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Effect of Proteolytic Enzymes and Protein Denaturing Agents on Normal and Dystrophic Myosins* (32817)

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The presence of increased amounts of proteolytic enzymes within the muscle cell in dystrophic animals is well established (1–4). Although a decrease in the concentration of contractile proteins in experimental muscular dystrophy is equally well documented (5–8), the role of the increased proteolytic enzyme activity in the mechanism of muscular dystrophy is not clear.

The aim of this work was to look for probable structural and functional differences between the normal and dystrophic proteins. Evidence was sought for the possibility that dystrophic contractile proteins are more sensitive to the action of proteolytic enzymes than normal contractile proteins. Also the reactivity of dystrophic and normal contractile proteins to denaturing agents was compared. Moreover the effect of denaturing agents on the sensitivity of dystrophic and normal contractile proteins to proteolysis and the action of these agents on the superprecipitation of normal and dystrophic muscle proteins was also studied. The results of this study are described below.

Materials and Methods. Adenosine triphosphate (ATP) and pronase (protease of B Subtilis type VIII) were purchased from Sigma Co. and trypsin was obtained from Worthington Biochemicals Co. All other reagents used were of reagent grade. Myosin A (MyA) and myosin B (MyB) were prepared as described by Mommaerts (9).

Rats were used 2 weeks after the bilateral dissection of the ischiadic nerves essentially as described by Kohn (7). The 2-year dystrophic hens (leghorn) were a generous gift of D. L. J. Pierro, University of Connecticut. Dystrophic mice (129/Re-dy) were purchased from The Jackson Laboratory, Bar Harbor, Maine.

The extent of trypsin and pronase digestion was followed by pH stat measurements essentially as described by Mihalyi and Harrington (10). A Radiometer TTT1 + SBU + SBR2 combination was used. Experiments were carried out in a water-jacketed cell under nitrogen gas in a total volume of 2 ml. Usually, 7.0 mg of myosin and 0.07 mg of trypsin or pronase were incubated and 10.0 m*M* NaOH was used for titration. The proteolytic enzymes were dissolved in 0.005 *M*/1 HCl and stored in the frozen state. The concentration was estimated by measuring their optical density at 280 mμ (10).

Superprecipitation experiments were measured with the turbidity increase method of Ebashi (11) in a Brice-Phoenix light scattering photometer connected to a 10-inch Beckman recorder. Usually, 0.7 mg of MyB was used for an experiment in the 25-mm quadratic turbidity cell. All experiments were performed in 15-ml total volume under constant mechanical stirring. The ATP was added to the mixtures from a microburette inserted in a hole on top of the photometer above the cell.

Results and Discussion. The results of a

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TABLE I. Effect of Trypsin and Pronase on Normal and Dystrophic Contractile Proteins.

Substrate for hydrolysis[a]	Proteolytic enzyme	Temperature (°C)	Additions	Normal[b] (μM NaOH /10 min)	Dystrophic[b] (μM NaOH /10 min)	No. of expt.	No. of animals per batch
MyB (rat)	trypsin	37		0.85	0.95	3	7–10
MyB (rat)	trypsin	37	urea (2 M)	1.28	1.32	2	7–10
MyB (rat)	trypsin	37	ethylene glycol (50%)	0.25	0.20	2	7–10
MyB (rat)	pronase	25		0.40	0.37	2	7–10
MyB (rat)	pronase	25	urea (2 M)	0.75	0.74	2	7–10
MyB (rat)	pronase	25	ethylene glycol (50%)	0.20	0.22	2	7–10
MyA (chicken)	trypsin	37		1.20	1.25	2	2
MyB (chicken)	trypsin	37		0.90	0.95	3	1
Myofibrills (chicken)	trypsin	37		0.80	0.85	2	1
MyB (chicken)	trypsin	37	urea (2 M)	1.30	1.25	2	1
MyB (mouse)	trypsin	37		1.25	1.20	2	10–20
MyB (mouse)	pronase	25		0.58	0.55	2	10–20

[a] Experiments performed in 0.6 M KCl conc at pH 8.0 in 2-ml total volume.

[b] Seven mg of substrate, 100:1 substrate–enzyme ratio.

typical trypsin and pronase digestion experiment are compiled in Table I. It may be seen that no significant difference was found between the dystrophic contractile proteins and their normal controls. The 2 M urea increased and the ethylene glycol inhibited the activity of the proteolytic enzymes with dystrophic and normal myosins almost equally. Repetition of the experiments (two times) presented in Table I gave identical results. The shape of the individual pH stat curves obtained with normal and dystrophic myosins as the substrate within the indicated 10-min reaction period was also similar. Trypsin usually produced an increased proteolysis in the first 1 or 2 min with both dystrophic and normal myosins and a slower reaction during the rest of the incubation time (10). Varying the substrate–enzyme ratio between 50 and 200 and repeating the experiments at 25 and 37°C produced similar changes with both dystrophic and normal contractile proteins.

Kohn (7) using viscosity as the indicator found no change in the trypsin sensitivity of MyA obtained from denervated muscles of rats. Our results support and extend this finding. There appears to be no significant difference in the trypsin and pronase sensitivity of the contractile proteins of chicken

TABLE II. Superprecipitation of Normal and Dystrophic MyB Suspensions.[a]

Source of MyB		Turbidity increase: Normal	Turbidity increase: Dystrophic	t numbers[b]
7–10 rats/batch	(1)	47	24	
	(2)	42	45	
	(3)	58	42	
	(4)	53	44	
	(5)	53	53	
	(6)	40	41	
	(7)	58	42	1.89
1 hen/batch	(1)	57	47	
	(2)	55	34	
	(3)	56	35	
	(4)	60	40	
	(5)	56	42	
	(6)	47	37	
	(7)	63	47	3.10
10 mice	(1)	50	53	
20 mice	(2)	48	44	

[a] Experiments performed at pH 7.5 for 5 minutes in 0.50 μM/ml of Tris, 0.66 μM/ml of KCl, 0.1 μM /ml of Mg^{2+} and 0.1 μM/ml of ATP. Total volume was 15 ml.

[b] The $t = \bar{x}_{norm} + \bar{x}_{dystr}/[(SEM_{norm})^2 + (SEM_{dystr})^2]^{-1/2}$; $\bar{x}$ = arithmetic mean; and SEM = standard error of mean.

TABLE III. Effect of Inhibitors on Superprecipitation.

Source of MyB	Added (to 15 ml total vol. of reaction mixture)[a]	Inhibition of turbidity increase (%)		No. of expt.
		Normal	Dystrophic	
Rat	PES (0.5 mg)	65	72	3
Chicken		70	68	2
Mouse		85	95	2
Rat	Urea (1.5 *M*)	60	70	3
Chicken		65	71	2
Rat	Dioxane (0.75 ml)	25	18	3
Chicken		15	25	2
Rat	Dioxane (1.5 ml)	75	85	3
Chicken		70	75	2
Rat	Ethylene glycol (4.5 ml)	30	35	2
Chicken		45	40	2
Chicken	EDTA[b] (0.5 μM)	37	40	2
Mouse		45	55	2
Rat	Trypsin (0.005 mg) (5-min incubation at pH 7.5	42	48	3
Chicken	and at 25°C)	55	58	2
Mouse		37	43	2
Chicken	MyB (7.5 mg/ml) (incubated with 25 m*M* DNP at	50	45	2
Mouse	pH 7.5 and at 25°C for 30 min); 0.75 mg of incubated MyB used for the experiment	60	63	2

[a] Experimental conditions as in Table II.

[b] Ethylenediaminetetraacetic acid.

and mice afflicted with genetically controlled muscular dystrophy. The results also showed that the digestion of dystrophic and normal myosins changed qualitatively and quantitatively in a similar way if ethylene glycol or urea was also incorporated into the reaction mixture. This latter finding may further support the conclusion that normal and dystrophic myosins are similar substrates for trypsin and pronase.

The turbidity increase method of Ebashi (11) offers two parameters to evaluate superprecipitation. One is the extent of turbidity increase (or decrease) and the second parameter is the time necessary for maximal turbidity change. Since the data regarding the time for maximal turbidity change were negative they are not included in Table II and III.

It may be seen from Table II that according to a statistical analysis (Student's *t* test) the observed turbidity increase differences between normal and dystrophic rat MyB are not significant. The same analysis (12) showed low significance (just above the 98% confidence limit) for the differences obtained with normal and dystrophic chicken MyB suspensions.

Superprecipitation was used to study actin–myosin interaction. The various denaturing agents studied act on different sites of the contractile proteins. Urea is known to interact with hydrogen bonds while dioxane and ethylene glycol attack primarily hydrophobic groups on the protein molecule. The DNP and PES probably compete with actin for the same myosin A sites. These agents were used to test the structural stability of the dystrophic contractile proteins in comparison with the normal.

The effect of various protein denaturing agents, interaction inhibitors and trypsin on the superprecipitation of normal and dystrophic MyB suspensions is summarized in

Table III. Urea, dioxane, glycol, polyethylene sulfonate (PES), 2,4-dinitrophenol (DNP), and trypsin inhibited superprecipitation to some extent. The individual effect of these agents on dystrophic MyB suspensions was similar to that of normal controls.

Barany *et al.* (8) found no changes in the amino acid composition, aderosine triphosphatase activity and actin binding ability of MyA obtained from dystrophic chicken and mouse. Our results indicate no alteration in the "test tube contraction" of dystrophic MyB suspensions obtained from three different sources.

Summary. The sensitivity of myosins obtained from dystrophic rats and chickens to trypsin and pronase digestion were identical. The effect of urea and ethylene glycol on this proteolysis was the same with normal and dystrophic myosins. The superprecipitation of MyB suspensions obtained from the same dystrophic and normal animals was also similar in the absence and presence of protein denaturing agents, interaction inhibitors, and trypsin.

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Inhibition of Arylamidase by Rabbit Antiarylamidase Antibody* (32818)

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It has been shown that specific antibodies to enzymes will inhibit enzymatic activity (1). This report concerns the effect of rabbit antibody on an arylamidase prepared from cell-free extracts of *Neisseria catarrhalis*. Arylamidases are widely distributed in nature; they hydrolyze amino acyl-β-naphthylamines to yield the constituent amino acid and β-naphthylamine (βNA). The antibodies to this enzyme were detected by gel diffusion and quantitative precipitin tests. The antibody effectively inhibited arylamidase when enzyme was preincubated with antibody prior to addition of substrate as well as when all three were combined simultaneously. This study analyzes the nature of the inhibition caused by the rabbit antiarylamidase.

Materials and Methods. The growth of *N. catarrhalis* (ATCC 8176) and preparation of cell-free extracts were as previously reported by Folds and Behal (2). Arylamidase activity was assayed by determining liberated βNA with a Bratton-Marshall type procedure described by Behal *et al.* (3). Purification of arylamidase was accomplished by salt fractionation, DEAE cellulose and calcium phosphate chromatography according to Behal and Folds (4).

Antisera were prepared by intramuscular injection of purified *N. catarrhalis* arylamidase, which had been incorporated into

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