[Jpn. J. Parasitol., Vol. 45, No. 3, 185–191, June, 1996]

Cleavage of Muscle Structural Proteins with Cysteinyl Protease from *Spirometra erinaceieuropaei* Plerocercoid

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(Accepted April 30, 1996)

Abstract

Enzyme activity has been observed in the plerocercoid of *spirometra erinaceieuropaei*, the causative parasite of human sparganosis, that cleaves the actin of the worm itself. The protease present in 0.1 M NaCl extracts from acetone-treated larvae was partially purified and concentrated by ammonium sulfate fractionation. The resulting protease rapidly cleaved molecules of native actin and myosin from vertebrates, which are muscle structural proteins, at neutral pH. In contrast to this phenomenon, the protease was unable to cleave albumin and globulin, two serum components. The actin cleavage reaction was completely inhibited by the presence of leupeptin. It was, however, not inhibited by pepstatin, phenylmethanesulfonyl fluoride or EDTA. Based on these findings, cleavage of native muscle structural proteins were considered to be the result of action by enzyme resembling cysteinyl protease.

Key words: *Spirometra*; cestode; protease; cysteinyl protease; actin; myosin.

Introduction

When the plerocercoid of *Spirometrid* cestodes is ingested into a host, the body casts off the scolex, which measures several millimeters, in the intestine of the host. In the case the host is a definitive host, the scolex grows into an adult. If the host is a paratenic host, the scolex penetrates the intestinal wall of the host and forms a sparganum in host tissue (Mueller, 1974). Thus, it is suspected that this plerocercoid most likely possesses enzymes that dissolve its own structural proteins as well as those of its host. Although previous reports have described the protease of *Spirometra erinaceieuropaei* (Kwa, 1972; Fukase *et al.*, 1985, Song and Chappell, 1993), the studies described in these reports do not provide an adequate discussion of the physiological function of that protease.

We attempted to isolate actin from plerocercoid in a study of the muscle contraction of *S. erinaceieuropaei* plerocercoid. However, the actin rapidly disappeared from the extract. Native muscle proteins, which were not denatured by exposure to acid or high temperature, are generally considered to be resistant to the action of digestive enzymes (Obinata *et al.*, 1981). We therefore surmised that protease is present in plerocercoid homogenate which is able to cleave native actin. Actin is an important protein that composes the cytoskeleton of various organisms (Pollard and Weihing, 1974). This paper describes an extraction method for protease from *S. erinaceieuropaei* plerocercoid and observation of its degradative action against molecules of native muscle structural proteins.

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Materials and Methods

Plerocercoids

Plerocercoids collected from *Elaphe quadrivirgata* or maintained in mouse were adequately washed with 0.15 M NaCl. After momentarily rinsing with distilled water, the worms were treated 3 times with 10–20 volumes of chilled acetone for 20–

30 min each time. They were then dried at room temperature. The dried worms were stored at –20°C until use.

Protease extraction

1 g of acetone-treated, dried worms were crushed into a powder using a porcelain mortar. The worm powder was homogenized five times for 5 sec each using a Polytron (Kinematica Co., Switzerland) in 20 ml of 10 mM Tris-HCl buffer (pH 7.6) containing 0.1 M NaCl, 1 mM dithiothreitol (DTT) and 1 mM EDTA. After extracting the homogenate overnight at 0°C, it was centrifuged at 170,000×g for 60 min. The protease was concentrated and partially purified by ammonium sulfate fractionation of the supernatant at 35–70% saturation. After adequately dialyzing the enzyme against 1 mM $NaHCO_3$ (pH 7.0), denatured protein was removed by centrifugation at 260,000×g for 60 min. As a result, approximately 3 g of acetone-treated, dried worm were obtained from 10 g of worm (wet volume), and approximately 20–30 mg of protein were obtained from 1 g of dry worm. A primary band corresponding to a molecular weight of about 23k was observed by electrophoresis for this enzyme fraction (Fig. 1).

Substrate protein and protein concentration:

Actin was obtained using the method of Pardee and Spudich (1982) from acetone-treated powder of rabbit skeletal muscle. Myosin was prepared according to the method of Kielley and Bradley (1965) from chicken skeletal muscle. Bovine albumin and globulin (Sigma Chemical Co., U.S.A.) were used as serum proteins. Protein concentration was determined by using a slightly modified reagent (Yamaguchi and Sekine, 1966) of the biuret reaction (Weichselbaum, 1946).

Reaction of protease with substrate protein

1 mg/ml of substrate protein and 10–20 μg/ml of enzyme were reacted in 5 mM Tris-maleate buffer (pH 7.0). In order to allow each substrate to be present in the reaction solution as monomer, 0.5 M NaCl was added to myosin, 0.15 M NaCl was added to globulin and albumin and nothing was added to the actin. After an adequate time had past at 37°C, the reaction was stopped by adding 1% sodium laulylsulfate (SDS) and 50 mM DTT to the reaction solution and heating for 1–2 min in a boiling water bath. The cleavage patterns of the substrate proteins were observed by electrophoresis. Dependency on pH was measured by performing the reaction with 10 mM phosphate-citrate buffer at each pH and then stopping with 5% perchloric acid 20 min later. The supernatant of the reaction solution obtained by centrifuging at 600×g for 5 min was then analyzed with a spectrophotometer (Hitachi 228A, Tokyo, Japan) for actin at an absorbance of 280 nm and for azocoll at an absorbance of 520 nm.

Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed using the method of Laemmli and Favre (1973), and the method of Fairbanks *et al.* (1971) with slight modifications (Nakamura *et al.*, 1979) when the sample contained myosin, a high molecular weight protein.

Results

Autolysis of G-actin from plerocercoid

A crude G-actin was extracted from plerocercoid. Acetone-treated worms were homogenized in 0.2 mM ATP (pH 7.0) both in the presence and absence of 100 μM leupeptin. Low molecular weight substances were then removed by dialysis against the same solution. SDS-gel electrophoresis of the supernatant obtained by centrifugation at 260,000×g for 60 min is shown in Fig. 1.

A band exhibiting a molecular weight of 42k that corresponded to actin was observed in protein extracted in the presence of leupeptin. This 42 kDa band was virtually undetected following actin extraction in the absence of leupeptin. Thus, it was suggested that protease is present in this actin extract that is able to digest actin of the worm itself but is able to be inhibited by leupeptin.

Reaction of protease against G-actin from rabbit skeletal muscle

Partially purified enzyme was reacted in purified actin from skeletal muscle. Fig. 2 shows the gel electrophoresis pattern after each reaction time following addition of 1/100 volume of that enzyme to actin. The actin was cleaved into a peptide having a molecular weight of approximately 35k and that

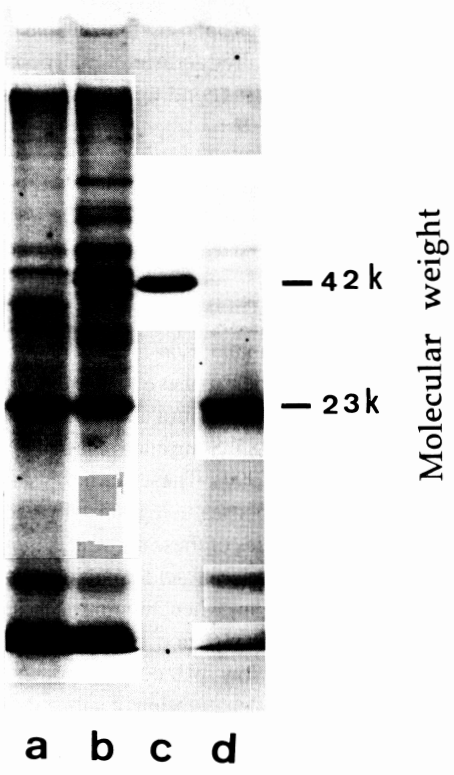

Fig. 1 SDS-polyacrylamide gel electrophoresis of crude extract of actin and protease fraction. Actin was extracted with 2 ml of 0.2 mM ATP and 2 mM Tris-maleate buffer (pH 7) in the (a) absence or (b) presence of leupeptin, from 0.1 g of worm powder. (c) Rabbit skeletal actin, (d) partial purified protease fraction from *S. erinaceieuropaei*. Proteins were applied to 20% acrylamide gel.

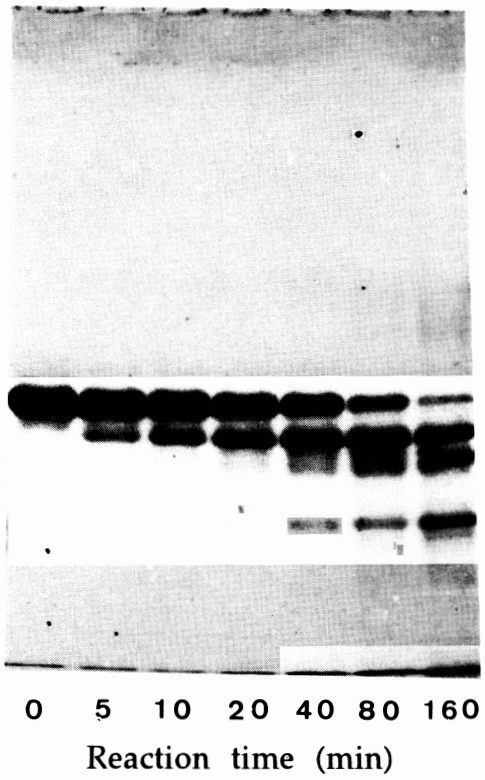

Fig. 2 SDS-polyacrylamide gel electrophoresis of rabbit skeletal actin reacted with protease from *S. erinaceieuropaei*. 1 mg/ml of actin containing 5 μg/ml of protease was incubated for adequate time at 37°C. Samples were applied to 15% acrylamide gel.

having a smaller molecular weight as time elapsed.

The effects of protease inhibitor and activator on the reaction were investigated to determine the properties of this protease (Fig. 3). Actin cleavage was completely inhibited by leupeptin at 10 μM. Although DTT, a sulfhydryl residue reducing reagent, had no effect, EDTA slightly activated protease activity. Pepstatin and phenylmethanesulfonyl fluoride (PMSF) had no effect whatsoever. In addition, in the case of performing chemical modification by alkylating agent and mercury reagent, having action that blocks sulfhydryl residue, enzyme activity decreased significantly, although not completely as in the case of leupeptin (data not shown).

Reaction of protease against native muscle and serum proteins from vertebrate

Next, Fig. 4 shows an investigation of the specificity of this protease with respect to various native protein substrates. Plerocercoid protease exhibited cleaving action against myosin, which together with actin is a major muscle protein. Myosin heavy chain having a molecular weight of 200k was cleaved into low molecular weight peptide by reacting with the protease at 37°C for 30 min. In contrast to the reactivity with these muscle proteins, the protease did not exhibit any reactivity with the major serum proteins of albumin and globulin.

Dependence of pH on protease activity

Previously, we found a high level of protease

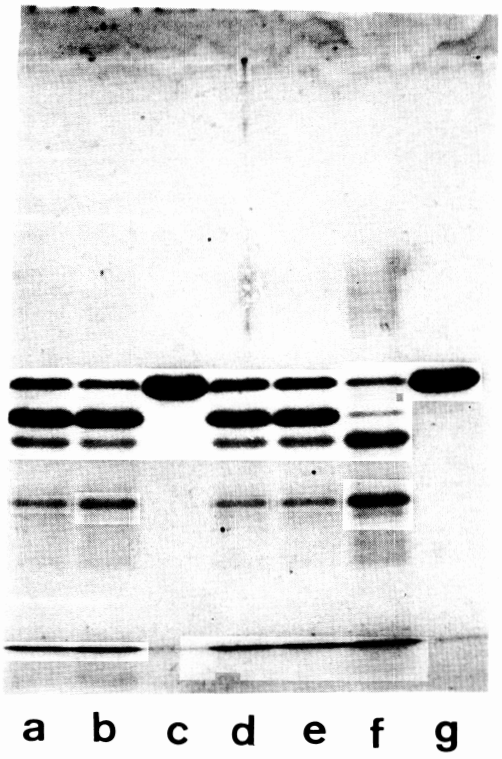

Fig. 3 Effect of various reagents on the actin cleavage reaction. The reaction mixture of 1 mg/ml of actin and 10 μg/ml protease containing (a) none reagent (control), (b) 10 mM DTT, (c) 10 μM leupeptin, (d) 10 μM pepstatin, (e) 10 μM PMSF and (f) 1 mM EDTA were incubated at 37°C for 20 min. (g) After heating, solution containing protease and 1% SDS was incubated with actin as a negative control. SDS electrophoresis was carried out as the same condition as Fig. 2.

activity against azocoll, a form of denatured collagen, in plerocercoid homogenate under acidic pH conditions (Nakamura and Yanagisawa, 1982). Therefore, the pH dependency of this enzyme with respect to actin degradation activity was measured. Fig. 5 shows the amounts of perchloric acid soluble peptide detected in centrifuged supernatant following reaction between actin or azocoll substrate and enzyme. Similar to azocoll, actin was strongly decomposed under acidic conditions. Maximum activity was observed at pH 5. Under acidic conditions of pH 5.5 and below, however, actin exhibited aggregation and irreversibly denatured. Thus, it was indicated that protease from plerocercoid of *S. erinaceieuropaei* has properties that enable it to cleave native and denatured actin into small peptides under a broad range of pH conditions from acidic to neutral pH.

Discussion

Following the initial study on proteolytic enzymes of *S. erinaceieuropaei* plerocercoid by Kwa (1972), the extraction of proteinase from tissue invasive *Pseudophyllidea* larvae along with the determination of its properties have been reported by several researchers (Nakamura and Yanagisawa, 1982; Fukase *et al.*, 1985; Song and Chappell, 1993; Polzer and Conradt, 1994). These studies involved determination of properties using non-physiological substrates. Examples of these substrates include synthetic substrate, gelatin, acid-soluble collagen and so forth. In addition, when attempting to assay enzyme activity, a long period of incubation for several hours under low pH conditions has the possibility of causing denaturation of substrate protein. We found an acidic protease in *S. erinaceieuropaei* plerocercoid that decomposes azocoll (Nakamura and Yanagisawa, 1982). However, azocoll is also a denatured collagen that conjugates with dye. These studies are insufficient in providing a theory of the physiological functions of the protease possessed by the plerocercoids of *S. erinaceieuropaei*.

We attempted to extract G-actin from acetone-treated plerocercoid using a low concentration of ATP. The 42 kDa band corresponding to actin on electrophoresis was completely absent in an extract to which leupeptin was not added (Fig. 1). The worm extract contains protease that is able to dissolve actin of the worm itself. Actin is a major structural protein that composes muscle filament and cytoskeleton (Pollard and Weihing, 1974; Pollard and Cooper, 1986). Native actin is resistant to the action of protease (Obinata *et al.*, 1981).

The reactivity of a protease fraction having for its main component an approximately 23 kDa protein was observed with respect to purified actin obtained from the skeletal muscle of rabbit serving as the host (Fig. 2). The actin was cleaved into small peptides with the passage of time. This protease cleaved actin

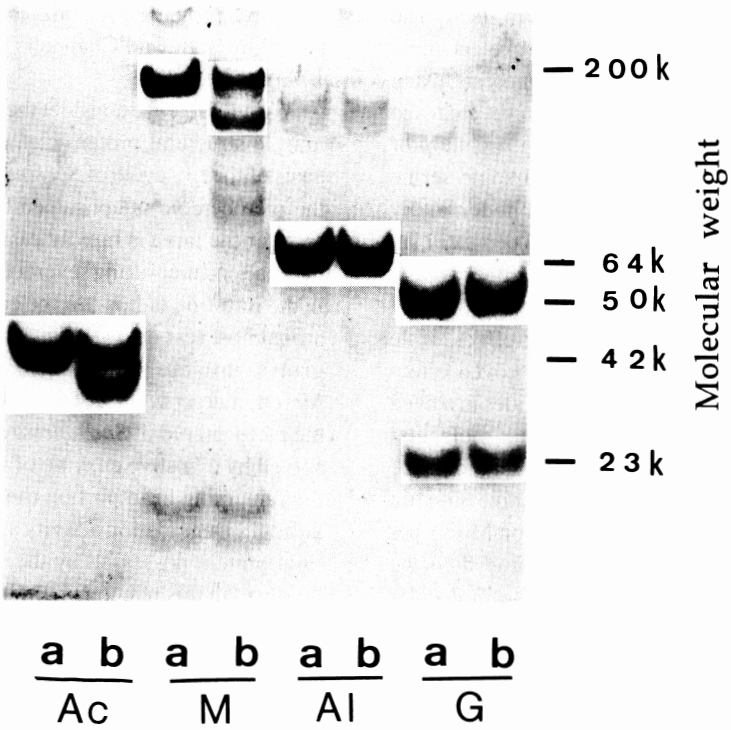

Fig. 4 Reaction of protease against muscle protein and serum proteins. (Ac) Rabbit skeletal actin (having molecular weight of 42k), (M) Chicken skeletal myosin (200K), (Al) bovine serum albumin (64k) or (G) bovine serum γ-globulin (heavy chain; 50k and light chain; 23k) was reacted with protease at 37°C for (a) 0 min or (b) 30 min. About 10 μg of each protein was applied to 5.6% polyacrylamide gel.

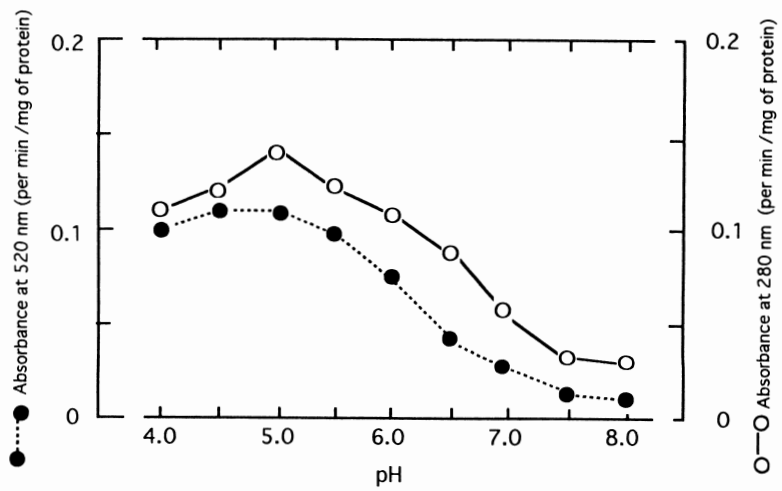

Fig. 5 Dependence of pH on protease activity against actin and azocoll. 0.1 mg/ml of protease was reacted with buffer solution containing 2 mg/ml of substrate at 37°C. For details, see Materials and Methods.

of the host as well as that of the worm itself. The properties of this protease against host skeletal muscle actin were observed by electrophoresis using typical protease inhibitors (Fig. 3). Actin cleavage was completely inhibited by leupeptin, an inhibitor of numerous cysteinyl proteases and some serine proteases. The protease was not inhibited at all by PMSF, a serine protease inhibitor, or pepstatin, an acid protease inhibitor. These results suggested that actin cleavage takes place due to the action of a thiol protease. Since addition of DTT had no effect, while sulfhydryl residue blocking reagent proved effective, it is assumed that sulfhydryl residues involved in protease activity maintain a stable molecular structure. This protease was also observed to be activated by addition of EDTA. Native G-actin binds a single divalent cation (Ca^{2+} or Mg^{2+}) per mole (Obinata *et al.*, 1981). Actin in which the cation has been chelated undergoes a molecular conformation change (denaturing) (Nagy, 1972). Activation resulting from addition of chelating agent to the reaction is considered to be the result of the effect on the substrate.

Cleavage of native serum protein and muscle structural protein were also observed (Fig. 4). The heavy chain of myosin, an important muscle structural protein together with actin, was cleaved into lower molecular weight peptides. In contrast, heavy and light chains of globulin as well as albumin were not changed by this protease. Myosin is a protein that has a complex structure consisting of six subunits and large molecular size of 480 kDa (Obinata *et al.*, 1981). The reactivity of protease against myosin has been reported in considerable detail by numerous researchers. Myosin is cleaved at specific regions by trypsin (Yagi and Yazawa, 1966), papain (Cooke, 1972), cathepsin B, D and L (Schwartz and Bird, 1977; Okitani *et al.*, 1980) and calpain (Hara *et al.*, 1983). Studies on the region of the myosin molecule that is cleaved by this enzyme are currently in progress.

Measurement of the pH dependency of actin cleavage activity revealed that maximum cleavage activity is exhibited in the weakly acidic range similar to when azocoll, a type of denatured collagen, is used for the substrate, and activity was observed over a broad pH range (Fig. 5). This enzyme is therefore considered to resemble the cysteinyl protease from *Spirometra mansoni* reported by Song and Chappell (Song and Chappell, 1993).

We attempted to consider the physioloical role of muscle structural protein cleavage activity on the basis of the life cycle of *S. erinaceieuropaei*. When the plerocercoid is consumed by a vertebrate, the body of the larva is rapidly cast off leaving behind the head end measuring several millimeters in length in the intestine of host. In the case the vertebrate is a final host (cat or dog), the scolex below the head grows into an adult form (Mueller, 1974). Marchiondo *et al.* (1987) reported that the body of the plerocercoid of *Spirometra mansonoides* is dissolved by digestive enzyme of the final host, while the remaining head portion most likely grows into an adult. Degradation activity against muscle structural protein possessed by the worm itself may be involved in this phenomenon. In the case the vertebrate is a reservoir host (frog, snake, bird, mouse, etc.), the head of the plerocercoid penetrates the smooth muscle of the host's intestine, passes through the body cavity and migrates into subcutaneous tissue, muscle and other organs. In the case of a human host, this phenomenon is known as "human sparganosis" (Mueller, 1974). Song and Chappel (1993) observed the presence of cysteinyl protease activity in the excretory-secretory products of *S. mansoni*. If the protease from plerocercoid of *S. erinaceieuropaei* described in this paper is also secreted, then the structural protein degradation action of this protease may also have a physiological role for migration into the host.

Additional experimentation on purification, localization using antibody and the regulatory mechanism of expression of activity is currently underway to verify these hypotheses.

References

1) Cooke, R. (1972): A new method for producing myosin subfragment-1. Biochem. Biophys. Res. Commun., 49, 1021–1028.
2) Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971): Electrophoretic analysis of the major polypeptide of human erythrocyte membrane. Biochemistry, 102, 2606–2617.
3) Fukase, T., Matsuda, Y., Akihama, S. and Itagaki, H. (1985): Purification and some properties of cysteine

protease of *Spirometra erinacei* plerocercoid (Cestode; Diphyllobothriiae). Jpn. J. Parasitol., 35, 351–360.
4) Hara, K., Ichihara, Y. and Takahashi, K. (1983): Purification and characterization of a calcium-activated neutral protease from monkey cardiac muscle. J. Biochem., 93, 1435–1445.
5) Kielley, W. W. and Bradley, L. B. (1965): The relationship between sulfhydryl groups and the activation of myosin adenosinetriphosphatase. J. Biol. Chem., 218, 653–659.
6) Kwa, B. H. (1972): Studies on the sparganum of *Spirometra erinacei*-II. Proteolytic enzyme(s) in the scolex. Int. J. Parasitol., 2, 29–33.
7) Laemmli, U. K. and Favre, M. (1973): Maturation of the head of bacteriophage T4. I. DNA packaging events. J. Mol. Biol., 80, 575–599.
8) Marchiondo, A. A., Weinstain, P. P. and Mueller, J. F. (1987): *In vitro* and *in vivo* enzymatic dissolution of the plerocercoid body of *Spirometra mansonoides*. Int. J. Parasitol., 17, 1313–1320.
9) Mueller, J. F. (1974): The biology of *Spirometra*. J. Parasitol., 60, 3–14.
10) Nagy, B. (1972): Difference spectral method for determination of native G-actin concentration. Anal. Biochem., 47, 371–377.
11) Nakamura, T., Yamaguchi, M. and Yanagisawa, T. (1979): Comparative studies on actins from various sources; Fragments of actin from *Ascaris* muscle cleaved at cysteinyl residues in comparison with those of other actins. J. Biochem., 85, 627–631.
12) Nakamura, T. and Yanagisawa, T. (1982): Activity of acid thiol proteinase from *Diphyllobothrium erinasei* plerocercoid [II]. Jpn. J. Parasitol., 31 (Suppl.), 36.
13) Obinata, T., Maruyama, K., Sugita, H., Kohama, K. and Ebashi, S. (1981): Dynamic aspects of structural proteins in vertebrate skeletal muscle. Muscle Nerve, 4, 456–488.
14) Okitani, T., Matsukura, U., Kato, H. and Fujimaki, M. (1980): Purification and some properties of myofibrillar protein-degrading protease, cathepsin L, from rabbit skeletal muscle. J. Biochem., 87, 1133–1143.
15) Pardee, J. D. and Spudich, J. A. (1982): Purification of muscle actin. Methods Cell Biol., 24, 271–289.
16) Pollard, T. D. and Cooper, J. A. (1986): Actin and actin-binding proteins. A critical evaluation of mechanisms and functions. Ann. Rev. Biochem., 55, 987–1035.
17) Pollard, T. D. and Weihing, R. R. (1974): Actin and myosin and cell movement. CRC Clit. Rev. Biochem., 2, 1–65.
18) Polzer, M. and Conradt, U. (1994): Identification and partial characterization of the proteases from different developmental stages of *Schistocephalus solidus* (Cestoda: Pseudophyllidae). Int. J. Parasitol., 24, 967–973.
19) Schwartz, W. N. and Bird, J. W. (1977): Degradation of myofibrillar proteins by cathepsins B and D. Biochem. J., 167, 811–820.
20) Song, C. Y. and Chappell, C. L. (1993): Purification and partial characterization of cysteine protease from *Spirometra mansoni* plerocercoids. J. Parasitol., 79, 517–524.
21) Weichselbaum, T. E. (1946): An accurate and rapid method for the determination of proteins in small amounts of blood serum and plasma. Am. J. Clin. Pathol. Tech. Sect., 10, 40–49.
22) Yagi, K. and Yazawa, Y. (1966): Isolation and characterization of a subfragment of myosin A. J. Biochem., 60, 450–458.
23) Yamaguchi, M. and Sekine, T. (1966): Sulfhydryl group involved in the active site of myosin adenosine triphosphatase. J. Biochem., 59, 24–33.