The Effect of Lipid on the Heat Denaturation of Actomyosin*¹

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Actomyosin (myosin B) prepared by the conventional method usually contains a considerable amount of lipid; it is suspected that the lipid has a significant effect on the properties of actomyosin. Therefore, studies were made on the effect of the former upon the heat denaturation of the latter.

Experimental methods: actomyosins of different lipid contents were prepared by delipidating or lipidating those of fatty fishes, yellowtail, and Pacific mackerel; their heat denaturation rate constants were calculated from the inactivation rates of their Ca^{2+} -ATPases.

Results: the denaturation rate of every actomyosin decreased with increasing lipid content; therefore, they were stabilized by lipid. Moreover, a linear correlation was established between the rate constants and the lipid contents. The stabilization by lipid was larger for mackerel actomyosin than for the yellowtail one.

These results suggest that we need to take account of the lipid content of actomyosin to study the heat denaturation of this protein.

In the conventional preparation method¹⁾ of actomyosin (myosin B) from fish flesh, lipid contained in the flesh binds to the protein and lipid thus bound is difficult to remove. Therefore, actomyosin preparation usually contains lipid as pointed out by TAGUCHI and IKEDA²⁾ (and may refer to Table 1). It is suspected that such lipid contained in actomyosin brings about significant effect on the properties of the latter. It is well known that lipid produces various effects on protein, but hardly any report has been published on the effect of lipid contained in actomyosin, excepting that of TAGUCHI and IKEDA³⁾ in which they indicated that lecithin activates actomyosin ATPase. Therefore, we investigated in the effects of lipid on several properties of actomyosin. This paper deals with the effect on the heat denaturation as one of the properties.

Experimentals

Materials

Cultured carp *Cyprinus carpio*, jack mackerel *Trachurus japonicus*, sardine *Sardinops melanosticta*, Pacific mackerel *Pneumatophorus japonicus* and cultured yellowtail *Seriola quinqueradiata* were purchased at market, and their dorsal and lateral

muscles were used for experiments.

Preparation of Delipidated Actomyosin

Washing under reduced pressure reported by NISHIOKA and SHIMIZU,*³ ultracentrifuging, and phospholipase treatment were applied to the conventional method of KAWASHIMA *et al.*¹⁾ to remove lipid.

The washing under reduced pressure was conducted twice by stirring minced flesh (3 mm in diameter) with 4-fold volumes of 20 mM phosphate buffer (pH 7.5) for 30 minutes at 5 mmHg, and floated lipid was removed. The ultracentrifugation was conducted by centrifuging actomyosin solution for one hour at $40,000 \times g$, fatty precipitate was discarded, and the supernatant was adopted. The phospholipase treatment was conducted by modification of the method of TAGUCHI and IKEDA,³⁾ that is, by treating actomyosin with 1 unit (0.2 mg) of phospholipase C (Boehringer Mannheim Co., from *Bacillus cereus*) per mg of the former for 7 hours at 4°C, by which lipid was hydrolyzed and eliminated. All actomyosin solutions were stored at 4°C in 0.6 M KCl-20 mM tris buffer (pH 7.0) at the concentration of 5 mg/ml.

Preparation of Lipidated Actomyosin

To the delipidated actomyosin was added lipid,

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which was extracted from the flesh with CHCl_3 - CH_3OH (2:1, v/v) and evaporated the solvents. In this case, KIMELBERG⁴⁾ suggested that it is important to disperse lipid homogeneously. Therefore, 2 g of lipid in 15 ml of 0.6 M KCl were sonicated with sonicator (Ultrasonics Co., Type W-375) for one minute at scale 4, and various volumes of the resulting suspension were mixed with the actomyosin solution under gently stirring for 30 minutes. To remove lipid unbound to actomyosin, each mixture was submitted twice to dilution with water and dissolution of the resulting actomyosin precipitate in KCl solution. Then, each actomyosin solution was centrifuged for one hour at $15,000 \times g$, and the supernatant was adjusted to 5 mg protein/ml with 0.6 M KCl-20 mM tris buffer (pH 7.0).

Determination of Lipid Content of Actomyosin

Lipid was extracted from known amount of actomyosin with CHCl_3 - CH_3OH (2:1, v/v) for 30 minutes under stirring, and the extract was filtered. After three times extractions the filtrates were joined and evaporated the solvents at 30°C under reduced pressure, the residue was dissolved in petroleum ether and filtered to remove contaminated KCl, and after the filtrate was evaporated at 30°C under reduced pressure the residual lipid was weighed. Lipid content was expressed as weight percent in actomyosin.

Measurement of Heat Denaturation of Actomyosin

By the method of ARAI *et al.*⁵⁾ actomyosin solution was heated at 30°C for the definite times, cooled in ice, and its Ca^{2+} -ATPase activity was measured at 25°C in 0.5 M KCl, and then the denaturation rate constant (k_d) was calculated by the following equation.

$$k_d = -2.303 \cdot \log (a_t/a_0)/t$$

In this equation a_0 and a_t are relative ATPase activities before heating and after heating for t second, respectively.

Determination of Protein Concentration

Protein concentration was determined by biuret method⁶⁾ standardized by micro-Kjeldahl method.

Results

Lipid Content of Actomyosin

Table 1 shows that all of the actomyosins prepared from several fishes by the conventional method contain lipid and those from fatty fishes such as sardine, Pacific mackerel, and yellowtail are high in lipid content, and that, as illustrated with yellowtail, the lipid content is high in winter when the body weight and lipid content of flesh are large. Moreover, the lipid content of actomyosin from cultured fish has been increasing for the last several years with the increase in the lipid content of flesh.

Delipidation of Actomyosin

It is necessary to remove lipid from actomyosin to study the effect of the former on the latter, but such solvents or surfactants that denature actomyosin cannot be used for this purpose. Therefore, washing under reduced pressure, ultracentrifuging, phospholipase treatment, and their combinations were examined as delipidation methods.

Table 2 is the results for yellowtail actomyosin.

Table 1. Lipid contents of actomyosins prepared by conventional method¹⁾ from several fishes

Species	Seasons	Body weights (g)	Lipid contents (%)
Carp	Winter	800	5.0
Jack mackerel	Summer	200	8.6
Sardine	Summer	150	18.7
Pacific mackerel	Summer	310	12.7
Yellowtail	Spring	1720	13.3
	Summer	1400	9.3
	Autumn	1500	12.4
	Winter	1810	19.3

Table 2. Removal of lipid from yellowtail actomyosin by several methods or their combinations

Methods	ATPase activities ($\mu\text{mol P}_i/\text{min/mg protein}$)	Yields of ATPase* (%)	Lipid contents (%)
Conventional	0.107	100.0	28.4
Washing under reduced pressure	0.100	60.0	17.0
Washing under reduced pressure-ultracentrifuging	0.100	55.9	12.1
Washing under reduced pressure-ultracentrifuging-hydrolyzing with phospholipase C	0.101	51.1	8.3

* Yields against conventional method.¹⁾

It was difficult to remove lipid completely, but about 40, 60, and 70% of the lipid were removed by the washing, by the washing-centrifuging, and by the washing-centrifuging-phospholipase treatment, respectively. By these methods the yields of ATPase decreased by about one half, but the activities were almost the same. In addition, all actomyosins obtained held superprecipitating activity and their ATP sensitivities were from 94 to 105.

Heat Denaturation of Actomyosin

Actomyosins with different lipid contents were prepared by the above delipidation methods and measured their heat denaturation rates. Fig. 1 is

Fig. 1. Heat denaturation of delipidated actomyosins from yellowtail. Lipid contents: 4.3 (○), 11.9 (●), and 23.8% (×).

Fig. 2. Heat denaturation of lipidated actomyosins from yellowtail. Lipid contents: 4.6 (○), 14.6 (●), and 24.4% (×).

the results for yellowtail actomyosins. It was shown that for every actomyosin, linear relationship exists between the logarithms of ATPase activities and the times of heating, and hence, the denaturation depends on the rate equation of

Fig. 3. Heat denaturation of delipidated actomyosins from Pacific mackerel. Lipid contents: 5.8 (○), 10.5 (●), and 12.7% (×).

Fig. 4. Heat denaturation of lipidated actomyosins from Pacific mackerel. Lipid contents: 9.4 (○), 13.1 (●), and 17.0% (×).

Table 3. Lipid contents and denaturation rate constants of actomyosins

Preparation methods	Lipid contents (%)	Denaturation rate constants $k_d (\times 10^{-4}/s)$
Yellowtail	Delipidated	4.3
		1.7
		11.9
		1.2
		23.8
		0.7
Pacific mackerel	Lipidated	4.6
		2.0
		14.6
		1.7
		24.4
		1.2
Pacific mackerel	Delipidated	5.8
		5.1
		10.5
		4.0
		12.7
		2.8
Pacific mackerel	Lipidated	9.4
		2.9
		13.1
		1.9
		17.0
		0.8

Fig. 5. Relationship between heat denaturation rate constants and lipid contents of actomyosin.

Open and solid symbols are delipidated and lipidated actomyosins from yellowtail (○, ●) and Pacific mackerel (□, ■), respectively.

first order, and that the slope of the straight line becomes gentle with increasing lipid content, and hence, actomyosin is stabilized by lipid.

It was considered that this stabilization might be caused by the differences in preparation methods of actomyosin. Then, lipid was added to the same actomyosin preparation in different amounts, and the lipidated actomyosins obtained were measured their heat denaturation. The results were, as illustrated in Fig. 2, the same as those for the delipidated actomyosins. Therefore, it may be concluded that lipid stabilizes actomyosin.

For Pacific mackerel also, results similar to

those for yellowtail were obtained either with the delipidated actomyosins (Fig. 3) and with the lipidated ones (Fig. 4).

As the denaturation of any actomyosins depended on the first order reaction, their denaturation rate constants were calculated by the equation cited in the experimentals. It is clear from Table 3 that the rate constants decrease with increasing lipid content. Moreover, on plotting these rate constants against the lipid contents, linear correlations were established as shown in Fig. 5. It is calculated from this figure that as the lipid content increases by 1%, the denaturation rate constant decreases by about 0.05 and $0.30 \times 10^{-4} s^{-1}$ for yellowtail and Pacific mackerel actomyosins, respectively.

Discussion

SHENOUDA *et al.*^{7,8)} indicated that lipid binds to actin and myosin, especially when these were stored, agitated, or heated, and the bound lipid is difficult to be removed. In our experiments also, considerable amount of lipid bound to actomyosin, especially when the lipid content of flesh was high, and it was troublesome to remove the bound lipid. Notwithstanding this difficulty, the experiments could be carried out on the effect of lipid upon the heat denaturation of actomyosin, by removing two thirds of the bound lipid with various delipidation methods, and by adding lipid to the delipidated actomyosin thus obtained.

Many papers have been published on the stability of actomyosin or myosin. CONNELL⁹⁾ studied on the susceptibilities of skeletal muscle myosins of some animals to aggregation, urea denaturation, and tryptic digestion, and suggested that adapting to the body temperatures of animals, their myosins can be divided into two groups and fish myosins form unstable one. WATABE *et al.*¹⁰⁾ reported that Ca^{2+} -ATPase of myosin from mackerel dark muscle is several times as stable as that from the white muscle. For fish myofibrils, JOHNSTON *et al.*¹¹⁾ pointed out that the thermostabilities of myofibrillar Mg^{2+} -ATPases are related to the environmental temperatures at which fishes live. Data that this is applicable also to actomyosins were presented by ARAI *et al.*^{5,12)} and by YABE *et al.*¹³⁾ and they described that the thermostability of actomyosin Ca^{2+} -ATPase of each fish species is native and proper to each species. However, no papers have ever been published on the effect of lipid upon the stability of actomyosin.

As illustrated in Table 1, lipid content of actomyosin varies with species, season, and body weight. Therefore, it is necessary to take the lipid content into consideration on the studies of actomyosin, if the former influences on the properties of the latter. In fact, these experiments revealed that lipid decreases the heat denaturation rate of actomyosin. Hitherto attention has not been paid to the lipid contained in actomyosin, but hereafter it needs to take account of the lipid content to study the heat denaturation of actomyosin, especially of fatty fish ones.

References

- 1) T. KAWASHIMA, K. ARAI, and T. SAITO: *Bull. Japan. Soc. Sci. Fish.*, **39**, 207-214 (1973).
- 2) T. TAGUCHI and S. IKEDA: *Bull. Japan. Soc. Sci. Fish.*, **34**, 339-343 (1968).
- 3) T. TAGUCHI and S. IKEDA: *Bull. Japan. Soc. Sci. Fish.*, **34**, 411-414 (1968).
- 4) H. K. KIMELBERG: *Biochim. Biophys. Acta*, **413**, 143-156 (1975).
- 5) K. ARAI, K. KAWAMURA, and C. HAYASHI: *Bull. Japan. Soc. Sci. Fish.*, **39**, 1077-1085 (1973).
- 6) A. G. GORNALL, C. J. BARDAWILL, and M. M. DAVID: *J. Biol. Chem.*, **177**, 751-766 (1949).
- 7) S. Y. K. SHENOUDA and G. M. PIGOTT: *J. Food Sci.*, **39**, 726-733 (1974).
- 8) S. Y. K. SHENOUDA and G. M. PIGOTT: *Adv. Exp. Med. Biol.*, **86A**, 657-686 (1977).
- 9) J. J. CONNELL: *Biochem. J.*, **80**, 503-509 (1961).
- 10) S. WATABE and K. HASHIMOTO: *J. Biochem.*, **87**, 1491-1499 (1980).
- 11) I. A. JOHNSTON, N. FREARSON, and G. GOLDSPINK: *Biochem. J.*, **133**, 735-738 (1973).
- 12) K. ARAI, A. HASNAIN, and Y. TAKANO: *Bull. Japan. Soc. Sci. Fish.*, **42**, 687-695 (1976).
- 13) K. YABE, K. NAKAMURA, M. SUZUKI, and Y. ITO: *Bull. Japan. Soc. Sci. Fish.*, **44**, 1345-1350 (1978).