Purification and Characterization of Calpastatin from Grass Prawn Muscle (*Penaeus monodon*)

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Calpastatin, a specific calpain inhibitor was purified to electrophoretical homogeneity from grass prawn (*Penaeus monodon*) muscle by 100 °C heat-treatment, DEAE-Sephacel, and Q-Sepharose chromatographs. No significant change in the inhibitory activity of crude calpastatin was observed even after 20 min incubation at 100 °C, pH 7.0. The purified prawn calpastatin had a molecular weight (M_r) of 80 and 88.7 kDa determined by SDS−PAGE and Sephacryl S-200 HR gel filtration, respectively. According to the active site titration, the purified calpastatin revealed four beef μ-calpain and two beef *m*-calpain binding domains, respectively. It was stable during 1 h of incubation at 30 °C under pH 4.5−10.0 and shown to be a highly specific inhibitor for calpain.

Keywords: *Calpastatin; calpain inhibitor; grass prawn*

INTRODUCTION

The indigenous proteinases that are involved in turnover and growth of muscle proteins contribute to the postmortem meat tenderization (Goll et al., 1992; Koohmaraie, 1992). Meat tenderness is found to be highly correlated with the proteolytic breakdown of myofibrillar proteins, disappearance of Z-lines, and disassembly of sarcomeric protein (Huang et al., 1998; Morton et al., 1999; Sargianos et al., 1996). According to Crouse and Koohmaraie (1990), postmortem beef became tender when calpastatin failed to regulate calpain hydrolytic activity. The calpain was mostly composed of 30 and 80 kDa subunits and could be classed as μ-calpain and *m*-calpain based on their calcium requirement (Murachi et al., 1981). From the study on yellowfin tuna (Watson et al., 1992), it is capable to hydrolyze isolated myofibrils at pH 7.5 and cause a significant loss of Z-disk integrity in skeletal muscle. Calcium-dependent calpains are, therefore, considered to be one of the major participating enzymes in meat tenderness (Koohmaraie, 1992). Postmortem proteolysis not only has a beneficial effect on meat tenderness but might also have an adverse effect on muscle softening of postmortem fish.

Calpastatin, a specific inhibitor of calpain, was ubiquitous in mammalian and avian cells and may play an important role in the intracellular Ca^{2+}-dependent regulation systems (Murachi, 1983). In general, there are two types of calpastatins, i.e., muscle (110 kDa) and erythrocyte (70 kDa) types, based on their molecular weights (M_r). The muscle calpastatin consisted of a nonhomologous sequence on the amino-terminal side (domain L) and four repetitive domains (domains 1−4) (Maki et al., 1988). These four repetitive sequence domains were identified as the functional units of proteolytic inhibitor (Maki et al., 1988; Kawasaki et al., 1989). The M_r of calpastatin from animal tissues varies with the species, from 22 to 180 kDa (Maki et al., 1991). Two calpastatins (78 and 37 kDa) have been detected from carp muscle (Yamada et al., 1985). These fragments of calapstatin suggested that the posttranslation proteolytic process by calpains occurred in carp muscle (Nakamura et al., 1989). Although many studies have been carried out on the calpastatin of red meat (Jiang, 1998), there is, thus far, only carp calpastatins reported among aquatic animals. The effect on calpain or its regulation by an endogenous inhibitor, calpastatin, on aquatic muscle tenderization or softness was still limited. The purpose of this study was to purify and characterize the calpastatin from grass prawn muscle.

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MATERIALS AND METHODS

Materials. Live grass prawn (*Penaeus monodon*) was obtained from a fish market in northern Taiwan (Keelung, Taiwan) and kept on ice water for about 30 min prior to use. After the heads, cuticle, hepatopancreas, and intestines were removed, the tail muscle was used for this study.

DEAE-Sephacel, Q-Sepharose, and Sepharyl S-200 resins were purchased from Pharmacia (Uppsala, Sweden). Papain, trypsin, β-chymotrypsin, cathepsin D, *N*α-benzoyl-L-arginine ethyl ester (BAEE), *N*α-benzoyl-L-tyrosine ethyl ester (BTEE), and β-mercaptoethanol (β-Me) were the product of Sigma Chemical Co. (St. Louis, MO). Purified beef μ- and *m*-calpains were obtained according to the procedures of Chang (1997). Acrylamide, bis(acrylamide), Coomassie brilliant blue G-250, and casein were obtained from Merck (Darmstadt, Germany). A dye-binding reagent to determine protein concentration was the product of Bio-Rad (Richmond, CA). Benzyloxycarbonylphenylalaninearginine-7-(4-methyl)coumarylamide (Z-Phe-Arg-MCA) was purchased from Peptide Institute Inc. (Osaka, Japan). Protein marker was the product of Life Technologies (Gaithersburg, MD). Other chemicals were of reagent grade.

Preparation of Prawn Meat Extract. Three hundred grams of prawn tail muscles were homogenized with 3 volumes of 50 mM Tris-HCl buffer, pH 8.3, containing 5 mM EDTA and 10 mM β-Me using a Waring blender subjoined with baffler for 3 min. After 1 h centrifugation at 100000*g*, the supernatant was filtrated through a filter paper (Toyo 5C) and its pH was adjusted to 7.5 with 1.0 M Tris-HCl buffer. The resulting sample was dialyzed against 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM NaN_3, 5 mM EDTA and 5 mM β-Me for 18 h and referred as crude calpastatin.

Determination of the Thermostability of Crude Calpastatin. The crude calpastatin in 50 mM Tris-HCl buffer (pH

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7.5) was incubated at 100 °C for 2−20 min in 2-min intervals and 25 and 30 min and then cooled immediately in ice water for 5 min. The remaining calpastatin activity against prawn meat calpain was then measured by using casein as a substrate in 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM β-Me, 5 mM $CaCl_2$, and 1.5 mM NaN_3 (Wang et al., 1993). The detail was described in the section of Determination of the Inhibitory Activity. The thermostability study for crude calpastatin from different batches of grass prawn was carried out three times.

Purification of a Calpastatin from Prawn Muscle Meat. The purification of a calpastatin from prawn muscle meat was carried out stepwise by heat treatment, DEAE-Sephacel chromatography, and then Q-Sephrose chromatography. The heat treatment of crude calpastatin was accomplished by 100 °C heating for 3 min, cooling in an ice water for 5 min immediately, and then removing the precipitates by centrifugation at 25000*g* for 3 min.

The crude heated calpastatin in 50 mM Tris-HCl buffer (pH 7.5) was then chromatographed on a DEAE-Sephacel column (2.6 × 10 cm) equilibrated with buffer A (20 mM Tris-HCl buffer, pH 7.5, containing 1 mM NaN_3, 5 mM EDTA and 10 mM β-Me). After the unabsorbed proteins were washed out using Buffer A, the calpastatin was eluted with a linear gradient of 0−0.7 M NaCl in buffer A. Five milliliter eluents were collected in each fraction at a flow rate of 0.5 mL per min.

Fractions containing calpastatin inhibitory activities on DEAE-Sephacel chromatography were pooled together and dialyzed against 20 mM sodium acetate buffer, pH 5.0 (buffer B), for 18 h. The resulting samples were loaded onto Q-Sepharose column (2.6 × 2.0 cm) equilibrated with buffer B. After washing out the unabsorbed proteins with buffer B, the calpastatin was eluted with a linear gradient of 0−0.7 M NaCl in buffer B. The flow rate was 0.5 mL/min and 2 mL/tube was collected. Fractions with calpastatin activity were collected and concentrated to minimal volume using an Amicon ultrafiltration with <10 000 cutoff membrane (Amicon Co., Danvers, MA). The purification procedures of calpastatin from different batches of prawn were carried out three times.

Determination of the Inhibitory Activity. The inhibitory activity of calpastatin against prawn meat calpain was analyzed by using casein (4 mg/mL) as a substrate in 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM β-Me, 5 mM $CaCl_2$, and 1.5 mM NaN_3 (Wang et al., 1993). The $CaCl_2$ was used to activate calpain and calpastatin activities. After 30-min incubation at 25 °C, the reaction was terminated by 0.6 mL of 10% trichloroacetic acid (TCA). The precipitates were removed by 15-min centrifugation at 4000*g*. The absorbance of supernatant (TCA-soluble peptides) at 278 nm (A_{278}) was determined by using a spectrophotometer (Hitachi U-2001, Japan). One unit of calpastatin inhibitory activity was defined as the amount of inhibitor that could inhibit 1 unit of prawn calpain activity. One unit of calpain activity was defined as the amount of proteinase that could hydrolyze caseins and obtain an A_{278} of 0.1 in the TCA-soluble peptides within 30-min reaction at 25 °C.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS−PAGE). To confirm the purity of prawn calpastatin and to determine its molecular weight, purified calpastatin in a dissociating buffer (62.5 mM Tris-HCl buffer, pH 8.3, containing 3% SDS, 5% β-Me, and 0.002% bromophenol blue) was heated at 100 °C for 5 min. The M_r of calpastatin was determined by 10% SDS−PAGE according to Laemmli (1970). Protein Ladders with M_r of 10 kDa (Life Technologies, Inc., Gaithersburg, MD) were used as protein marker.

Sephacryl S-200 Column Chromatography. To determine the molecular weight and whether calpastatin is a monomer in its native form, purified calpastatin solution (1 mg) was applied onto Sephacryl S-200 column (1.6 × 90 cm) which was equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 5 mM EDTA and 1 mM NaN_3. The proteins were then eluted with the same buffer at a flow rate of 0.5 mL/min. Five milliliters of eluents were collected. A high molecular weight calibration kit (myosin, 212 kDa; macroglobulin, 170 kDa; galactosidase, 116 kDa; transferrin, 78 kDa; and glutamic dedydrogenase, 53 kDa) (Pharmacia, Uppsala, Sweden) was used as protein marker.

Protein Concentration. Protein concentrations of calpastatin or purified μ- and *m*-calpain during purification were determined by a dye-binding method of Bradford (1976). Bovine serum albumin was used as a standard protein.

pH Stability. Purified calpastatin in 50 mM citric acid−Na_2HPO_4 (pH 3.5−4.5), Goods (pH 4.5−10.0), or phosphate (pH 11.0−11.9) buffers with different pH values was incubated at 30 °C. After 1 h incubation, an equal volume of 1.0 M sodium phosphate buffer (pH 6.5) was added to adjust the pH to 6.5. The remaining inhibitory activity was then measured according to Wang et al. (1993). The pH stability study for purified calpastatin from different batches of grass prawn was carried out three times.

Active Site Titration. Molar ratios of beef *m*-calpain (0.91 μmol/mL) and μ-calpain (0.68 μmol/mL) to the purified calpastatin were determined by the titration of both calpains against various concentration of prawn calpastatin. The casein was used as substrate and dissolved in 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM β-Me, 5 mM $CaCl_2$, and 1.5 mM NaN_3. Purified *m*-calpain and μ-calpain were obtained according to Jiang et al. (1998). The active site titration for purified calpastatin from different batches of grass prawn was carried out three times.

Inhibitory Effect of Purified Calpastatin against Different Proteinases. *Cysteine Proteinases.* The inhibitory effect of calpastatin on papain and cathepsin B was evaluated according to Lee et al. (2000). Purified calpastatin was preincubated with 45 μmol/mL papain or 50 μmol/mL cathepsin B in 0.2 M sodium phosphate buffer, pH 6.5, containing 4 mM EDTA and 4 mM cysteine for 5 min at 40 °C. Z-Phe-Arg-MCA (20 μM) in the same buffer was added to the reaction mixture in a final volume of 1.0 mL and then incubated for another 10 min. The reaction was terminated by adding 1 mL of a mixture of 100 mM sodium monochloroacetate, 30 mM sodium acetate, and 70 mM acetic acid (pH 4.3). The fluorescence of the reaction mixture was measured at an emission of 460 nm with an excitation of 350 nm using a fluorescence spectrophotometer (model F-2000, Hitachi, Japan).

Serine Proteinases. The inhibitory effect of calpastatin on trypsin, α- or β-chymotrypsins was determined according to the method of Jiang et al. (1991). The reaction mixture contained 5 μmol/mL trypsin, 20 μmol/mL α- or 10 μmol/mL β-chymotrypsins, appropriate amount of purified calpastatin in 1.0 mL of 50 mM sodium phosphate buffer (pH 7.6 for trypsin; pH 7.8 for chymotrypsins), 1 mM $CaCl_2$, and 10 μM substrate (BAEE for trypsin; BTEE for chymotrypsins). The absorbance at 253 nm for each reaction mixture was monitored continually at 25 °C by using a spectrophotometer.

Aspartic Proteinase. The inhibitory activity of calpastatin against 8 μmol/mL cathepsin D in 1.0 mL of 50 mM sodium citrate buffer (pH 3.0) was determined using acid-denatured hemoglobin as substrate (Makinodan and Ikeda, 1976). After 60 min incubation at 37 °C, 1.0 mL of 10% TCA was added to stop the reaction. TCA-soluble peptides were then measured according to the method of Lowry et al. (1951). The inhibitory effect study of purified calpastatin from different batches of grass prawn was carried out three times.

Statistical Analysis. All measurements were made in triplicate with two sample replication for a total of six measurements. Standard deviation for sample treatment was calculated with a spreadsheet program (Excel 97, Microsoft).

RESULTS AND DISCUSSION

Thermal Stability. The thermostable calpastatin has reported from several sources, including beef (Chang, 1997), carp (Toyohara et al., 1983), human erythrocyte (Takano and Murachi, 1982), and chicken muscles (Ishiura et al., 1982). Carp calpastatin activity displayed no significant decrease even after 5 min heating at 100 °C (Toyohara et al., 1983). Also Murachi (1983) indicated

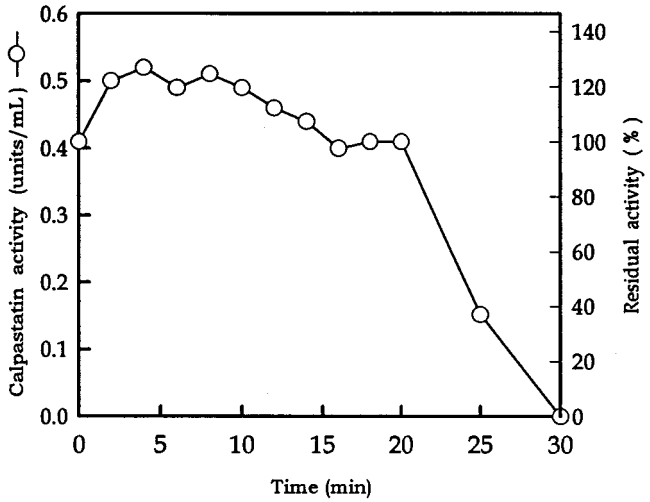

Figure 1. Thermostability of prawn calpastatin. The crude prawn calpastatin in 50 mM Tris-HCl buffer (pH 7.5) was incubated at 100 °C for 30 min.

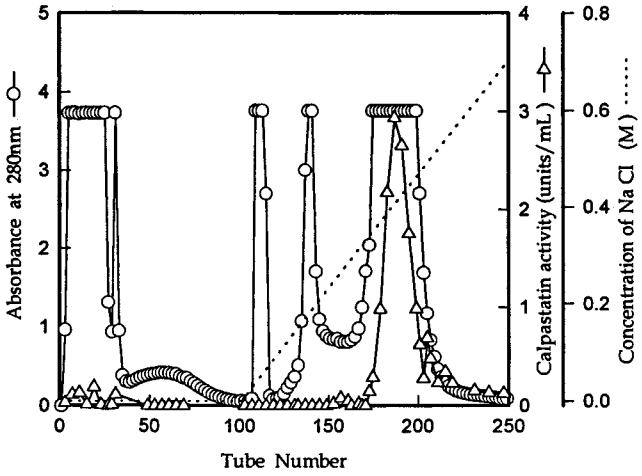

Figure 2. Chromatogram of prawn calpastatin on DEAE-Sephacel. The column was equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 10 mM β-Me, 5 mM EDTA, and 1 mM NaN_3, eluted with a linear gradient of 0−0.7 M NaCl in the same buffer.

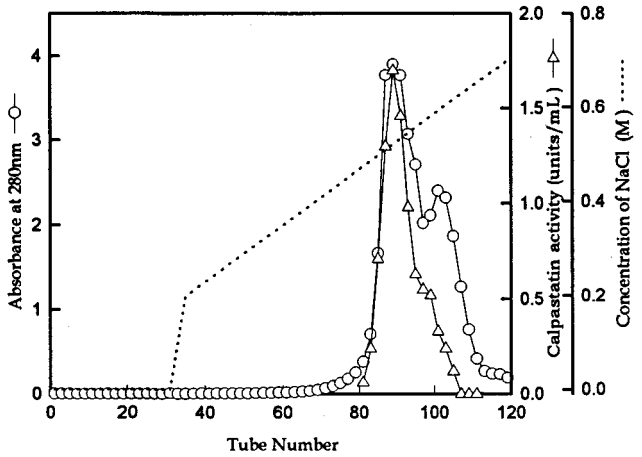

Figure 3. Chromatogram of prawn calpastain on Q-Sepharose. The column was equilibrated with 20 mM sodium acetate buffer, pH 5.0, and eluted with a linear gradient of 0−0.7 M NaCl in the same buffer.

that the calpastatin inhibitory activity in bovine heart muscle, rabbit liver, rat liver, or brain remained constant during 5−20 min heating at 100 °C. As indicated in Figure 1, the inhibitory activity of crude prawn calpastatin against calpain increased at earlier stage and then decreased to almost the same level as that before incubation during further heating at 100 °C up to 20 min. Increase in inhibitory activity during 3−12 min incubation might be due to the inactivation of the contaminant calpain, while slight decrease in inhibitory activity, compared with the highest level during heating, was because of the inactivation of calpain during further incubation. It was almost completely inactivated after 30 min incubation at 100 °C (Figure 1).

Purification of Calpastatin. Calpastatin in prawn crude extract exhibited a very thermal stable inhibitor (Figure 1). The heat treatment was, therefore, employed to precipitate the contaminant proteins during the purification of prawn calpastatin. After 3-min incubation at 100 °C, almost 70% of contaminant proteins were removed and more than 6-fold calpastatin activity was recovered (Table 1). This result suggested that the heat treatment not only could remove most of the heat-liable proteins but also inactivate the endogenous calpain, which can consequently simplify the purification procedures.

As shown in Figure 2, the calpastatin on DEAE-Sephacel chromatography was eluted at 0.4 M NaCl in 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM NaN_3, 5 mM EDTA, and 10 mM β-Me. It was further purified to electrophoretical homogeneity by Q-Sepharose at around 0.5 M NaCl in 20 mM sodium acetate buffer, pH 5.0 (Figures 3 and 4). Using the total activity after heat treatment as 100%, about 45% and 80% activity was lost during DEAE-Sephacel and Q-Sepharose chromatographs, respectively. About 247-fold of purification was achieved after Q-Sepharose chromatography, compared with that after heat treatment (Table 1).

Molecular Weight. The M_r of purified calpastatin was 80 kDa (Figure 4), which was similar to that from carp, 78 kDa (Yamada et al., 1985) estimated by SDS−PAGE. However, it was about 88.7 kDa when determined by Sephacryl S-200 column chromatography (Figure 5), suggesting the native form of prawn calpastatin was a monomer. According to Ishiura et al. (1982), calpastatin from chicken skeletal muscle was also a monomer with M_r of 68 kDa. However, that from carp was a tetramer with M_r of 300 kDa, determined by gel filtration chromatography (Toyohara et al., 1983). The M_r of calpastatin from various tissues ranged from 24 to 172 kDa in SDS−PAGE (Parkes, 1986). However,

Table 1. Summary of the Purification of Calpastatin from Grass Prawn Muscle

procedure	total protein, mg	total activity, units	specific activity (x 10^{-3}), units/mg	yield,[a] %	purification,[b] fold
crude extract	15961.0	24.7	1.6	100	1
heat treatment (100 °C/3 min)	4983.0	167.6	33.9	679 (100)[a]	22 (1)[b]
DEAE-Sephacel	12.5	90.6	7248.0	367 (54)	4530 (214)
Q-Sepharose	3.8	31.8	8368.4	129 (19)	5230 (247)

[a] Values in parentheses are the percentage ratios compared to the yield after heat treatment. [b] Values in parentheses are the percentage ratios compared to the purification fold after heat treatment.

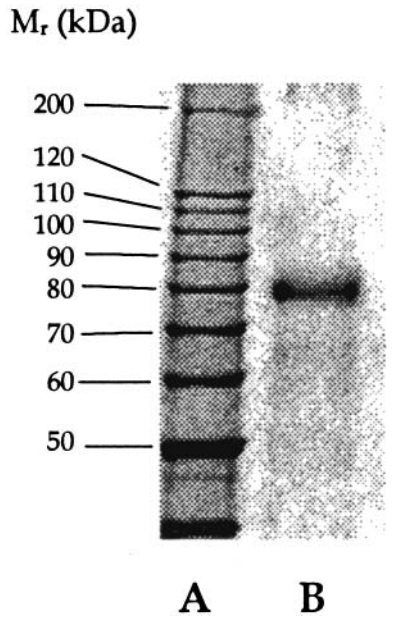

Figure 4. SDS−PAGE analysis of the purified calpastatin from grass prawn. Polyacrylamide concentration of stacking and resolving gels were 3.75 and 10.0%, respectively. Lane A is referred as a protein marker of Life Protein Ladder (10−200 kDa) and B as a purified calpastatin.

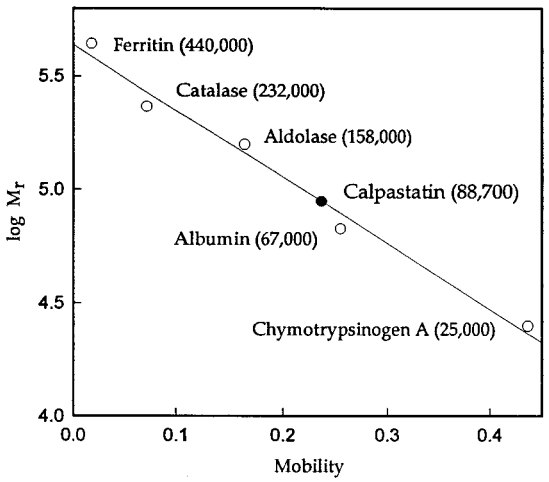

Figure 5. Calibration curve for the determination of native prawn calpastatin on gel filtration chromatography of Sephacryl S-200. The chromatography was eluted with 20 mM Tris-HCl buffer, pH 7.5, containing 5 mM EDTA and 1 mM NaN_3 and the flow rate was 0.5 mL/min. The standard proteins (i.e., Ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa; albumin, 67 kDa; chymotrypsinogen A, 25 kDa) were used to determine the molecular weight of native prawn calpastatin.

that estimated by SDS−PAGE was higher than that by the deduced primary sequence (Maki et al., 1990). The M_r of bovine skeletal muscle calpastatin was about 76 kDa calculated from amino acid residues (Killefer and Koohmaraie, 1994) and 70 kDa determined by Superose 12 HR gel filtration (Jiang et al., 1997).

pH Stability. The inhibitory activity of calpastatin was relatively stable at pH range from 4.5 to 10.0 during 1 h incubation at 30 °C. There was a dramatic reduction in calpastatin activity in citric acid−disodium phosphate buffer, pH 3.5. Similar phenomenon was also observed at pH 11−12 in phosphate buffer. The inhibitory activity was almost completely lost at pH 11.9 (Figure 6).

Active Site Titration. The purified calpastatin showed inhibitory activity toward purified beef μ- or

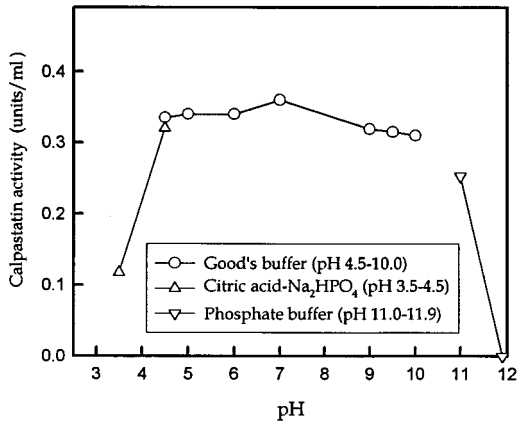

Figure 6. pH stability of the purified prawn calpastatin. The calpastatin was dialyzed against desired pH buffer (e.g., 50 mM citric acid−Na_2HPO_4 buffer for pH 3.5−4.5, 50 mM Good's buffer for pH 4.5−10.0, and 50 mM phosphate buffer for pH 11.0−11.9) and incubated at 30 °C for 1 h. The pHs of resulting samples were then adjusted to 7.5 by 1 M Tris-HCl buffer before determining the residual activity.

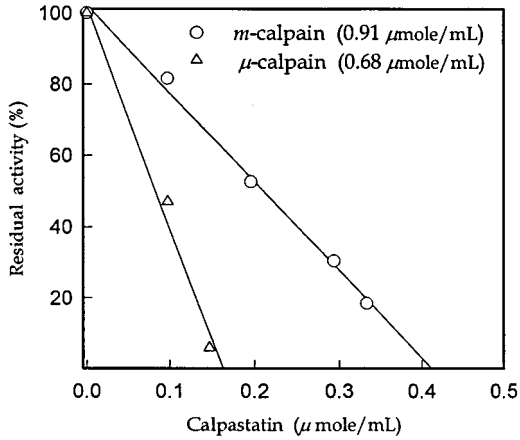

Figure 7. Active site titration of purified prawn calpastatin with beef μ- and m-calpains.

m-calpains while using casein as a substrate. The caseinolytic activities of both calpains reduced linearly with increase of calpastatin concentration (Figure 7). The amounts of purified calpastatin required to completely inhibit 0.91 μmol/mL m-calpain and 0.68 μmol/mL μ-calpain activity were calculated to be 0.41 and 0.16 μmol/mL, respectively (Figure 7). These data indicated that the purified prawn calpastatin contains two and four repetitive domains for beef m-calpain and μ-calpain, respectively. According to Imajoh et al. (1987a), 1 mol of rabbit liver calpastatin could interact with 4 mol of m-calpain. Although the repetitive domain against m-calpain between rabbit and prawn calpastatin is different, the stoichiometry of m- and μ-calpain inhibition indicated that the prawn calpastatin is a multidomain inhibitor. Calpastatin usually possesses three or four repetitive inhibitory domains (Imajoh et al., 1987b; Maki et al., 1987; Takano et al., 1988). Each domain may bind one calpain in the presence of calcium, but the inhibitory activity on the repetitive domains is not identical. Therefore, the stoichiometry of the enzyme−inhibitor complex is little consensus. A ratio of 1−9 molecules of calpain to one calpastatin had been reported (Cottin et al., 1983; DeMartino and Croall, 1984; Imajoh et al., 1984; Lepley et al., 1985; Nakamura et al., 1984). The result from the molar ratio of prawn

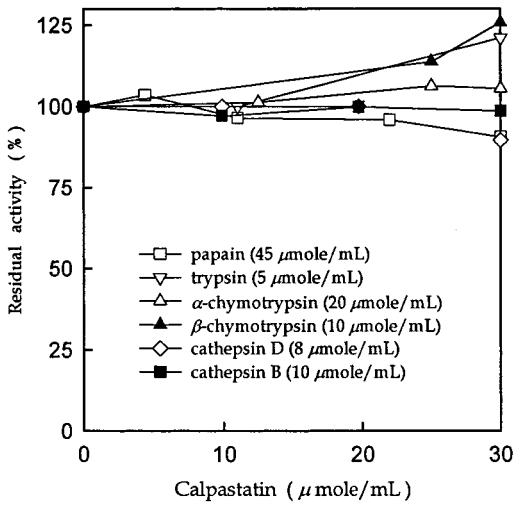

Figure 8. Inhibitory specificity of purified prawn calpastatin against various proteinases (e.g., papain, 45 μmol/mL; cathepsin B, 50 μmol/mL; trypsin, 5 μmol/mL; α-chymotrypsin, 20 μmol/mL; and β-chymotrypsin, 10 μmol/mL; and cathepsin D, 8 μmol/mL). The remaining proteinase activities were determined as described in Materials and Methods.

calpastatin to beef calpains in this study also supports this viewpoint.

Inhibitory Specificity. From this study, calpastatin revealed a specific inhibitory activity against beef *m*- or μ-calpain (calcium-dependent papain-like proteinases). No inhibitory activity against other cysteine proteinases such as papain and cathepsin B, serine proteinases such as trypsin, α-, and β-chymotrypsins, and aspartic proteinases such as cathepsin D was observed (Figure 8). Pig and chicken calpastatins also were found to be calpain-specific (Ishiura et al., 1982; Takano et al., 1988). Although calpain belongs to cysteine proteinase superfamily with homologous center motif of other cysteine proteinases (Berti and Storer, 1995; Johnson and Guttmann, 1997), calpastatin bears calpain-specific inhibitory activity and has no influence on papain and cathepsin B activities. On the other hand, calpastatin has no any sequence similar to kininogen (known as a cysteine proteinase inhibitor) which can also inhibit calpain activity (Maki et al., 1991). This unique property implies that calpastatin is a different cysteine proteinase inhibitor the others of the cystatin superfamily.

In summary, the purified calpastatin from grass prawn muscle was very heat stable and the pH stability ranged from 4.5 to 10. It exists as monomer with M_r of 80−89 kDa and was also a specific inhibitor for calpains. From the data obtained, the prawn calpastatin might be a good candidate to control the tenderization or softening of postmortem fish.

LITERATURE CITED

Bradford, M. M. A rapid and sensitive method for the quantition of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

Berti, P. J.; Storer, A. C. Alignment/phylogeny of the papain superfamily of cysteine proteases. *J. Mol. Biol.* **1995**, *246*, 273−283.

Chang, T. A Stepwise separation and continuous activity measurement for μ- and *m*-calpains and calpastatin. Master dissertation, National Taiwan Ocean University, Keelung, 1997, pp 34−43, 84−92.

Cottin, P.; Vidalenc, P. L.; Merdaci, N.; Ducastaing, A. Evidence for noncompetitive inhibition between two calcium-dependent activated neutral proteinases and their specific inhibitor. *Biochim. Biophys. Acta* **1983**, *743*, 299−302.

Crouse, J. D.; Koohmaraie, M. Effect of freezing of beef on subsequent postmortem aging and shear force. *J. Food Sci.* **1990**, *55*, 573−574.

DeMartino, G. N.; Croall, D. E. Purification and characterization of a protein inhibitor of calcium-dependent proteases from rat liver. *Arch. Biochem. Biophys.* **1984**, *232*, 713−720.

Goll, D. E.; Thompson, V. F.; Taylor, R. G.; Christiansen, J. A. Role of the calpain system in muscle growth. *Biochimie* **1992**, *74*, 225−237.

Huang, J.; Forsberg, N. E. Role of calpain in skeletal-muscle protein degradation. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 12100−12105.

Imajoh, S.; Kawasaki, H.; Kisaragi, M.; Mukai, M.; Sugita, H.; Suzuki, K. A 107-kDa inhibitor for calcium-activated neutral proteinase (CANP): purification from the human liver. *Biomed. Res.* **1984**, *5*, 481−488.

Imajoh, S.; Kawasaki, H.; Emori, Y.; Ishiura, S.; Minami, Y.; Sugita, H.; Imahori, K.; Suzuki, K. A fragment of an endogenous inhibitor produced in *Escherichia coli* for calcium-activated neutral protease (CANP) retains an inhibitory activity. *FEBS Lett.* **1987a**, *215*, 274−278.

Imajoh, S.; Kawasaki, H.; Emori, Y.; Suzuki, K. Calcium-activated neutral protease inhibitor from rabbit erythrocytes lacks the N-terminal region of the liver inhibitor but retains three inhibitory units. *Biochem. Biophys. Res. Commun.* **1987b**, *146*, 630−637.

Ishiura, S.; Tsuji, S.; Murofushi, H.; Suzuki, K. Purification of an endogenous 68000-dalton inhibitor of Ca^{2+}-activated neutral protease from chicken skeletal muscle. *Biochim. Biophys. Acta* **1982**, *701*, 216−223.

Jiang, S. T. Contribution of Muscle Proteinases to Meat Tenderization (a review). *Proc. Natl. Sci. Counc., Part B: Life Sci.* **1998**, *22*, 97−107.

Jiang, S. T.; Moody, M. W.; Chen, H. C. Purification and characterization of proteases from digestive tract of grass prawn (*Penaeus monodon*). *J. Food Sci.* **1991**, *56*, 322−326.

Jiang S. T.; Wang, J. H.; Chang, T.; Chen, C. S. A continuous method for measuring calpain activity. *Anal. Biochem.* **1997**, *244*, 233−238.

Jiang, S. T.; Ho, M. L.; Chang, T.; Kurth, L. B. Effects of elution conditions on the separation of calpastatin, μ- and *m*-calpain on DEAE-Sephacel chromatography. *Biosci., Biotechnol., Biochem.* **1998**, *62*, 2270−2272.

Johnson, G. V.; Guttmann, R. P. Calpains: intact and active? *Bioessays* **1997**, *19*, 1011−1018.

Kawasaki, H.; Emori, Y.; Imajoh, S.; Minami, Y.; Suzuki, K. Identification and characterization of inhibitory sequences in four repeating domains of the endogenous inhibitor for calcium-dependent protease. *J. Biochem.* **1989**, *106*, 274−281.

Killefer, J.; Koohmaraie, M. Bovine skeletal muscle calpastatin: cloning, sequence analysis, and steady-state mRNA expression. *J. Anim. Sci.* **1994**, *72*, 606−614.

Koohmaraie, M. The role of Ca^{2+}-dependent protease (calpains) in post mortem proteolysis and meat tenderness. *Biochimie* **1992**, *74*, 239−245.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature* **1970**, *277*, 680−685.

Lee, J. J.; Tzeng, S. S.; Jiang, S. T. Purification and characterization of low molecular weight kininogen from pig plasma. *J. Food Sci.* **2000**, *65*, 81−86.

Lepley, R. A.; Pampusch, M.; Dayton, W. R. Purification of a high-molecular-weight inhibitor of the calcium-activated proteinase. *Biochim. Biophys. Acta* **1985**, *828*, 95−103.

Lowry, H.; Rosebrough, N. J.; Farr, A. L.; Randall, J. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

Maki, M.; Takano, E.; Mori, H.; Kannagi, R.; Murachi, T.; Hatanqka, M. Repetitive region of calpastatin is a functional

unit of the proteinase inhibitor. *Biochem. Biophys. Res. Commun.* **1987**, *143*, 300−308.

Maki, M.; Takano, E.; Osawa, T.; Ooi, T.; Murachi, T.; Hatanaka, M. Analysis of structure−function relationship of pig calpastatin by expression of mutated cDNAs in *Escherichia coli*. *J. Biol. Chem.* **1988**, *263*, 10254−10261.

Maki, M.; Hatanaka, M.; Takano, E.; Murachi, T. Structure−function relationships of calpastatin. In *Intracellular Calcium-Dependent Proteolysis*; Mellgren, R. L., Murachi, T., Eds.; CRC Press: Boca Raton, FL, 1990; Chapter 3, pp 37−54.

Maki, M.; Ma, H.; Takano, E.; Adachi, Y.; Lee, W. J.; Hatanaka, M.; Murachi, T. Calpastatins: Biochemical and molecular biological studies. *Biomed. Biochim. Acta* **1991**, *50*, 509−516.

Makinodan, Y.; Ikeda, S. Studies on fish muscle proteinases VI: separation of carp muscle cathepsins A and D, and some properties of carp muscle cathepsin A. *Bull. Jpn. Soc. Sci. Fish.* **1976**, *42*, 239−247.

Morton, J. D.; Bickerstaffe, R.; Kent, M. P.; Dransfield, E.; Keeley, G. M. Calpain-calpastatin and toughness in *M. longissimus* from electrically stimulated lamb and beef carcasses. *Meat Sci.* **1999**, *52*, 71−79.

Murachi, T. Calpain and calpastatin. *Trends Biochem. Sci.* **1983**, *8*, 167−169.

Murachi, T.; Tanaka, K.; Hatanaka, M.; Murakami, T. Intracellular Ca-dependent protease (calpain) and its high molecular weight endogenous inhibitor (calpastatin). *Adv. Enzyme Regul.* **1981**, *19*, 407−424.

Nakamura, M.; Inomata, M.; Hayashi, M.; Imahori, K.; Kawashima, S. Purification and characterization of an inhibitor of calcium-activated neutral protease from rabbit skeletal muscle: purification of 50,000-dalton inhibitor. *J. Biochem.* **1984**, *96*, 1399−1407.

Nakamura, M.; Inomata, M.; Imajoh, S.; Suzuki, K.; Kawashima, S. Fragmentation of an endogenous inhibitor upon complex formation with high and low-calcium requiring forms of calcium-activated neutral proteases. *Biochemistry* **1989**, *28*, 449−455.

Parkes, C. Calpastatins. In: *Proteinase Inhibitors*; Barrett, A. J., Salvesen, G., Eds.; Elsevier: Amsterdam, 1986; Chapter 19.

Sargianos, N.; Gaitanaki, C.; Dimitriadis, B.; Beis, I. Proteolytic degradation of isolated myofibrils and myofibrillar proteins by *m*-calpain from the skeletal muscle of the amphibian *Rana ridibunda*. *J. Exp. Zool.* **1996**, *276*, 30−42.

Takano, E.; Murachi, T. Purification and some properties of human erythrocyte calpastatin. *J. Biochem.* **1982**, *92*, 2021−2028.

Takano, E.; Kaki, M.; Mori, H.; Hatanaka, M.; Marti, T.; Titani, K.; Kannagi, R.; Ooi, T.; Murachi, T. Pig heart calpastatin: identification of repetitive domain structure and anomalous behavior in polyacrylamide gel electrophoresis. *Biochemistry* **1988**, *27*, 1964−1972.

Toyohara, H.; Makinodan, T.; Ikeda, S. Detection of calpastatin and a trypsin inhibitor in carp muscle. *Agric. Biol. Chem.* **1983**, *47*, 1151−1154.

Wang, J. H.; Ma, W. C.; Su, J. C.; Chen, C. S.; Jiang, S. T. Comparison of the properties of *m*-calpain from tilapia and grass prawn muscles. *J. Agric. Food Chem.* **1993**, *41*, 1379−1384.

Watson, C. L.; Morrow, H. A.; Brill, R. W. Proteolysis of skeletal muscle in yellow fin tuna (*Thunnus albacares*): Evidence of calpain activation. *Comp. Biochem. Physiol.-B: Comp Biochem.* **1992**, *103*, 881−887.

Yamada, Y.; Sakamoto, S.-I.; Seki, N. Two calpain inhibiting proteins (calpastatin) in carp skeletal muscle. *Bull. Jpn. Soc. Sci. Fish.* **1985**, *51*, 1021−1028.

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