophy increases the number of mast cells in the muscle tissue during the process of muscular dystrophy. The number of mast cells in 4-month-old MDX-mouse is approximately 4-fold higher than in controls (*8*). At this stage, the amount of purified myonase in MDX-mouse skeletal muscle is increased to approximately 5- to 10-fold that in control skeletal muscle (unpublished data). The increased amount of myonase

coincides with the increased number of mast cells. However, it is uncertain whether myonase is derived from mast cells, because anti-serum against myonase recognizes myocytes but not mast cells (Fig. 6).

Degenerated and regenerated fibers co-exist in the skeletal muscles of MDX-mouse throughout life. Muscular dystrophy in MDX-mouse does not progress because an equal balance is maintained between degeneration and regeneration of muscle fibers. MDX-mouse thus lives for the same duration as normal mouse. The mechanism of this equal balance between degeneration and regeneration of muscle fibers is unknown. Myonase in regenerating fibers might play a role in the constructive process of muscle structure. Further study on this point is in progress.

Thanks are due to Dr. H. Kido, Tokushima University, Tokushima, for the gift of anti-rat mast cell chymase rabbit IgG, to A. Okamoto, A. Shirakawa, and T. Hosaka for their technical assistance. We are also grateful to Dr. V. Wray, Gesellschaft fuer Biotechnologische Forschung, Braunschweiz, Germany, for reading the manuscript.

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