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PHOSPHORYLATION AND IMMUNOLOGICAL CROSS-REACTIVITY OF PARAMYOSIN: A COMPARATIVE STUDY

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Abstract—1. Paramyosins isolated from nematode *Caenorhabditis elegans*, clam *Meretrix meretrix* and *Mercenaria mercenaria*, scallop *Patinopecten yessoensis*, abalone *Notohaliotis discus*, lobster *Homarus vulgaris* and sea cucumber *Holothuria leucospilata* were examined for phosphorylation rate and immunological reactivity against anti-*Meretrix* α-paramyosin antiserum.

2. A high phosphorylation was only observed with the α-forms of bivalve paramyosins from *Meretrix*, *Mercenaria* and *Patinopecten*.

3. The enzyme-linked immunosorbent assay data also revealed that phosphorylatable paramyosins of catch muscle differed in structure from those of non-catch muscle.

INTRODUCTION

Molecular mechanism of catch contraction exhibited by molluscan smooth muscles still remains unresolved, in spite of extensive contributions from many laboratories for the past three decades (Johnson, 1962; Twarog, 1976: Cohen, 1982; Rüegg, 1986). Whether paramyosin plays a significant role in the catch contraction of molluscan smooth muscles also has not been clarified without doubt (Cohen and Castellani, 1988).

To elucidate the functional role of paramyosin, different approaches have been attempted in the past. Some researchers (Cohen *et al.*, 1971; Elfvin *et al.*, 1976; Melson and Cowgill, 1976; Winkelman, 1976) have studied the comparative aspects of paramyosin molecule and immunological cross-reactivity patterns. Subsequently, Achazi (1979) and Cooley *et al.* (1979) have reported the role played by paramyosin in the phosphorylation regulatory mechanism of muscle in *Mytilus edulis* and *Mercenaria mercenaria*, respectively. However, the phosphorylation and immunological cross-reactivity of paramyosin have not been investigated simultaneously.

Based on their estimations of immediate stiffness and static-elastic modulus in skinned and living common mussel (*Mytilus edulis*) smooth muscle during active contraction and catch state, Pfitzer and Rüegg (1982) concluded that there exists a dual intracellular regulation for active contraction and catch state. While the active contraction depends on calcium ion concentration, a catch state could be produced in a calcium ion-free solution. Relaxation of this catch state could be brought out by the addition of cAMP or the catalytic subunit of cAMP-dependent protein kinase type I. The view that paramyosin is involved in catch contraction gained strength from the observation that a cAMP-dependent protein kinase can phosphorylate paramyosin (Achazi, 1979).

In the present study, we report the phosphorylation rates of paramyosin preparations isolated from seven species belonging to phyla Nematoda, Mollusca, Arthropoda and Echinodermata. We have also surveyed the immunological cross-reactivity patterns exhibited by different paramyosin antigens to anti-*Meretrix* α-paramyosin antiserum. A preliminary report has been presented previously (Watabe *et al.*, 1988).

MATERIALS AND METHODS

Animals

Live specimens of clam (*Meretrix meretrix*), scallop (*Patinopecten yessoensis*), abalone (*Notohaliotis discus*) and lobster (*Homarus vulgaris*) were purchased at the Tokyo Central Wholesale Fish Market and transported to the Tokyo laboratory. The sea cucumber (*Holothuria leucospilata*) was obtained from Sesoko Island, Okinawa Prefecture. Clams *Mercenaria mercenaria* were purchased from a commercial dealer. Soil worms *Caenorhabditis elegans* were cultured in the Okayama laboratory, by the method of Brenner (1974).

Isolation of paramyosins

Approximately 30–40 g of relevant muscle portions (Table 1) were dissected from the rest of the tissues and used for isolation of paramyosin. α-Paramyosin was isolated from *Caenorhabditis*, *Meretrix*, *Mercenaria*, *Patinopecten*, *Notohaliotis* and *Holothuria* species by the method of Merrick and Johnson (1977). All the procedures were carried out at 0–4°C to minimize proteolytic degradation. All solutions and buffers contained 0.5 mM dithiothreitol (DTT) to maintain sulfhydryl residues in the reduced state

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Abbreviations used—BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol-bis (β-aminoethyl ether)-*NNN'N'*-tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris (hydroxymethyl) aminomethane.

and to prevent proteolysis (Levine *et al.*, 1982). The yield varied between 10–20 mg paramyosin/g of muscle (wet wt). β-Paramyosins from *Meretrix* and *Homarus* were isolated, according to Johnson *et al.* (1959). The yield was approximately 7.5 mg paramyosin/g of muscle (wet wt).

While efforts were made to conduct the assays immediately after isolation of each paramyosin, the remaining portion of each purified paramyosin was kept frozen at −80°C with sucrose at a 20% final concentration.

Determination of molecular weight

SDS–polyacrylamide gradient (7.5–20%) slab gels were prepared using the discontinuous buffer systems of Laemmli (1970). Protein molecular weight markers used for calibration were: rabbit muscle myosin heavy chain (mol. wt 205,000), β-galactosidase (mol. wt 116,000), rabbit muscle phosphorylase b (mol. wt 97,400), bovine serum albumin (mol. wt 66,000), ovalbumin (mol. wt 45,000) and carbonic anhydrase (mol. wt 29,000), purchased from Sigma Chemicals, MO.

Polyclonal antiserum for α-paramyosin

By standard procedures (Hurn and Chantler, 1980), rabbit anti-α paramyosin antiserum was raised by injecting subcutaneously 2 mg of clam *Meretrix* paramyosin, emulsified in Freund's complete adjuvant (1:1 v/v ratio with the antigen). Booster doses were given at 2-week intervals in Freund's incomplete adjuvant (1:1 v/v ratio with the antigen). Serum was collected from the blood via jugular artery incision after two months of initial injection of antigen, and stored −80°C until use.

Ouchterlony immunodiffusion

Ouchterlony immunodiffusion was carried out at room temperature in 1 mm thick, 1.0% (w/v) agarose gels (Wako Chemicals) in 0.4 M KCl, 0.01 M sodium phosphate buffer pH 7.4, containing 1 mM sodium azide (Ouchterlony and Nilsson, 1978). The center well was filled with anti-*Meretrix* α-paramyosin antiserum and the peripheral wells with paramyosin antigen solutions (5 μg). The plates were incubated in a moist chamber for 36 hr before observing the diffusion patterns. They were then washed, dried and stained with Coomassie Brilliant Blue.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA for paramyosin preparations was carried out according to Engvall (1980). Ten-fold serial dilutions of paramyosin antigens made in phosphate-buffered saline (PBS) were coated in a plastic microtiter plate of high binding capacity (Nunc, No. 439454, Denmark) and incubated for 2 hr at room temperature. After removing the antigen solution, the wells were washed three times with PBS containing 0.05% Tween-20 (PBS-Tween). The wells were then treated with a blocking solution, PBS (pH 7.2) containing 1.0% bovine serum albumin (BSA), for 2 hr at room temperature. Following the removal of BSA, and triple washing of wells with PBS-Tween, 100 μl of 100-fold dilution of *Meretrix* α-paramyosin antiserum in PBS was added to each well and incubated for 2 hr. After subsequent removal of antiserum, the wells were washed with PBS-Tween three times and filled with 100 μl of alkaline phosphatase-conjugated anti-rabbit IgG goat antibody (Sigma Chemicals) diluted 1:2000 fold with PBS-Tween and incubated for another 2 hr. Following the removal of second antibody and triple washing with PBS-Tween, 100 μl substrate solution was assayed for phosphatase activity 0.1% *p*-nitrophenylphosphate disodium salt in 1 M diethanolamine–HCl buffer, pH 9.8, was added to start the reaction. After 15 min, the reaction was stopped by the addition of 25 μl of 5N NaOH and the absorbance of each well was read at 405 nm in a MicroELISA reader (Toyo Soda MPR A4, Tokyo).

Phosphorylation of α-paramyosin

α-Paramyosin phosphorylation was assayed according to the method of Mrwa and Hartshorne (1980), at 25°C in 25 mM Tris–HCl (pH 7.5), 4 mM $MgCl_2$, 1 mM [γ^{32}P]ATP (approximately 5000 cpm/nmol; Amersham International, Buckinghamshire, England) and 1 mM ethylene glycol-bis (β-aminoethyl ether)-*NNN'N'*-tetraacetic acid (EGTA), containing 0.35 M KCl. cAMP-dependent protein kinase catalytic subunit, type I (Sigma Chemicals, MO) was used as the enzyme for phosphorylation at an enzyme-substrate ratio of 1:400. The concentration of paramyosin in the reaction mixture was maintained at 1 mg/ml. Following a 2-min pre-incubation period, the reaction was initiated by the addition of ATP. Aliquots of 150–170 μl were removed from the reaction mixture after 30 min, unless otherwise specified, and treated with 25% trichloracetic acid (TCA) and 2% sodium pyrophosphate to stop the reaction. The precipitate formed was collected on the filter discs of polypropylene columns (Seikagaku, mini PP, Tokyo) fixed with gauze. Subsequently, the precipitate was washed with a mixture containing 5% TCA and 1% sodium pyrophosphate and boiled at 90°C for 10 min. After another washing with the same mixture, the washed precipitate trapped in the gauze was counted for radioactivity by Cherenkov method in a liquid scintillation counter (Beckman LS 1801).

Autoradiography

The prepared SDS–PAGE gel was dried in an automatic electric vacuum gel drier for 6–8 hr before submitting it to autoradiography. Evaluation of the incorporation of ^{32}P into paramyosin was done by permitting contact between the radiolabelled gel and a Kodak X-omat AR film (Bonner and Laskey, 1974; Stein and Yanishevsky, 1979). After exposure between 2 days and 2 weeks, depending on the intensity of the ^{32}P label, the film was developed for 5 min with a Kodak X-ray developer, rinsed briefly with 4% acetic acid and immersed for 2 min in a Kodak X-ray fixer before final rinsing with running water.

Table 1. Comparison of molecular weight and phosphorylation rate of paramyosin among seven invertebrates

Species	Muscle portion	Paramyosin type	Approximate mol. wt	Phosphorylation rate*
Nematoda:				
Caenorhabditis elegans	Body wall	α	97,000	0.02
Mollusca:				
Notohaliotis discus	Foot	α	110,000	0.21
Meretrix meretrix	White adductor	α	110,000	0.87
		β	99,000	0
Mercenaria mercenaria	White adductor	α	110,000	0.98
Patinopecten yessoensis	White adductor	α	99,000	1.02
Arthropoda:				
Homarus vulgaris	Claw	β	105,000	0
Echinodermata:				
Holothuria leucospilata	Longitudinal	α	98,000	0.03

*mol ^{32}P/mol paramyosin/30 min; mean of duplicate determinations.

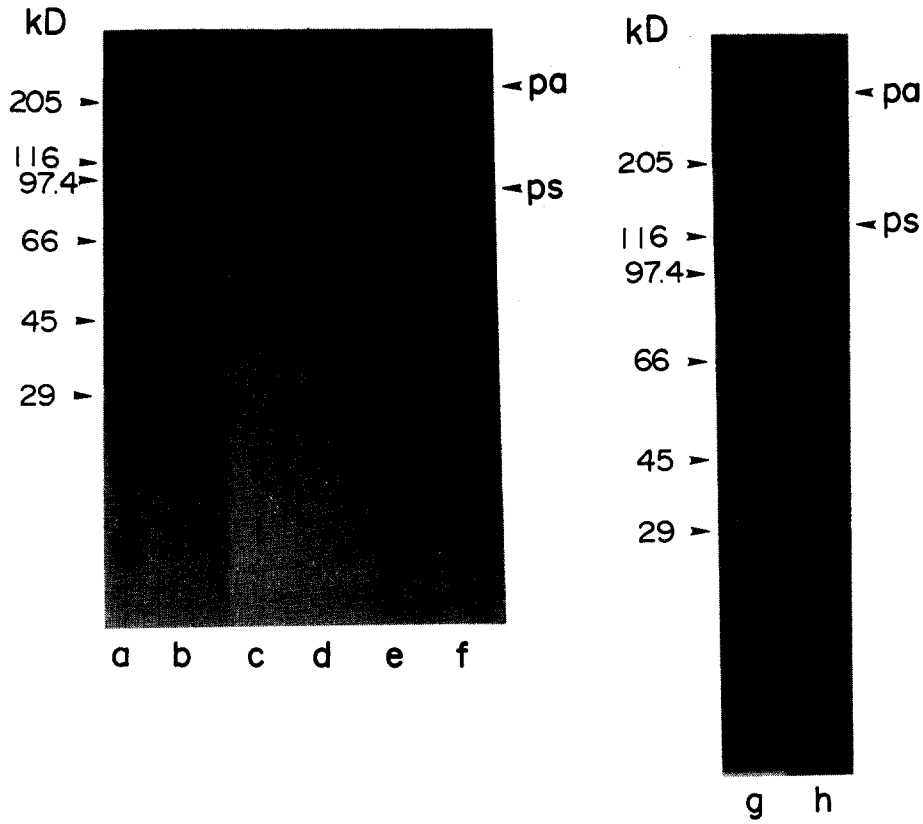

Fig. 1. SDS–PAGE of purified paramyosins from seven species: *Caenorhabditis elegans* α (a), *Homarus vulgaris* β (b), *Meretrix meretrix* α (c), *Meretrix meretrix* β (d), *Mercenaria mercenaria* α (e), *Patinopecten yessoensis* α (f), *Notohaliotis discus* α (g), *Holothuria leucospilata* α (h). The bands of aggregated paramyosin (pa) and paramyosin (ps) are marked. Apparent molecular weights are provided in kDa.

Determination of protein

Protein concentration of the paramyosin was determined by the method of Lowry *et al.* (1951), using BSA as the standard. Since sucrose interferes with the Lowry's colorimetric method, whenever the paramyosins were stored at −80°C with 20% sucrose, the protein concentration of the retrieved sample was measured by u.v. absorbances at 280 and 260 nm (Kalckar, 1947).

RESULTS

Molecular weight

Figure 1 shows the SDS–PAGE patterns of paramyosin preparations. While samples (a) to (f), representing the paramyosin preparations of *Caenorhabditis*, *Homarus*, *Meretrix*, *Mercenaria* and *Patinopecten* were electrophoretically analyzed in one slab gel, samples (g) and (h) representing the paramyosins of *Notohaliotis* and *Holothuria* were analyzed separately, soon after purification. Due to high susceptibility of paramyosin molecule to proteolytic degradation (even in the presence of antimicrobial agents), it was extremely difficult to electrophoretically analyze all the prepared samples of paramyosin in a single gel for comparative purposes.

The molecular weights of paramyosins from seven species ranged from mol. wts 97,000–110,000 (Table 1). The aggregated paramyosin bands (mol. wt over 220,000) were also visible, as shown in Fig. 1; *Caenorhabditis* (lane a), *Meretrix* α and β (lanes c and d), *Mercenaria* (lane e) and *Notohaliotis* (lane g). Other distinct bands which were seen in the paramyosins of *Caenorhabditis* and *Meretrix* β were those of low mol. wt proteoloytic degradation products. Another prominent, thick band which was seen immediately below the β-paramyosin subunit of *Meretrix* was identified as the γ-paramyosin (Fig. 1, lane d), a proteolytic derivative of α-paramyosin.

Phosphorylation

To identify the optimal ionic concentration in which phosphorylation occurs, as a preliminary experiment, phosphorylation rates of *Meretrix* α-paramyosin were measured under varying KCl concentrations, namely 0.2, 0.35 and 0.5 M, at 25°C, in 25 mM Tris–HCl (pH 7.5), 4 mM $MgCl_2$, 1 mM [γ ^{32}P] ATP (approximately 5000 cpm/nmol) and 1 mM EGTA for 30 min. The highest phosphorylation for *Meretrix* α-paramyosin occurred at KCl concentration over 0.3 M. β-Paramyosin of *Meretrix* did not show any appreciable amount of ^{32}P incorporation. Therefore 0.35 M KCl concentration was used to survey the comparative phosphorylation rates of various paramyosin preparations.

The α-paramyosin preparations of clams *Meretrix* and *Mercenaria* as well as scallop *Patinopecten* showed higher phosphorylation rates of 0.87–1.02 mol

^{32}P/mol paramyosin (Table 1). But the abalone paramyosin was phosphorylated at a lower rate of 0.21 mol ^{32}P/mol paramyosin. The phosphorylation rates of *Caenorhabditis* and *Holothuria* paramyosin preparations were relatively insignificant. β-Paramyosin preparations of *Meretrix* and *Homarus* were not phosphorylated.

The SDS–PAGE patterns of phosphorylated α-paramyosins of *Meretrix*, *Patinopecten* and *Caenorhabditis* and their corresponding autoradiograms are shown in Fig. 2. The absence of ^{32}P incorporation into the *Caenorhabditis* paramyosin was distinctly visible. Whereas, in *Meretrix* paramyosin, the ^{32}P incorporation had occurred at the aggregated component of the molecule, the *Patinopecten* paramyosin showed ^{32}P incorporation in its subunit component.

To confirm that phosphorylation rates vary between the paramyosins of catch and non-catch muscles, an experiment was conducted to compare the phosphorylation rates between the α-paramyosins of *Meretrix* and *Notohaliotis* (both of the phylum Mollusca), along the time course of 30-min incubation. The results obtained are provided in Fig. 3. The phosphorylation rates for *Meretrix* paramyosin after 1- and 5-min incubations were 0.60 and 0.80 mol ^{32}P/mol paramyosin, respectively; corresponding figures for *Notohaliotis* paramyosin were 0.08 and 0.13 mol ^{32}P/mol paramyosin, respectively. Whereas the *Meretrix* paramyosin reached its peak phosphorylation rate at 5–10 min, for the *Notohaliotis* paramyosin, the peak phosphorylation rate was reached gradually after 30 min.

Immunological cross-reactivity

Phylogenetically related paramyosin preparations of *Meretrix*, *Patinopecten* and *Notohaliotis* showed immunological cross-reactivity to anti-*Meretrix* α-paramyosin antiserum. But the cross-reactivity shown by paramyosins of *Homarus* and *Holothuria* (belonging to phyla Arthropoda and Echinodermata, respectively) were distinctly insignificant at the concentration of antigen-antiserum used in the immunodiffusion test (Fig. 4).

Comparative ELISA cross-reactivity patterns of different paramyosins to anti-*Meretrix* α-paramyosin antiserum are shown in Fig. 5. The immunobinding performances revealed were as follows: (a) between 0.1 and 100 μg antigen concentrations, the *Meretrix* α-paramyosin showed a higher immunobinding response in comparison to that of *Meretrix* β-paramyosin. (b) The half maximal binding for *Meretrix* α-paramyosin occurred at 1 ng concentration of antigen and for *Meretrix* β-paramyosin, at a concentration slightly lower than 1 ng. (c) The half maximal binding for *Patinopecten*, *Notohaliotis* and

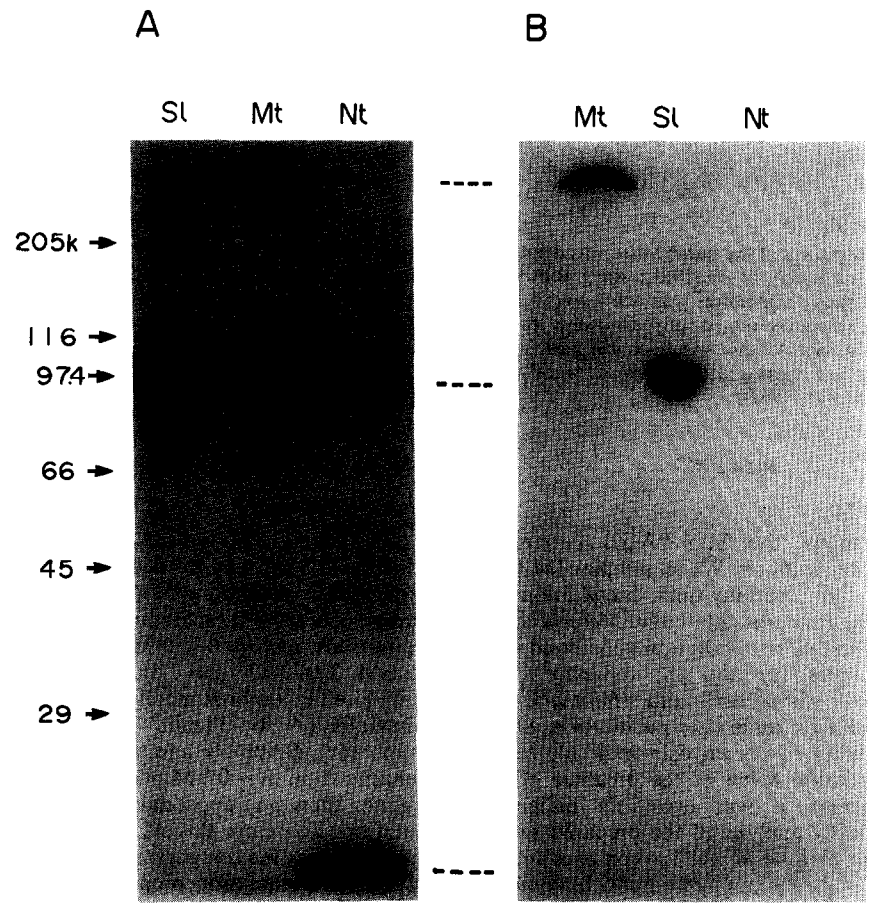

Fig. 2. SDS–PAGE patterns of phosphorylated α-paramyosins of *Meretrix*, *Patinopecten* and *Caenorhabditis* (A) and their corresponding autoradiograms (B). Sl, *Patinopecten*; Mt, *Meretrix* and Nt, *Caenorhabditis*.

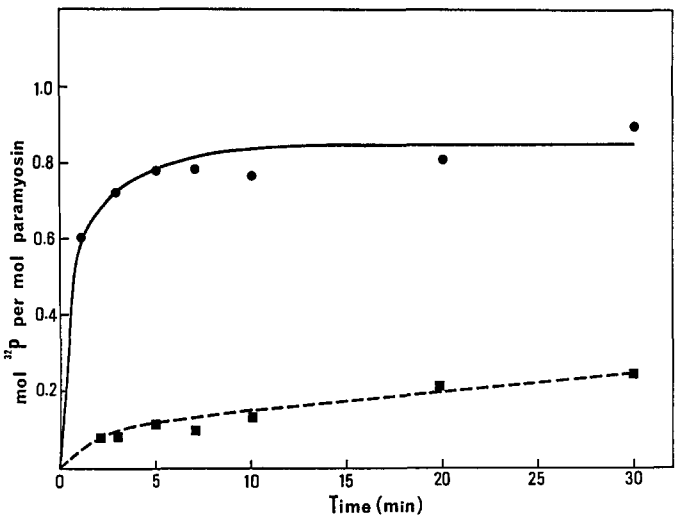

Fig. 3. Comparison of phosphorylation rate between α-paramyosin preparations of *Meretrix* (●) and *Notohaliotis* (■). Phosphorylation assay conditions, as described in the Materials and Methods; reaction was terminated at 2, 3, 5, 7, 10, 20 and 30 min durations.

Holothuria paramyosin antigens occurred at 5 ng, 0.1 μg and 5 μg concentrations, respectively.

DISCUSSION

Comparison of the molecular weights of paramyosin subunits of seven species showed that the paramyosin of *Caenorhabditis elegans* had the lowest molecular weight of 97,000. Previously, Waterston *et al.* (1974) have reported the molecular weight of purified paramyosin subunit of *C. elegans* as 91,000 and commented that degradation of protein occurred despite the use of phenylmethylsulfonyl fluoride (PMSF) and EDTA or EGTA in buffer throughout the procedures. Also in the present study, the paramyosin of *C. elegans* showed proteolytic degradation, as seen by the low mol. wt fractions in Fig. 1. This was expected since during maceration of the tissues of the worm, endogenous proteases in the body could degrade the paramyosin component.

Though the paramyosin has been reported to be present in the obliquely striated muscle of body wall and the muscles of pharyngeal walls of *C. elegans*, its functional significance has not been elucidated. Waterston *et al.* (1974) have postulated that paramyosin in *C. elegans* may permit the growth of longer thick filaments by influencing the muscle assembly and by increasing the tensile strength of such long filaments. It should be noted that the adult worms are only 1 mm in length (Brenner, 1974) and the amino acid sequence of the paramyosin of *C. elegans* has recently been determined (Kagawa *et al.*, 1989).

Unlike in *C. elegans*, paramyosin was selectively distributed among the muscle types of the studied macro-marine species (Table 1). The molecular weight of *Homarus* paramyosin obtained in this study was favorably comparable with the previously reported value for the same species (Winkelman, 1976). Of the seven paramyosins isolated in this study, the lobster paramyosin was comparatively difficult to isolate and purify. This had been observed by Goldfine (1985) as well. One possible reason is that the claw muscle of a lobster is relatively smaller in weight than the adductor or foot muscles of bivalves or gastropods. In addition, it appears that the chances of paramyosin from claw muscle getting proteolyzed during dissection are higher relative to the molluscan muscles.

The mol. wt of 110,000 for the *Mercenaria* α-paramyosin subunit reported here corroborates the similar figures obtained previously by Lowey *et al.* (1963), Stafford and Yphantis (1972) and Cowgill

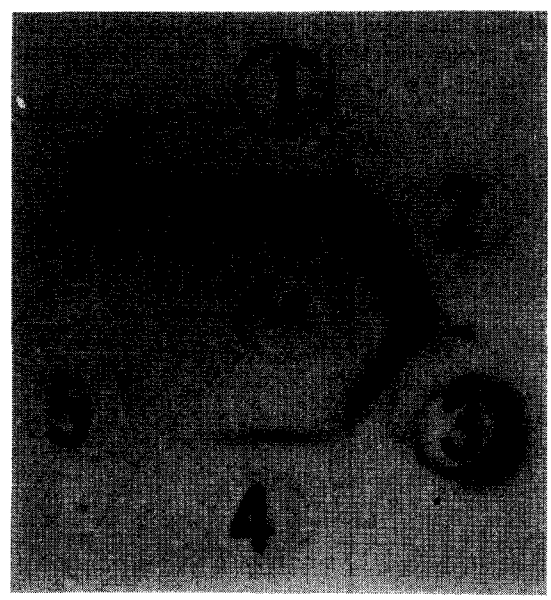

Fig. 4. Ouchterlony immunodiffusion patterns of different paramyosin antigens against anti-*Meretrix* α-paramyosin rabbit antiserum. Antigens in the peripheral wells: *Mercenaria* (1), *Meretrix* α (2), *Patinopecten* (3), *Notohaliotis* (4), *Holothuria* (5) and *Homarus* (6). Antiserum (As) is in the center well. Protein concentration of antigen 5 μg in each well; antiserum concentration, as *per se*, without dilution. An arrow indicates the spur.

(1974). The evidence that the paramyosin of *Mercenaria* is attacked by at least two hydrolytic enzymes under the usual isolation procedures was shown by Stafford and Yphantis (1971). They also identified the EDTA-inhibited and PMSF-inhibited hydrolase activities. When extracting the paramyosin into high salt at pH 7.5, in the presence of 0.01 M EDTA, Stafford and Yphantis (1971) isolated a new molecular species of paramyosin with a mol. wt 5,000 higher than the paramyosins isolated previously by Johnson *et al.* (1959). This new, relatively higher mol. wt subunit was designated as the α-paramyosin form, and the proteolytic derivative with lower mol. wt subunit was identified as the β-paramyosin form (Cowgill, 1972: Merrick and Johnson, 1977). Another proteolytic derivative, γ-form of *Mercenaria* paramyosin has also been recorded (Yeung and Cowgill, 1976). Additionally in the present study, the α, β and γ-forms of *Meretrix* paramyosin were identified (Fig. 1). It was shown that there was a difference of approximately 10,000 in the mol. wt between the *Meretrix* α-paramyosin and its proteolytic derivative, β-paramyosin.

It was also demonstrated in this study that the α-paramyosin of *Meretrix* was phosphorylatable but the β-paramyosin of the same species was not. The varying phosphorylation rates ranging from 0.02 to 1.02 mol ^{32}P/mol paramyosin (Table 1), could be due to the following reasons. Firstly, the paramyosins isolated from the muscles of different species of four phyla have different molecular structures and therefore vary in their ability to be phosphorylated. Secondly, in their natural state, different specific protein kinase enzymes activate the different paramyosin components present in these invertebrates. Thirdly, the paramyosin preparations could already have been phosphorylated during the extraction procedures. However the α-paramyosin preparations of clams *Meretrix* and *Mercenaria* as well as scallop *Patinopecten* showed a high phosphorylation rate of 0.87–1.02 mol ^{32}P/mol paramyosin. These species also exhibit catch mechanisms. Fourthly, the fragility of the paramyosin molecule's *C*-terminal region against endogenous proteases should also contribute to the varying phosphorylation rates among the paramyosin preparations of different species. Chen *et al.* (1988) had reported that the amino acid phosphorylated was a serine residue at the *C*-terminal region of the paramyosin molecule. *In vitro* phosphorylation was also found to be inhibited markedly by actin (Tan *et al.*, 1987; Chen *et al.*, 1988).

Previously, Achazi (1979) and Cooley *et al.* (1979) had studied the phosphorylation patterns of the paramyosin in mussel *Mytilus edulis* and clam *Mercenaria mercenaria*. Achazi's (1979) data on the phosphorylation of *M. edulis* paramyosin showed that, (a) maximal phosphorylation occurred within 10–12 min, at 30°C and pH 6.5 in 0.6 M KCl; (b) maximal ^{32}P incorporation was 0.50 mol/mol paramyosin. In comparison to the finding of Achazi (1979), the present investigation has recorded almost twice this level of ^{32}P incorporation in the α-paramyosins of *Meretrix*, *Mercenaria* and *Patinopecten*. However, the present data in the *Meretrix* α-paramyosin phosphorylation agrees with the observation of Achazi (1979) that maximal ^{32}P incorporation occurred within 10–12 min.

Cooley *et al.* (1979), while studying the phosphorylation of *Mercenaria* paramyosin showed that, (a) the paramyosin contained 3–5 phosphate groups/molecule, depending on the method of extraction; (b) dephosphorylation resulted in an increase in the solubility of paramyosin near pH 7 and near physiological ionic strength. From these observations, they inferred that the number of phosphates/molecules

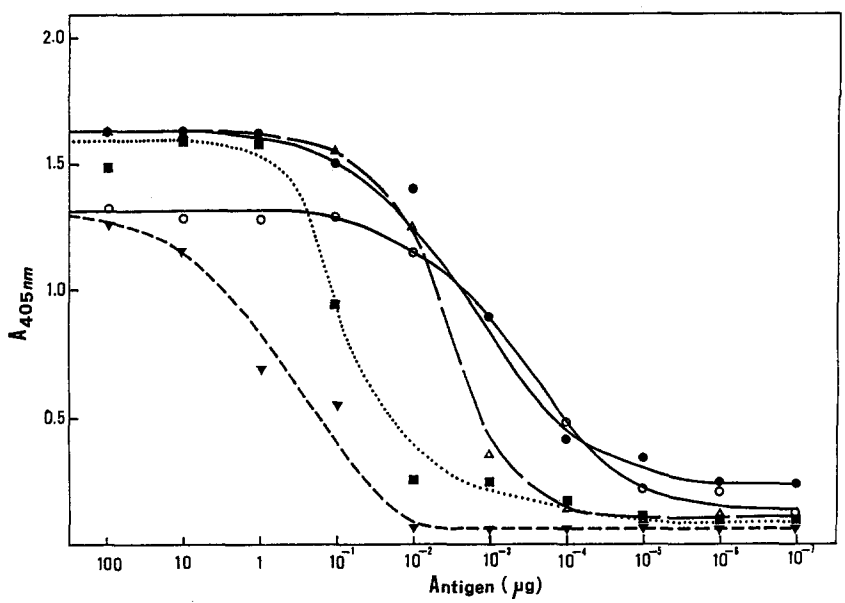

Fig. 5. Cross-reactivity of different paramyosin antigens with anti-*Meretrix* α-paramyosin rabbit antiserum in ELISA. Antigens used are *Meretrix* α (●), *Meretrix* β (○), *Patinopecten* α(△), *Notohaliotis* α (■) and *Holothuria* α (▼). Anti-*Meretrix* α-paramyosin antiserum, 1:100 dilution; Anti-rabbit IgG alkaline phosphatase conjugate, 1:2000 dilution.

may be a determining factor in the aggregation behavior of paramyosin-containing filaments. And, since correlations exist between the catch contraction of molluscan muscles and aggregation properties of paramyosin, phosphorylation may be involved in catch contraction.

From the phosphorylation rate data in the present study, it could be inferred that the phosphorylation of α-paramyosin has some functional significance in the exhibited catch mechanism of bivalves since the paramyosins of catch muscles showed distinctly higher phosphorylation rates than the paramyosins extracted from non-catch muscles. The function of paramyosin in non-catch muscles such as those of nematodes and holothurians has not been clarified yet.

Though paramyosin's involvement in the catch mechanism of bivalve smooth muscle was emphasized by the phosphorylation studies of Achazi (1979) and Cooley *et al.* (1979), recent investigations have shown that, apart from paramyosin, other phosphorylatable proteins could also be involved in the catch contraction of bivalve smooth muscle. For instance, Castellani and Cohen (1987) had observed that myosin heavy chain was also a target for phosphorylation in the anterior byssus retractor muscle of *M. edulis*, by an endogenous myosin heavy chain kinase. These researchers also commented that whether paramyosin is phosphorylated by this endogenous kinase is not known. Subsequently Sohma *et al.* (1988) showed that, in the smooth adductor muscle of *Patinopecten yessoensis*, myosin regulatory light chain phosphorylation is catalyzed by a regulatory light chain-*a* myosin kinase, which is regulated by the cAMP-dependent regulatory chain.

Based on the immunological cross-reactivity studies of *Homarus* and *Mercenaria* paramyosins to specific anti-*Limulus* paramyosin antiserum, Elfvin *et al.* (1976) inferred that there must be considerable homology in the amino acid sequence and/or the tertiary configuration of the different paramyosins. In the present study, the results of immunodiffusion experiments on various paramyosin antigens to anti-*Meretrix* α-paramyosin antiserum indicated that immunological cross-reactivity was present within the molluscan species. *Homarus* and *Holothuria* paramyosin antigens did not show distinct immunological cross-reactivity to the anti-*Meretrix* α-paramyosin antiserum in the Ouchterlony immunodiffusion test (Fig. 4). The immunological cross-reactions depend on the precise concentration of antigen and antiserum. A negative response cannot be considered as a conclusive proof for absence of immunological cross-reactivity. Therefore, one of the possible inferences in this data is that the protein concentrations of *Homarus* and *Holothuria* paramyosin antigens were too low to elicit a cross-reaction in the immunodiffusion test.

The ELISA immunological cross-reactivity profiles of *Meretrix* α and *Meretrix* β-paramyosin preparations in the present study resembled each other in comparison to the profiles of three other α-paramyosin preparations of *Patinopecten*, *Notohaliotis* and *Holothuria* (Fig. 5). However, whereas *Meretrix* α-paramyosin was phosphorylatable, *Meretrix* β-paramyosin lacked phosphorylatability. This difference suggests that the antigenic sites responsible for distinct immunological cross-reactivity patterns of different species are located at a different segment other than the phosphorylatable site of the protein molecule. Lack of additional reports on the immunological cross-reactivity among various paramyosin antigens to a molluscan paramyosin antiserum makes it difficult to provide a confirmational inference.

From the results obtained in this study, it is concluded that: (a) the immunological cross-reactivity trends elicited by immunodiffusion and ELISA experiments reveal a close relationship among paramyosins at an intra-phyletic level and (b) the phosphorylatable paramyosin of catch muscle differs in structure from that of non-catch muscle.

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REFERENCES

Achazi R. K. (1979) Phosphorylation of molluscan paramyosin. *Pflügers Arch.* **379,** 197–201.

Bonner W. M. and Laskey R. A. (1974) A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46,** 83–88.

Brenner S. (1974) The genetics of *Caenorhabditis elegans*. *Genetics* **77,** 71–94.

Castellani L. and Cohen C. (1987) Myosin rod phosphorylation and the catch state of molluscan muscles. *Science* **235,** 334–337.

Chen G. X., Tan R. Y. Gong Z. X. Huang Y. P., Wang S. Z. and Cao T. G. (1988) Paramyosin and the catch mechanism. *Biophys. Chem.* **29,** 147–153.

Cohen C. (1982) Matching molecules in the catch mechanism. *Proc. natn. Acad. Sci. USA* **79,** 3176–3178.

Cohen C. and Castellani L. (1988) New perspectives on catch. *Comp. Biochem. Physiol.* **91C,** 31–33.

Cohen C., Szent-Györgyi A. G. and Kendrick-Jones J. (1971) Paramyosin and the filaments of molluscan "catch" muscles—I. Paramyosin; structure and assembly. *J. molec. Biol.* **56,** 223–237.

Cooley L. B., Johnson W. H. and Krause S. (1979) Phosphorylation of paramyosin and its possible role in the catch mechanism. *J. biol. Chem.* **254,** 2195–2198.

Cowgill R. W. (1972) Susceptibility of paramyosin to proteolysis and the relationship to regions of different stability. *Biochemistry* **11,** 4532–4539.

Cowgill R. W. (1974) Location and properties of sulfhydryl groups on the muscle protein paramyosin from *Mercenaria mercenaria*. *Biochemistry* **13,** 2467–2474.

Elfvin M., Levine R. J. C. and Dewey M. M. (1976) Paramyosin in invertebrate muscles. I. Identification and localization. *J. cell Biol.* **71,** 261–272.

Engvall E. (1980) Enzyme immunoassay ELISA and EMIT. *Meth. Enzym.* **70,** 419–439.

Goldfine S. (1985) Comparative studies on paramyosin, an invertebrate muscle protein. Ph.D. Thesis, State University of New York at Stony Brook, 141 pp.

Hurn B. A. L. and Chantler S. M. (1980) Production of reagent antibodies *Meth. Enzym.* **70,** 104–142.

Johnson W. H. (1962) Tonic mechanisms in smooth muscles. *Physiol. Rev.* **42** (suppl 5), 113–159.

Johnson W. H., Kahn J. S. and Szent-Györgyi A. G. (1959) Paramyosin and contraction of "catch muscles". *Science* **130,** 160–161.

Kagawa H., Gengyo K., MacLachlan A., Brenner S. and Karn J. (1989) The paramyosin gene (unc-15) of *Caenorhabditis elegans*; molecular cloning nucleotide sequence and models for thick filament assembly. *J. molec. Biol.* **207**, 311–333.

Kalckar H. M. (1947) Differential spectrophotometry of purine compounds by means of specific enzymes. III. Studies of the enzymes of purine metabolism. *J. biol. Chem.* **167**, 461–475.

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Levine R. J. C., Elfvin M. J. and Sawyna V. (1982) Preparation and assay of paramyosin. *Meth. Enzym.* **85**, 149–160.

Lowey S., Kucera J. and Holtzer A. (1963) On the structure of the paramyosin molecule. *J. molec. Biol.* **7**, 234–244.

Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

Melson G. L. and Cowgill R. W. (1976) Comparison of the muscle protein paramyosin from different molluscan species. *Comp. Biochem. Physiol.* **55B**, 503–510.

Merrick J. P. and Johnson W. H. (1977) Solubility properties of α-paramyosin. *Biochemistry* **16**, 2260–2264.

Mrwa U. and Hartshorne D. J. (1980) Phosphorylation of smooth muscle myosin and myosin light chains. *Fed. Proc.* **39**, 1564–1568.

Ouchterlony O. and Nilsson L. A. (1979) Immunodiffusion and immunoelectrophoresis. In *Handbook of Experimental Immunology* (Edited by Weir D. M.), Vol. 1, pp. 1–44. Blackwell Scientific, Oxford.

Pfitzer G. and Rüegg J. C. (1982) Molluscan catch muscle; Regulation and mechanics in living and skinned ABRM of *Mytilus edulis*. *J. comp. Physiol.* **147**, 137–142.

Rüegg J. C. (1986) *Calcium in Muscle Activation; a Comparative Approach*. pp. 155–164. Springer, Berlin.

Sohma H., Inoue K. and Morita F. (1988) A cAMP-dependent regulatory protein for RLC-*a* myosin kinase catalyzing the phosphorylation of scallop smooth muscle myosin light chain. *J. Biochem., Tokyo* **103**, 431–435.

Stafford W. F. and Yphantis D. A. (1972) Existence and inhibition of hydrolytic enzymes attacking paramyosin in myofibrillar extracts of *Mercenaria mercenaria*. *Biochem. biophys. Res. Comm.* **49**, 848–854.

Stein G. H. and Yanishevsky R. (1979) Autoradiography. *Meth Enzym.* **58**, 279–292.

Szent-Györgyi A. G., Cohen C. and Kendrick-Jones J. (1971) Paramyosin and the filaments of molluscan "catch" muscles; II. Native filaments: Isolation and characterization. *J. molec. biol.* **56**, 239–258.

Tan R. Y., Huang Y. P., Kung T. H. and Cao T. Q. (1987) The phosphorylation of paramyosin. *Acta biochim. Biophys. Sinica* **19**, 501–508 (in Chinese).

Twarog B. M. (1976) Aspects of smooth muscle function in molluscan catch muscle. *Physiol. Rev.* **56**, 829–838.

Watabe S., Sri Kantha S., Hashimoto K. and Kagawa H. (1988) Immunological species-specificity of paramyosin in association with its phosphorylation *J. muscle Res. Cell Motil.* **9**, 282–283 (abstr).

Waterston R. H., Epstein H. F. and Brenner S. (1974) Paramyosin of *Caenorhabditis elegans*. *J. molec. Biol.* **90**, 285–290.

Winkelman L. (1976) Comparative studies of paramyosins. *Comp. Biochem. Physiol.* **55B**, 391–397.

Yeung A. T. and Cowgill R. W. (1976) Structural difference between α-paramyosin and β-paramyosin of *Mercenaria mercenaria*. *Biochemistry* **15**, 4654–4659.