EXISTENCE AND INHIBITION OF HYDROLYTIC ENZYMES ATTACKING PARAMYOSIN IN MYOFIBRILLAR EXTRACTS OF MERCENARIA MERCENARIA

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Summary: Paramyosin is attacked by at least two hydrolytic enzymes in extracts of the adductor muscle of M. mercenaria under the usual isolation conditions. EDTA inhibited and phenylmethanesulfonyl fluoride inhibited hydrolase activities have been identified. When paramyosin is extracted into high salt at pH 7.5 in the presence of 0.01M EDTA, we isolate a new molecular species of paramyosin with a molecular weight ~5,000 daltons higher than material isolated by previous procedures. A high resolution acrylamide gel electrophoresis technique employing split gels was used to detect small differences in apparent sub-unit molecular weight.

The general use of proteinase inhibitors during the isolation of myofibrillar proteins usually has not been considered necessary*, probably because of the widely held opinion that such enzymes do not exist in significant amounts in the myofibril (1,2,3). Although neutral proteinase activity has been amply demonstrated in muscle homogenates (4-13), hydrolysis in such extracts of individually identifiable or purified contractile proteins has not been observed (9). We have demonstrated the existence of a probable metalloproteinase activity inhibited by EDTA and a presumptive seryl proteinase activity inhibited by phenylmethanesulfonyl fluoride (PMSF), both of which partially cleave paramyosin (PM) in extracts of both red and white portions of the adductor muscle of Mercenaria mercenaria. PM was isolated by two different procedures, in the presence or absence of either EDTA or PMSF or both. The material obtained was then examined by electrophoresis on Tris-dodecylsulfate gels

* A recent exception to this is the use of pepstatin during the isolation of troponin by D. J. Hartshorne, Cold Spring Harbor Symposium on Quantitative Biology, (1972).

MATERIALS-Phenylmethanesulfonyl fluoride was obtained from Schwartz/Mann, Tris-(hydroxymethyl) aminomethane was Trizma grade from Sigma Chemical Co. Sodium dodecylsulfate was MC and B, USP grade.

PREPARATIVE PROCEDURES- Proteinase Inhibition with PMSF: PMSF (0.03M) in propylene glycol was made up freshly for each preparation. Aliquots were added to all solvents, except the chromatographic buffer, to a final concentration of 3×10^{-4}M. Use of PMSF as a seryl enzyme inhibitor was reported originally by Fahrney and Gold (14) and was suggested to us by A. G. Szent-Gyorgyi, to whom we are grateful.

Method I, Chromatography on Controlled Pore Glass: The red or white portion of the adductor muscle was minced and washed in 0.1M NaCl, extracted with 0.6M NaCl, 0.04M Tris, pH 7.5 for one hour and centrifuged at 48,000 x g for 30 min. The supernatant was applied to a 2.54 X 100 cm column of Corning Controlled Pore Glass CPG-10-1250 and PM was collected after recycling three times at either 4° or 22°. The entire procedure was carried out either with or without 0.01M EDTA in all solutions. Details of the procedure have been presented (15), and a manuscript is in preparation.

Method II, Ethanol Precipitation: The preparation of PM was carried out also according to the procedure of Johnson et al. (16) at 4° with and without 0.01M EDTA in all solutions.

MOLECULAR WEIGHT DETERMINATION BY TRIS-DODECYLSULFATE GEL ELECTROPHORESIS-

Polyacrylamide gel electrophoresis was carried essentially according to the procedure of Shapiro et al. (17) using Tris-acetate buffer, 0.05M, pH 8.1, instead of phosphate. To each sample of protein (0.3-2.0 mg/ml), 0.2 ml 0.01M EDTA (if the PM had been prepared without EDTA), 0.2 ml of 10% SDS and 1.0 ml of 0.5M Na_2SO_3 were added in the order listed. Initially, PMSF at a final concentration of 3×10^{-4}M was added. Later, however, this was found to be unnecessary when split gels were used. The mixtures were allowed to react at

40° for one hour and then dialyzed against electrode buffer. Small differences in M.W. were detected by comparing samples on the same gel (6 X 120 mm, 4% acrylamide, 0.1% bis-acrylamide). When mixtures of samples were run, it was noted that hydrolytic activity contaminating some samples was still effective in the presence of detergent. (A similar observation was made by Pringle (18) with yeast hexokinase preparation.) The resulting difficulty of interpreting gel patterns was eliminated by using split gels (19,20). A silicone rubber septum (0.5 X 6.2 X 8 mm) was inserted 1 mm into the top of each gel after the acrylamide had polymerized. At least one sample in each run was dansylated (21) to serve as a fluorescent marker. Initial runs were performed with bovine serum albumin, ovalbumin, and β-PM as molecular weight standards (22), to determine the slope of the calibration curve. Thereafter, longer runs allowing the PM subunits to migrate nearly to the end of the gel were used to determine relative mobility and M.W. of the PM subunit obtained by each procedure.

RESULTS- PM isolated either by method I or method II in the absence of EDTA yielded material having the usual electrophoretic properties in dodecylsulfate: there was a main band (β-PM) with a M.W. of 1.00×10^5 (22) and another band (α-PM) of lower staining intensity representing a few per cent of the main band with a M.W. of 0.94×10^5 (23). When PM was prepared by method I with chromatography at 22° in the absence of EDTA, the main component had a M.W. of 0.94×10^5, and small amounts of material with slightly lower M.W.'s were also seen. If PM was isolated by method I at 22° in the absence of EDTA but with PMSF, two bands were seen in roughly equal amounts with M.W.'s of 1.00 and 0.94×10^5, and the bands with slightly lower M.W.'s were no longer seen. PM isolated by either method I or method II in the presence of 0.01M EDTA showed one band (α-PM) with a M.W. of 1.05×10^5. None of the lower M.W. PM bands could be seen at a load of 75 μg. Lastly, PM isolated in the presence of both EDTA and PMSF also yielded a single band with a M.W. of 1.05×10^5 (not shown in figure 1). It should be noted that with 0.01M EDTA present in either procedure, extraction

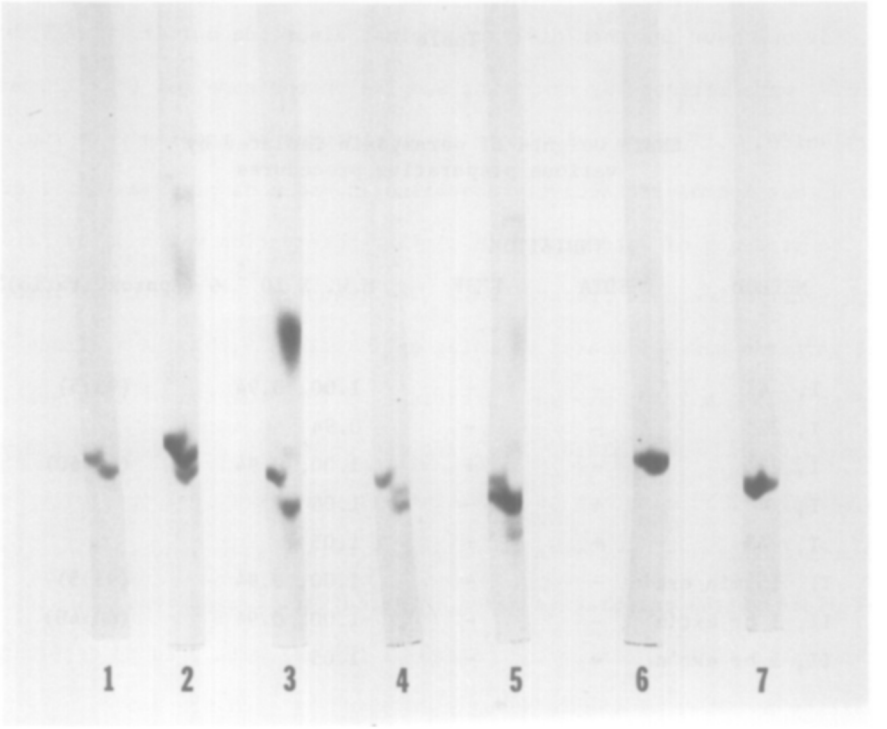

FIGURE 1

Tris-dodecylsulfate polyacrylamide gel electrophoresis patterns of various paramyosin preparations: (1) Method I with EDTA, 4°/without EDTA, 4°C, (2) Method II (1 hour extraction), with EDTA/without EDTA, (3) Method I with EDTA, 4°/without EDTA, 22°, (4) Method I, with EDTA, 4°/with PMSF, 22°, (5) Method I with PMSF, 22°/without PMSF, 22°, (6) Method I with EDTA, 4°/Method II with EDTA, (7) Method I with EDTA, 4°, PM from red and white mucles. [PMSF] = 3 X 10^{-4}M; [EDTA] = 1 X 10^{-2}M. (The diffuse band on the right side of gel #3 is probably a result of air oxidation of γ-PM in SDS. The sample was run ∿18 hrs after sulfonation. Compare this to the right side of gel #5, which is the same material run immediately after sulfonation.) The gels are not all from the same run.

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times of up to 90 min could be used without any evidence for hydrolysis. These results are summarized in Table 1, and the split gels are shown in Figure 1.

CONCLUSION- We have shown that previously used procedures for the isolation of PM from the adductor of M. mercenaria yield an enzymatically degraded form of the molecule. In the presence of 0.01M EDTA, we isolate a new molecular species of PM, α-PM, with a M.W. ∿5,000 daltons higher than that of the main component, β-PM, found by the previous procedures. There appear to be at least two hydro-

Table I

Chain weights of paramyosin isolated by various preparative procedures

METHOD	INHIBITOR EDTA	INHIBITOR PMSF	M.W. X 10^{-5} & (Approx. ratio)*
I, 4°	-	-	1.00, 0.94 (95:5)
I, 22°	-	-	0.94
I, 22°	-	+	1.00, 0.94 (40:60)
I, 4°	+	-	1.05
I, 4°	+	+	1.05
II, 15 min ext'n	-	-	1.00, 0.94 (95:5)
II, 1 hr ext'n	-	-	1.00, 0.94 (60:40)
II, 1 hr ext'n	+	-	1.05

* In most samples faint bands with approximately twice the M.W. of the main bands were seen. This material probably represents either incomplete sulfonation or recombination of chains after sulfonation (24).

lytic activities which attack PM when it is extracted into high salt at pH 7.5. An EDTA inhibited hydrolase activity acts so rapidly at 4° when EDTA is absent that conversion to β-PM is well completed within the usual extraction times (5-15 min.). However, the presumptive seryl hydrolase appears to act slowly enough at 4° that its existence can be detected by carefully examining the usual preparations of PM: it seems to require β-PM for substrate since it does not hydrolyze α-PM even with extraction times of up to 90 min. in the presence of EDTA.

A neutral proteinase from rat skeletal muscle (9) is completely inhibited by EDTA at 0.0015M but not at 0.001M; therefore, the concentration of EDTA required for complete inhibition of the M. mercenaria enzyme activity may be quite critical. EDTA at an unspecified concentration has been used in the

isolation of oyster PM by Woods (24), ostensibly to inhibit heavy metal ion catalyzed oxidation of thiol groups. He found a subunit M.W. of 0.97×10^5 daltons using high speed ultracentrifugation in guanidine hydrochloride. It is difficult to compare his value with our apparent subunit size because of the limited accuracy of absolute M.W. values determined by either method. Species differences may also preclude meaningful comparisons. Further characterization of α-PM is currently underway.

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