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Glucose-6-phosphate Isomerase Is an Endogenous Inhibitor to Myofibril-Bound Serine Proteinase of Crucian Carp (*Carassius auratus*)

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Glucose-6-phosphate isomerase (GPI) was purified to homogeneity from the skeletal muscle of crucian carp (*Carassius auratus*) by ammonium sulfate fractionation, column chromatographies of Q-Sepharose, SP-Sepharose, and Superdex 200 with a yield of 8.0%, and purification folds of 468. The molecular mass of GPI was 120 kDa as estimated by gel filtration, while on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), two subunits (55 and 65 kDa) were identified, suggesting that it is a heterodimer. Interestingly, GPI revealed specific inhibitory activity toward a myofibril-bound serine proteinase (MBSP) from crucian carp, while no inhibitory activity was identified toward other serine proteinases, such as white croaker MBSP and crucian carp trypsin. Kinetic analysis showed that GPI is a competitive inhibitor toward MBSP, and the K_i was 0.32 μM. Our present results indicated that the multifunctional protein GPI is an endogenous inhibitor to MBSP and may play a significant role in the regulation of muscular protein metabolism *in vivo*.

KEYWORDS: Crucian carp; glucose-6-phosphate isomerase; myofibril-bound serine proteinase; inhibitor; purification; immunoblotting

INTRODUCTION

Glucose-6-phosphate isomerase (GPI, EC 5.3.1.9) is a housekeeping cytosolic enzyme that works as the second enzyme in glycolysis. GPI catalyzes the reversible isomerization of glucose-6-phosphate to fructose-6-phosphate and participates in gluconeogenesis and the pentose phosphate pathway (*1*). Molecular cloning and sequencing analysis have identified mammalian GPIs as neuroleukin (NLK), autocrine motility factor (AMF), and maturation factor (MF) (*2*−*6*). Other functions of GPI, such as sperm agglutination (*7*) and lysyl aminopeptidase (*8*), have also been reported.

In comparison to detailed research on GPI from mammalians and microorganisms, less information was available for GPI from fish. Previously, we have identified that a specific endogenous inhibitor toward myofibril-bound serine proteinase (MBSP) in the skeletal muscle of marine fish white croaker (*Argyrosomus argentatus*) was actually GPI (*9*). The evidence that GPI acts as a MBSP inhibitor (MBSPI) in marine fish white croaker implied that the multifunctional protein GPI may commonly play an inhibitor role to MBSPs in other fish species. However, GPI from white croaker specifically inhibited the activity of MBSPs from marine fish white croaker and lizard fish (*Saurida wanieso*), while no inhibitory activity was identified toward MBSP from freshwater fish, such as common carp (*9*). MBSPs are unique serine proteinases, which revealed activity in degrading myofibrillar proteins, such as myosin heavy chain (MHC), α-actinin, actin, and tropomyosin at neutral pH (*9*, *10*). More recently, we described the purification of MBSP from fresh water fish crucian carp (*Carassius auratus*) muscle and determination of its full-length primary protein sequence (*11*). Identification the endogenous inhibitor of crucian carp MBSP and if GPI plays the MBSPI role are of much interest to us. In the present study, we described a detailed study on the purification of GPI from the skeletal muscle of crucian carp and detected its MBSPI character with an attempt to clarify the inhibition mechanism between GPI and MBSP.

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MATERIALS AND METHODS

Fish. Crucian carp (*C. auratus*) (body weight of approximately 0.3 kg) were purchased alive from the fish market of Jimei, Xiamen, China. The fish were subdued in iced water and sacrificed instantly. Skeletal muscle was collected and used for the experiment immediately.

Chemicals. Q-Sepharose, SP-Sepharose, and Superdex 200 10/300G were products of Pharmacia (Uppsala, Sweden). Porcine trypsin, fructose-6-phosphate, β-nicotin amideadenine dinucleotide phosphate (NADP), and glucose-6-phosphate dehydrogenase were purchased from Sigma (St. Louis, MO). MBSP from the skeletal muscle of crucian carp was

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prepared as described (*11*). White croaker MBSP, crucian carp trypsin and chymotrypsin, and trypsin A and trypsin B from the pyloric ceca of mandarin fish (*Siniperca chuatsi*) were purified in our laboratory according to the previous reports (*12*, *13*). Substrates of *t*-butyloxy-carbonyl-Phe-Ser-Arg-4-methyl-coumaryl-7-amide (Boc-Phe-Ser-Arg-MCA) for trypsin-type serine proteinases and succinyl-Ala-Ala-Pro-Phe-4-methyl-coumaryl-7-amide (Suc-Ala-Ala-Pro-Phe-MCA) for chymotrypsins were purchased from Peptide Institute, Inc. (Osaka, Japan). Protein markers for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) were from Fermentas (Vilnius, Lithuania). Prestained protein markers for immunoblotting were from New England Biolabs (Richmond, CA). Molecular-weight calibration markers for gel filtration and dithiothreitol (DTT) were from Bio-Rad (Hercules, CA). Rat anti-white croaker MBSPI polyclonal antibody was prepared as described (*9*). Rat anti-red sea bream actin polyclonal antibody was a kind gift from Dr. Tachibana of the Faculty of Fisheries, Nagasaki University, Japan. Rat anti-common carp tropomyosin was prepared in our own laboratory. The second antibodies of rabbit anti-rat IgG−horseradish peroxidase (HRP) and goat anti-rabbit IgG−HRP were from DAKO (Denmark). 3,3′-Diaminobenzidine tetrahydrochloride (DAB) was from Pierce (Rockford, IL). All other chemicals used were of analytical grade.

Enzyme Assay and Kinetics. GPI activity was assayed according to the second reaction of the glycolytic pathway. The enzyme activity was measured in the reverse direction (fructose-6-phosphate → glucose-6-phosphate) by coupling with excess glucose-6-phosphate dehydrogenase and nicotinamide adenine dinucleotide phosphate (NADP). The production of NADPH was monitored at 340 nm using a Unico UV-2600 spectrophotometer (*14*, *15*). Routine assays were performed at room temperature. The standard assay mixture was 0.5 mL of 50 mM Tris-HCl (pH 8.0) containing 1 mM fructose-6-phosphate, 0.36 mM NADP, 7 mM $MgCl_2$, and 1 unit of glucose-6-phosphate dehydrogenase (*14*). The reaction was initiated by the addition of glucose phosphate isomerase, and the value was recorded in the first 2 min. One unit of enzyme activity is defined as that amount catalyzing the isomerization of 1 μmol of substrate/min under the above conditions (*14*, *15*).

For a kinetic study, velocities were recorded at the first 2 min of the reaction. Briefly, 0.75 unit of GPI was added to six concentrations of fructose-6-phosphate (0.1, 0.2, 0.4, 0.6, 1, and 2 mM) in a 0.5 mL reaction system containing 50 mM Tris-HCl (pH 8.0), 0.8 mM NADP, 7 mM $MgCl_2$, and 1 unit of glucose-6-phosphate dehydrogenase. Kinetic parameters, including V_{max} and K_m, were evaluated on the basis of Lineweaver−Burk plots (*14*).

Purification of GPI. All procedures were performed at 4 °C. Briefly, 200 g of minced skeletal muscle was homogenized in 4-fold of 20 mM Tris-HCl (pH 8.5) using a homogenizer (Kinematica, PT-2100, Switzerland), followed by centrifugation at 10000*g* for 20 min. The supernatant was fractionated with ammonium sulfate between 40 and 80% saturation. The precipitate was dissolves in a minimum volume of 20 mM Tris-HCl (pH 8.5) and dialyzed against the same buffer. The dialyzed solution was subsequently applied to a Q-Sepharose column (2.6 × 18 cm) equilibrated with 20 mM Tris-HCl (pH 8.5). After the column was washed until the absorbance at 280 nm reached the baseline, bound proteins were eluted at a flow rate of 1.0 mL/min with a 0−0.5 M linear NaCl gradient and active fractions were collected. Collected fractions adjusted pH to 5.0 by 1 M HCl, were concentrated by ultrafiltration using a 30 kDa cutoff membrane of YM-30 (Millipore, MA), and exchanged Tris-HCl buffer with 20 mM HAc−NaAc (pH 5.0). Samples were then applied to a SP-Sepharose column (1.6 × 12 cm) equilibrated with 20 mM HAc−NaAc buffer (pH 5.0). Elution was carried out using a stepwise method with NaCl concentrations of 0, 0.2, 0.3, 0.4, and 0.6 M, respectively, in the same buffer. The pooled active fractions were further concentrated by ultrafiltration and applied to the gel-filtration column of Superdex 200 10/300 GL (1.0 × 30 cm), which was connected to an AKTA design system (UPC-10, GE) and equilibrated with 50 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl at a flow rate 0.4 mL/min.

Protein Concentration Determination. The protein concentration was determined by measuring the absorbance at 280 nm of the sample solution from column chromatographies or with the method of Lowry et al. (*16*) using bovine serum albumin as a standard.

Electrophoresis. SDS−PAGE was performed on a 12% gel according to the method of Laemmli (*17*) and stained with Coomassie Brilliant Blue R-250 (CBB) or silver. Native−PAGE was also performed according to the procedure of Laemmli (*17*), while samples were not heated and without the addition of SDS or any reducing agent.

Estimation of the Molecular Mass. The molecular mass of the purified enzyme was estimated by SDS−PAGE. Gel filtration was also applied to estimate the molecular mass of the native enzyme by a Superdex 200 10/300 GL (1.0 × 30 cm) column using thyroglobulin (670 000 Da), bovine γ-globulin (158 000 Da), chicken ovalbumin (44 000 Da), equine myoglobin (17 000 Da), and vitamin B_{12} (1350 Da) as molecular-mass standards.

Immunoblotting. Immunoblotting was carried out as described by Towbin et al. (*18*). Briefly, after SDS−PAGE, proteins on acrylamide gel were electrophoretically transferred to a nitrocellulose membrane. Nonspecific protein sites were blocked with 5% nonfat milk in Tris-HCl-buffered saline (TBS = 20 mM Tris-HCl at pH 7.5 containing 0.145 M NaCl). The membrane was incubated with polyclonal antibodies, respectively, at room temperature for 2 h and washed with TBST (TBS and 0.05% Tween 20). After incubation for 1 h with HRP-labeled second antibodies, membranes were further washed extensively with TBST. Immunodetection was carried out using DAB as a substrate.

Inhibition on Crucian Carp MBSP. Approximately 0.03 unit of MBSP was preincubated with two different concentrations of GPI (0.04 and 0.08 μM) in 950 μL of 20 mM Tris-HCl buffer at pH 8.0 and room temperature for 15 min. After preincubation, 50 μL of 10 μM Boc-Phe-Ser-Arg-MCA was added to initiate the reaction at 55 °C for 30 min. The remaining activity was determined as described above. Control tests were performed under the same conditions, except GPI was replaced with Tris-HCl buffer.

Inhibition on Different Serine Proteinases. Approximately 0.03 unit of crucian carp MBSP, pig trypsin, crucian carp trypsin, crucian carp chymotrypsin, mandarin fish trypsin A and trypsin B, and white croaker MBSP were preincubated individually with 0.15 nmol of purified GPI in 1 mL of 0.1 M Tris-HCl at pH 8.0 and room temperature (28 °C) for 15 min. Fluorogenic substrates, Boc-Phe-Ser-Arg-MCA for MBSP and trypsins, and Suc-Ala-Ala-Pro-Phe-MCA for chymotrypsins were then added to the reaction mixture, respectively, to a final concentration of 0.5 μM and further incubated at 37 °C for 30 min, while MBSP was incubated at 55 °C. The reaction was terminated by the addition of 1.5 mL of stopping agent [35:30:35 methyl alcohol/*n*-butyl alcohol/distilled water (v/v/v)]. The fluorescence intensity of liberated 7-amino-4-methylcoumarin (AMC) was measured by a fluorescence spectrophotometer (Jasco, FP-6200, Japan) at the excitation wavelength of 380 nm and the emission wavelength of 450 nm. An inhibitory activity assay was performed in duplicate, and variation between duplicate samples was always less than 10%.

Determination of the Inhibition Type of GPI on MBSP. For the determination of the inhibition type of GPI on MBSP, MBSP (0.03 unit) was preincubated with GPI at four levels (0, 0.045, 0.09, and 0.15 μM) and five substrate concentrations (1, 1.5, 2, 2.5, and 5 μM), resulting in 20 different substrate−inhibitor combinations. Each assay was performed in quadruplicate. Kinetic parameters, including V_{max} and K_m, were evaluated on the basis of Lineweaver−Burk plots. The K_i value was calculated according to the method of Suder et al. (*19*).

Inhibition on the Degradation of Myofibrillar Proteins by MBSP. Crucian carp myofibril proteins were prepared and treated with serine proteinase inhibitor Pefabloc SC to inhibit the activity of endogenous MBSP as described (*20*). MBSP (0.03 unit) were preincubated with various amounts of GPI in a total volume of 20 μL at room temperature for 20 min. Then, 80 μL of myofibrils (2.5 mg/mL) was added into the mixtures individually and further incubated at 55 °C for 1 h. A control test was performed by incubating myofibrils without the addition of MBSP and GPI. After the reaction, samples were immediately analyzed by SDS−PAGE or immunoblotting to detect the degradation of proteins.

RESULTS

Purification of GPI. GPI was purified from the skeletal muscle of crucian carp by ammonium sulfate fractionation and column chromatographies of Q-Sepharose (**Figure 1A**), SP-Sepharose (**Figure 1B**), and Superdex 200 (**Figure 1C**). In ionic column Q-Sepharose, GPI active fractions were obtained at a NaCl concentration of 0.2 M, while in cationic column SP-Sepharose,

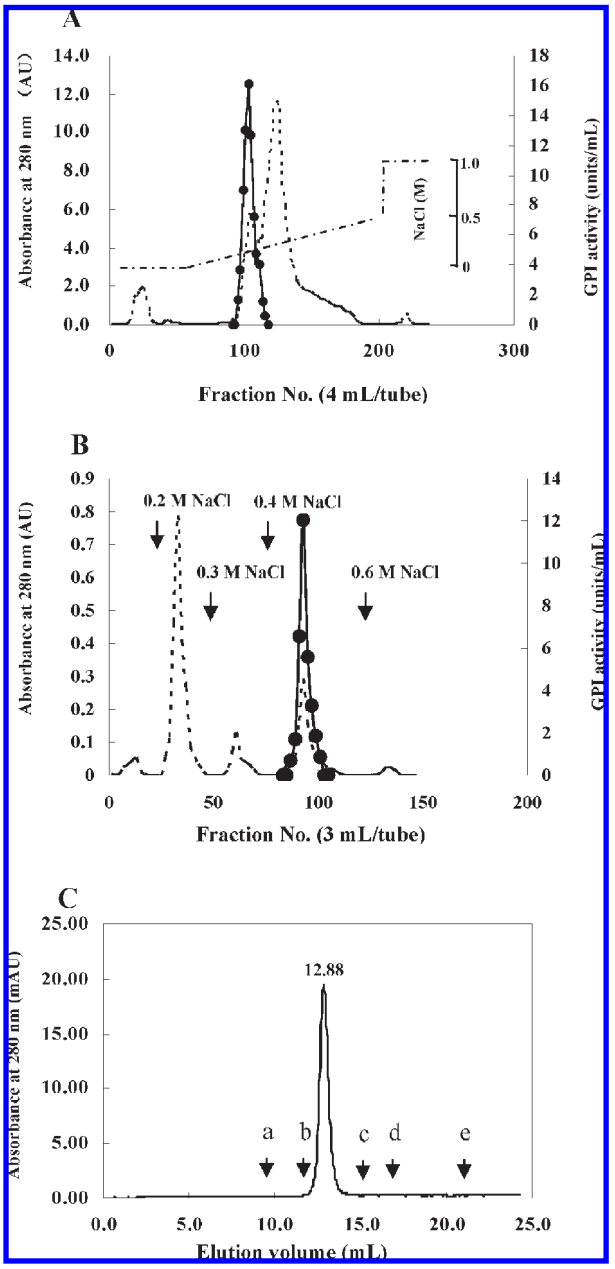

Figure 1. Chromatographic purification of GPI from crucian carp skeletal muscle: (A) Q-Sepharose chromatography, (B) SP-Sepharose chromatography, and (C) Superdex 200 10/300GL gel-filtration chromatography. Arrowheads indicate the elution volumes of molecular-mass markers: (a) thyroglobulin (670 000 Da), (b) bovine γ-globulin (158 000 Da), (c) chicken ovalbumin (44 000 Da), (d) equine myoglobin (17 000 Da), and (e) vitamin B_{12} (1350 Da). Absorbance at 280 nm (---), and GPI activity (●).

Table 1. Purification Results of GPI

step	total protein (mg)	total activity (units)	specific activity (units/mg)	purification (fold)	yield (%)
crude extract	2880	875	0.3	1	100
Q-Sepharose	232	565	2.4	8	65
SP-Sepharose	1.2	145	121	402	17
Superdex	0.5	70	140	468	8

GPI was eluted at 0.4 M NaCl. After subjecting fractions with highest activity to a gel-filtration column of Superdex 200, GPI was finally purified to homogeneity. A summary of the purification is shown in **Table 1**. Approximately 0.5 mg of GPI was obtained from 200 g of muscle with a recovery of 8.0% and a 468-fold increase in specific activity.

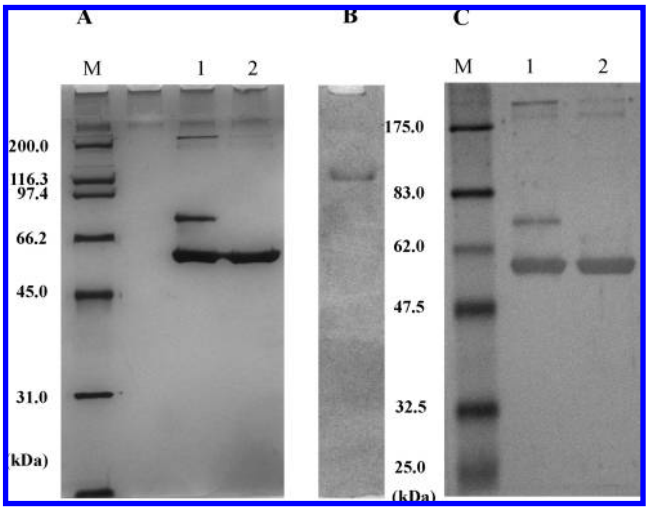

Figure 2. SDS−PAGE, Native−PAGE, and Immunoblotting of GPI. (A) SDS−PAGE. The gel was silver-stained. M, protein marker; lane 1, under nonreducing conditions; lane 2, under reducing conditions. (B) Native−PAGE. (C) Immunoblotting. M, prestained protein marker; lane 1, under nonreducing conditions; lane 2, under reducing conditions.

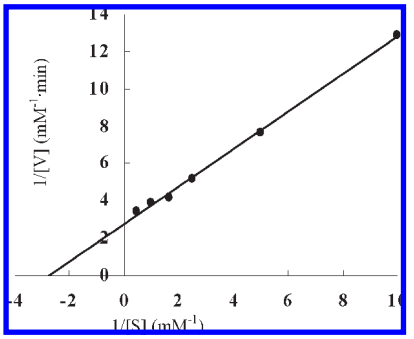

Figure 3. Lineweaver−Burk plot of GPI for fructose-6-phosphate. Velocities were recorded at the first 2 min of the reaction as described in the Materials and Methods. Kinetic parameters of V_{max} and K_m were evaluated on the basis of the plot. Data were collect at pH 7.5 and 25 °C.

Molecular Mass and Purity of GPI. On the basis of **Figure 1C**, the molecular mass of GPI as determined by gel filtration was 120 kDa. On SDS−PAGE under nonreducing conditions, two major bands (55 and 66 kDa) and a minor band (250 kDa) were detected, while mainly a single band at 55 kDa was observed under reducing conditions (**Figure 2A**). On native−PAGE, only a single band was identified, suggesting that GPI was highly purified (**Figure 2B**). Immunoblotting analysis using rat against white croaker MBSPI (GPI) polyclonal antibody also revealed positive reaction bands (**Figure 2C**), suggesting that the present protein is GPI and having a high homogeneity with GPI from white croaker (*9*).

Kinetic Parameters of GPI. The Lineweaver−Burk plots of GPI using fructose-6-phosphate as a substrate were shown in **Figure 3**. The K_m and k_{cat} values of crucian carp GPI for fructose-6-phosphate were 0.36 mM and 18 s^{-1}, respectively. These results were similar to GPIs from other reports (*14*, *15*, *21*).

Inhibition on Different Serine Proteinases. The inhibition effect of GPI on different serine proteinases was investigated. Similar to MBSPI (GPI) from white croaker muscle (*9*), crucian carp GPI revealed a specific inhibitory effect to crucian carp MBSP, while no inhibitory effect could be detected to other serine proteinases, including crucian carp trypsin and crucian carp chymotrypsin,

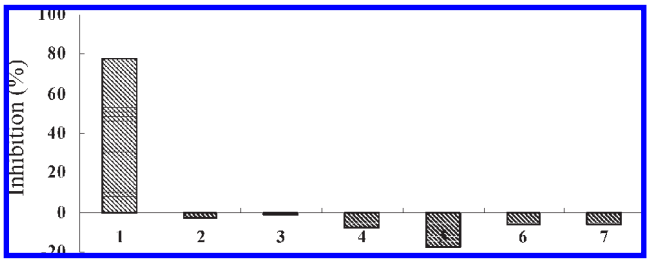

Figure 4. Inhibition of GPI on various serine proteinases. To each proteinase (0.03 unit), 0.15 nmol of GPI was added and preincubated at room temperature for 15 min and the remaining activity was determined, respectively. Proteolytic activity in the absence of GPI was taken as a control. 1, crucian carp MBSP; 2, crucian carp trypsin; 3, crucian carp chymotrypsin; 4, mandarin fish trypsin A; 5, mandarin fish trypsin B; 6, porcine trypsin; 7, white croaker MBSP.

trypsin A and trypsin B from the pyloric ceca of mandarin fish, pig trypsin, and white croaker MBSP (**Figure 4**).

Inhibition on MBSP and Inhibition Kinetic Studies. As shown in **Figure 5A**, the activity of crucian carp MBSP decreased with the increasing addition of GPI. The interaction between GPI and MBSP as shown by Lineweaver−Burk plots fulfills the model of competitive inhibition (**Figure 5B**), and the K_i was 0.32 μM (**Figure 5C**).

Inhibition on the Degradation of Myofibrillar Proteins. The inhibition effect of GPI on the degradation of myofibrillar proteins by MBSP was also studied. Crucian carp MBSP degrades myofibrillar proteins, such as myosin heavy chain (MHC), actin, and tropomyosin, effectively at pH 8.0 (**Figure 6A**). However, with the increasing addition of GPI, the degradation of these proteins was significantly suppressed. Immunoblotting analysis further exhibited that the degradation of actin and tropomyosin was also effectively inhibited (**Figure 6B**). These results strongly suggest that GPI is the endogenous target-oriented inhibitor for MBSP.

DISCUSSION

As an important enzyme in glycolysis, GPI has been studied extensively. Because the function of GPI as a NLK was identified (*2*, *3*), other functions of GPI, such as AMF and MF, were reported in succession (*5*, *22*, *23*). Our previous study for the first time identified GPI as an endogenous inhibitor for MBSP in marine fish white croaker (*9*). All of these results revealed the fact that GPI is a multifunctional protein. Various methods for purification of GPI from different origins have been described (*9*, *14*, *15*, *24*). In the present study, we combined ammonium sulfate precipitation, ion-exchange chromatographies, and gel filtration to purify crucian carp GPI. To check the purity of GPI, SDS−PAGE, Native−PAGE, and immunoblotting were used (**Figure 2**). Furthermore, phenyl-Sepharose and hydroxyapatite chromatography, which isolate proteins according to different principles, were also performed. All of these results showed that a single peak is synchronous with the single activity peak (data not shown), suggesting that GPI was highly purified.

The molecular mass of native GPI as determined by gel filtration was 120 kDa, which was similar to GPIs from other species, such as teleost fish (110 ± 5 kDa) (*14*), catfish muscle (132 kDa), catfish liver (131 kDa), conger muscle (132 kDa), conger liver (135 kDa) (*15*), bovine heart (118 kDa) (*21*), and rabbit muscle (122 kDa) (*25*). SDS−PAGE of crucian carp GPI under nonreducing conditions yielded two major bands of 55 and 65 kDa and a weak band with a size of 250 kDa. However, under

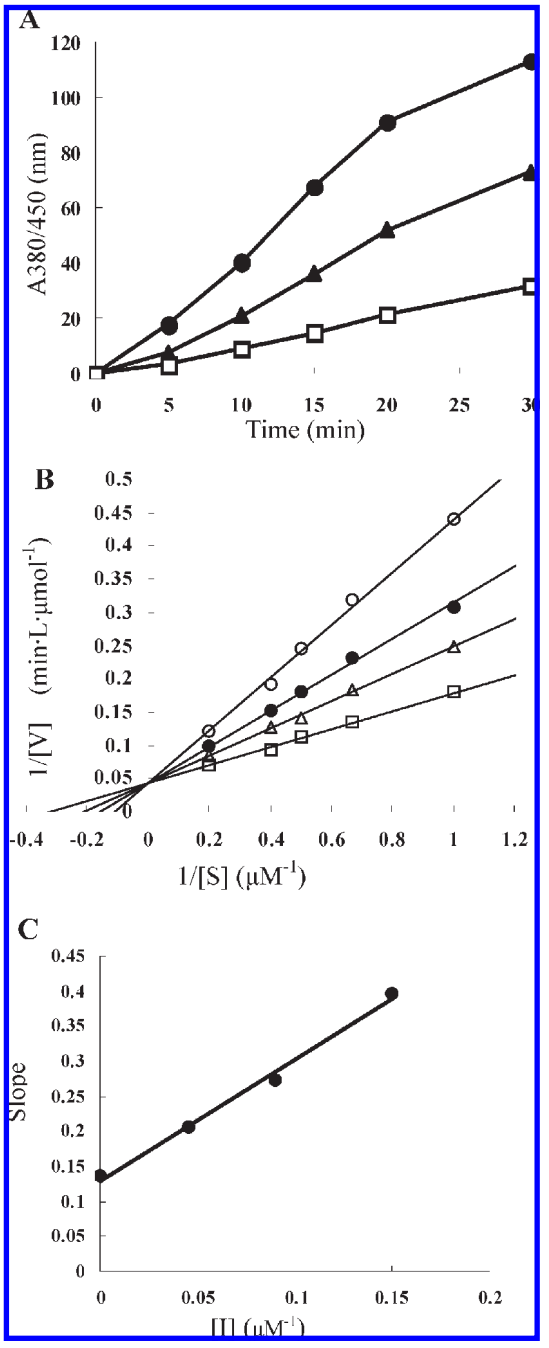

Figure 5. Inhibition on MBSP and inhibition kinetics. (A) Approximately 0.03 unit of MBSP was preincubated with different amounts of GPI in a 1 mL reaction system, respectively, at room temperature for 30 min. The absorbance values of liberated AMC at A380/450 were recorded: (●) 0 μM, (▲) 0.04 μM, and (□) 0.08 μM GPI. (B) Lineweaver−Burk plots of MBSP-hydrolyzing activity in the presence of different concentrations of GPI. MBSP (0.03 unit) was preincubated with GPI at room temperature for 15 min, and different amounts of substrate Boc-Phe-Ser-Arg-MCA were added. The mixture was then incubated at 55 °C for 30 min, and the remaining activity was determined. Final concentrations of GPI used were (□) 0 μM, (△) 0.045 μM, (●) 0.09 μM, and (○) 0.15 μM. (C) Replots of slopes calculated from Lineweaver−Burk plots against the concentrations of GPI.

reducing conditions, only one band with a size of 55 kDa was identified (**Figure 2**). These results suggested that GPI from crucian carp skeletal muscle is quite possibly a heterodimer that consists of two different subunits: α (65 kDa) and β (55 kDa). This was in accordance with the molecular mass of native GPI

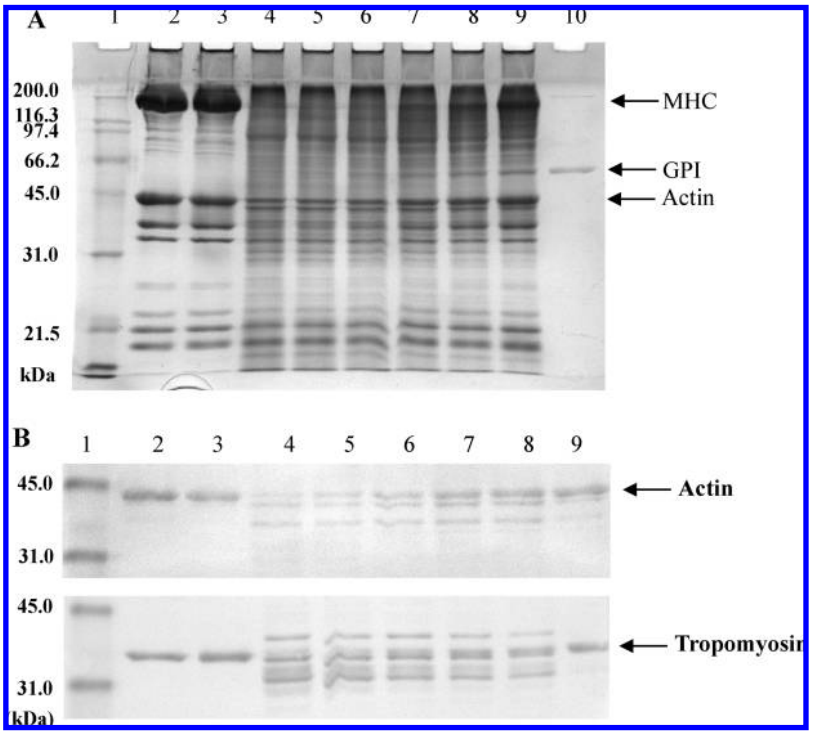

Figure 6. Inhibition effect of GPI on the degradation of myofibrillar proteins by MBSP. Pefabloc SC-treated crucian carp myofibril (0.25 mg) was incubated with 0.03 unit of MBSP in 0.1 mL Tris-HCl (pH 8.0) containing 0.5 NaCl at 55 °C for 1 h in the presence or absence of various amounts of GPI followed by SDS−PAGE and immunoblotting. (A) SDS−PAGE. Lane 1, protein marker; lane 2, non-incubated myofibril; lane 3, incubated myofibril (control); lanes 4−9, MBSP in the presence of different concentrations of GPI: 0, 0.07, 0.15, 0.2, 0.3, and 0.45 μM. (B) Immunoblotting to detect the degradation of actin and tropomyosin. Lane 1, prestained protein marker; lanes 2−9, similar to A.

(120 kDa), as determined by gel filtration. Such results of the heterodimer have also been reported in GPIs from human placentae (*24*), and the existence of heterodimer forms for GPI has been found in wildflower genus *Clarkia* (*26*) and mouse (*27*).

It has been reported that most mammalian GPIs are homodimers that are composed of two identical subunits intracellularly, while they perform AMF and neurokine functions extracellularly as a monomer (*28*, *29*). Our present result is different from the GPI from marine fish white croaker, which is a 55 kDa monomer (*9*), suggesting that GPIs are species-dependent. It is also different from GPIs from teleost fish (*14*), catfish, and conger (*15*), which exhibited as homodimers in both muscle and liver. According to **Figure 2A**, we speculated that subunit β may migrate to 65 kDa under nonreducing conditions and to 55 kDa under reducing conditions. A similar result was also found in NLK, which migrated as a 66 kDa polypeptide under nonreducing conditions and as a 55 kDa band under reducing conditions (*5*, *30*). However, tumor cell AMF and rabbit heart GPI migrated as a 65 kDa band under reducing conditions and as a 55 kDa band under nonreducing conditions (*5*). These results implied that subunit β of crucian carp GPI and NLK is similar and, quite possibly, the 250 kDa band is a dimer form of native GPI with a composition of $\alpha_2\beta_2$.

It has been proposed that the formation of the dimer may enhance the stability of protein and a fast formation of the oligomeric structure might avoid a random interaction of its subunits with other proteins *in vivo* (*31*). GPI subunit formation may include SH oxidation (*32*), subunit aggregation (*33*), or other cross-link effects (*28*, *29*). Obviously, crucian carp GPI dimer is not formed by SH oxidation, because the heterodimer can easily be separated to two subunits (65 and 55 kDa) under nonreducing conditions. Crystal structure research on *Bacillus stearothermophilus* GPI indicated that the dimer is formed by a monomer through an arm-to-arm hug and these interactions were stabilized by a total of 76 intermolecular hydrogen bonds as well as van der Waals interactions (*22*). The presence of SDS can damage the hydrogen bonds and other noncovalent bindings; therefore, the dimer forms of GPI cannot be detected by SDS−PAGE.

Identification of proteinases and their endogenous inhibitors is essential for the understanding of their biological functions as well as their possible applications in food processing. Although serine proteinases have been widely identified in animals, plants, and microorganisms and serine proteinase inhibitors are the most studied among the four classes of inhibitors, most of them were purified using trypsin or chymotrypsin as target enzymes. In the present study, crucian carp MBSP was used for investigation and was significantly inhibited by GPI (**Figure 5**). It is interesting to notice that GPI did not show any inhibitory activity against trypsin and chymotrypsin and other serine proteinases, including a myofibril-bound serine proteinase from marine fish white croaker (**Figure 4**). These results strongly suggested that the endogenous MBSP inhibitor is species-dependent. Previously, we speculated that GPI has some sites that can be recognized by MBSP and form the enzyme−inhibitor complex (*9*). In the present study, we found that the interaction between GPI and MBSP fulfills the competitive model of inhibition, and its K_i was 0.32 μM (**Figure 5**). MBSPI from white croaker exhibited high homology to porcine and rabbit GPIs and revealed the monomer form under native conditions (*9*). In contrast, crucian carp GPI is a heterodimer, indicating that the MBSPI activity of GPIs may not be dependent upon the dimer structure. A substrate specificity study exhibited that MBSP specifically hydrolyzed substrates with arginine and lysine residues at the P_1 site (*11*, *12*, *34*). MBSP

may recognize the arginine or lysine residues in GPI and form a MBSP−GPI complex. This enzyme−inhibitor interaction may affect the enzyme structure and finally suppress the activity of MBSP.

Similar to our previously studies, MBSP degraded myofibrillar proteins, such as myosin heavy chain (MHC), actin, and tropomyosin, effectively at neutral pH, and such degradations were effectively inhibited by the addition of GPI (**Figure 6**). Although the mechanism by which the activity of MBSP is regulated by GPI remains unclear, our previous results (*9*, *11*) together with the present study indicated that the amount of GPI is in excess of MBSP. As the endogenous target-oriented proteinase inhibitor, the multifunctional protein GPI was thus considered to play a significant role on the regulation of fish muscular protein turnover *in vivo*. Furthermore, during the production of fish cake, sarcoplasmic proteins (including GPI) were often discarded in the washing processes. As the specific endogenous inhibitor of MBSP, our present study strongly suggested the fact that GPI can be regenerated from the discarded water and applied to fish cake production to suppress the activity of MBSP.

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