Comparative Studies on the Tryptic Digestion of Actomyosins and Myosins from Several Fish Species—II

Changes in Ca^{2+} and EDTA-ATPases

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Ca^{2+} - and EDTA-ATPase of actomyosins from several fish species and rabbit were assayed during digestion with trypsin. In typical experiments, the initial activation as well as the inactivation with increasing trypsin concentration were confined to the shorter durations. During digestion, Ca^{2+} -ATPase of heat denatured samples of bigeye tuna and carp actomyosins showed patterns which were identical with those of their respective fresh preparations.

A higher initial activation was a general feature of the tuna actomyosins, if the assay of Ca^{2+} -ATPase was performed in 60 mM KCl. The initial activation of tilapia actomyosin Ca^{2+} -ATPase was of a lesser extent, whereas it was absent from carp, yellowtail and flatfish actomyosins. At the higher KCl concentration (500 mM) in the ATPase assay, the response of Ca^{2+} -ATPase was altogether monophasic *i.e.* devoid of any initial activation even in the case of rabbit actomyosin. EDTA-ATPase pattern of actomyosin digestion by trypsin closely resembled the pattern of Ca^{2+} -ATPase at the higher KCl concentration.

The activation of myosin Ca^{2+} -ATPase of tuna species occurred to a lesser extent than that of actomyosin ATPase, and the reversal of the order of stability observed in case of actomyosin ATPase of tilapia and skipjack tuna was no longer noted. In the light of published work, the reasons for these discrepancies have been discussed.

In a previous study¹⁾, it was shown that the relative stability of various actomyosins and myosins, as expressed by the velocity of viscosity fall during the first rapid phase of tryptic digestion, is in good agreement with that established by heat or urea²⁾ inactivation of Ca^{2+} -ATPase. So far, the exposure of ATPase to denaturing agents caused a decline in the activity, but wide variations were observed during its tryptic modification. The available literature shows that an initial activation of this ATPase during the proteolysis is not exception³⁻⁵⁾. When assayed at millimolar concentration of KCl (60 mM), Ca^{2+} -ATPase activity of actomyosins and myosins from tilapia and tuna species also showed a biphasic response, the first phase being an initial activation. On the contrary, carp, yellowtail and flatfish ATPases were devoid of the first phase, *i.e.* the initial activation. However, at higher KCl concentration (500 mM), the response of Ca^{2+} -ATPase of all actomyosins became invariably monophasic. In this way the changes in the present case differed

from those noted during thermal and urea inactivation of Ca^{2+} -ATPase of various fish actomyosins, where first order kinetics were followed throughout the course of inactivation²⁾. Actin, so far, played only a stabilizing role and did not alter the interspecies order of relative stability as in case of tryptic digestion. Keeping in view the discrepancy in the pattern of Ca^{2+} -ATPase, investigations were further extended to EDTA-ATPase of actomyosins.

Materials and Methods

Both frozen and fresh muscles of the following fish species were used in the present study: tilapia (*Tilapia mossambica*), carp (*Cyprinus carpio*), yellowtail (*Seriola quinqueradiata*) and flatfish (*Limanda angustirostris*). Only frozen muscle samples of skipjack tuna (*Katsuwonis pelamis*), yellowfin tuna (*Thunnus albacares*) and bigeye tuna (*Thunnus obesus*) were utilized. Rabbit actomyosin and myosin have been included for comparison.

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In this case also both, frozen and fresh muscle samples, were utilized.

Preparation of actomyosin was made as already referred to^{1,21}. The conditions for tryptic digestion have also been reported elsewhere¹⁷. ATPase activity was assayed as described under the legends of respective figures. The enzymatic properties of some actomyosins and myosins used in the present study are typically summarized in Table 1 and 2.

Table 1. Specific ATPase activities of some fish actomyosins

Name of Species	Conditions of assay		
	60 mM KCl, 5 mM CaCl ₂	500 mM KCl, 5 mM CaCl ₂	500 mM KCl, 1 mM EDTA
Rabbit	0.721	0.158	0.202
Tilapia	0.663	0.103	0.112
Skipjack tuna	0.707	0.193	0.153
Yellowfin tuna	0.582	0.152	0.124
Bigeye tuna (Fresh)	0.240	0.052	0.070
(Denatured)	0.057	—	—
Carp	0.650	0.136	0.151
Yellowtail	0.565	0.113	0.171
Flatfish	0.288	0.058	0.088

ATPase activity was expressed as $\mu\text{mol Pi/min}\cdot\text{mg}$ of protein.

Table 2. Specific ATPase activities of some fish myosins

Name of Species	Conditions of assay	
	60 mM KCl, 5 mM CaCl ₂	
Rabbit	0.941	
Tilapia	0.371	
Skipjack tuna	0.937	
Yellowfin tuna	0.601	
Bigeye tuna	0.250	
Carp	0.270	
Yellowtail	0.237	

ATPase activity was expressed as $\mu\text{mol Pi/min}\cdot\text{mg}$ of protein.

Results

Changes in Ca^{2+} -ATPase of Actomyosin during Assay in 60 mM KCl

The changes in this ATPase of bigeye tuna actomyosin (Fig. 1-A), at various concentrations of trypsin, reveal that both the initial activation as well the inactivation are confined to shorter durations, with the increasing concentration of trypsin. The remarkable point is about 100% increase in the ATPase during the first few minutes, while such activation is totally absent from carp actomyosin ATPase (Fig. 1B). The extent of initial activation,

Fig. 1. Changes in Ca^{2+} -ATPase activity of actomyosin during digestion with varying amounts of trypsin at 25°C.

A, 5.65 mg/ml of bigeye tuna actomyosin, having specific Ca^{2+} -ATPase activity of $0.240 \mu\text{mol Pi/min}\cdot\text{mg}$ of protein, was digested with 1/25 (—), 1/50 (---) and 1/100 (-----) part of trypsin (w/w).

B, 5.50 mg/ml of carp actomyosin, having specific Ca^{2+} -ATPase activity of $0.653 \mu\text{mol Pi/min}\cdot\text{mg}$ of protein, was digested with 1/50 (—), 1/500 (---) and 1/2,000 (-----) part of trypsin (w/w). ATPase activity was assayed at 25°C, in a reaction medium containing final concentration of 60 mM KCl, 25 mM Tris-maleate buffer (pH 7.0), 5 mM CaCl₂ and 1 mM ATP.

however, varies to some extent from preparation to preparation. In case of carp actomyosin Ca^{2+} -ATPase, distinct differences in the inactivation rate can be noted after 30 minutes of digestion (Fig. 1-B), and the higher is the trypsin concentration, the faster is the inactivation of ATPase.

Another set of experiments was performed to determine if *in situ* denaturation of actomyosin has any bearing upon the changes induced during tryptic digestion of Ca^{2+} -ATPase. An actomyosin preparation of bigeye tuna of the description given under Fig. 2-A was thermally denatured, and then subjected to tryptic digestion. As shown in Fig. 2-A, no remarkable differences can be noted in comparison with the pattern of fresh actomyosin ATPase. Similar results were obtained on fresh and thermally denatured carp actomyosin also (Fig. 2-B). Thus, this finding is in agreement with the observations made during viscosity changes¹¹, where tryptic digestion of fresh and thermally denatured samples was found to give identical

relative $\log Z\eta$ (Viscosity number) plots.

Results obtained after digesting actomyosins of various species at a trypsin concentration of 1/50

Fig. 2. Changes in Ca^{2+} -ATPase activity of fresh (—) and denatured (---) actomyosin during digestion with trypsin at 25°C .

A, 5.65 mg/ml of bigeye tuna actomyosin was digested with 1/50 part of trypsin (w/w). The fresh preparation had a $\log \eta_r/C$ value of 1.57 dl/g and ATP sensitivity of 59, which after thermal treatment at 35°C for 60 minutes became 0.87 dl/g and 9, respectively.

B, 5.50 mg/ml of carp actomyosin was digested with 1/2,000 part of trypsin. The fresh preparation had a $\log \eta_r/C$ value of 1.56 dl/g and ATP sensitivity of 99, which after thermal treatment at 35°C for 60 minutes became 1.26 dl/g and 31, respectively. Conditions of ATPase assay were the same as in Fig. 1.

Fig. 3. Changes in Ca^{2+} -ATPase activity of actomyosins from various fish species and rabbit, during digestion with 1/50 part of trypsin (w/w) at 25°C . ATPase assay was performed in 60 mM KCl with other conditions were the same as in Fig. 1.

1, Rabbit; 2, Skipjack tuna; 3, Yellowfin tuna; 4, Tilapia; 5, Bigeye tuna; 6, Carp; 7, Yellowtail and 8, Flatfish.

part of actomyosin, are shown in Fig. 3. As evident, the actomyosin ATPase of all tuna species shows a marked initial activation, but it starts to decline within the first hour. Contrary to them the initial activation of rabbit actomyosin Ca^{2+} -ATPase lasts for several hours.

Changes in Ca^{2+} -ATPase of Actomyosins during Assay in 500 mM KCl

As shown in Fig. 4, at this concentration of KCl no initial activation could be noticed either in rabbit, tilapia, or tuna actomyosin ATPase. Rabbit actomyosin ATPase remained unchanged for several hours, whereas that of fish species showed a progressive decrease only. A comparison can easily be made by noting the duration marking the loss of 50% of activity which shows the inter-species order of stability to be, skipjack tuna, tilapia, yellowfin tuna, bigeye tuna, carp, yellowtail and flatfish. Just as in case of the initial activation, the duration required for 50% loss is subject to variations within reasonable limit for a particular species. The most remarkable point, however, is the improvement in the stability of tilapia actomyosin ATPase.

Fig. 4. Changes in Ca^{2+} -ATPase activity of actomyosins from various fish species and rabbit during digestion with 1/50 part of trypsin (w/w) at 25°C . Assay conditions were the same as in Fig. 1, excepting the molarity of KCl, which was 500 mM here instead of 60 mM.

1, Rabbit; 2, Skipjack tuna; 3, Tilapia; 4, Yellowfin tuna; 5, Bigeye tuna; 6, Carp; 7, Yellowtail and 8, Flatfish.

Changes in EDTA-ATPase of Actomyosins during Assay in 500 mM KCl

As shown in Fig. 5, general pattern of this ATPase of various species resembles closely the pattern of Ca^{2+} -ATPase in 500 mM KCl. But, even this result shows tilapia actomyosin to be less trypsin resistant than that of skipjack tuna actomyosin.

Fig. 5. Changes in EDTA-ATPase activity of actomyosins from various fish species and rabbit, during digestion with 1/50 part of trypsin (w/w), at 25°C. Assay was performed at 25°C in a reaction mixture containing 500 mM KCl, 25 mM Tris-maleate buffer (pH 7.0), 1 mM EDTA and 1 mM ATP.

1, Rabbit; 2, Skipjack tuna; 3, Tilapia; 4, Yellowfin tuna; 5, Bigeye tuna; 6, Carp; 7, Yellowtail and 8, Flatfish.

Changes in Ca^{2+} -ATPase of Myosins during Assay in 60 mM KCl

If these changes are taken into consideration then the order of relative stability of various species as established on the basis of earlier studies^{1,21} can be observed again (Fig. 6). That is, in the decreasing order of stability it becomes: tilapia, skipjack tuna, yellowfin tuna, bigeye tuna, carp and yellowtail. However, the differences between the pattern of tilapia and skipjack tuna myosin ATPase are not quite distinct.

Fig. 6. Changes in Ca^{2+} -ATPase activity of myosins from various fish species and rabbit during digestion with 1/50 part of trypsin (w/w) at 25°C. ATPase was assayed in 60 mM KCl with all other conditions were the same as in Fig. 1.

1, Rabbit; 2, Tilapia; 3, Skipjack tuna; 4, Yellowfin tuna; 5, Bigeye tuna; 6, Carp and 7, Yellowtail.

Discussion

On the basis of variations in Ca^{2+} -ATPase of various myosins at millimolar concentrations of KCl during tryptic hydrolysis, they have been classified into 3 groups: A) where the ATPase remains unchanged, B) where a marked activation occurs; and C) where only the loss of activity is apparent³⁻⁵¹. So far group B included mammalian actomyosins or myosins only; but the present study shows that activation as high as 120–160% might occur during tryptic digestion of tuna proteins (Figs. 3 and 6). Nevertheless, such classification does not distinguish between stable and unstable ATPase. A comparison of rabbit actomyosin (or myosin) Ca^{2+} -ATPase with that of actomyosins (or myosins) from tilapia or tuna species clearly indicates that the rate of decline should be the measure of interspecies differences in the susceptibility (Figs. 1 and 6).

One of the most obvious reason to cause either the reversal of the order of stability between tilapia and skipjack tuna actomyosin ATPases, or the exceptional increase in activity, seems to be the presence of actin. It is because myosin Ca^{2+} -ATPase of tuna species (Fig. 6) is not elevated to the extent shown by their actomyosin ATPase (Fig. 3), and the interspecies order of stability is also unchanged. An initial activation might be the result of the increase in relative value of the specific ATPase activity of a trypsin digested fraction (which is almost water soluble and contains acto-heavy meromyosin) against an undigested fraction (which is precipitable actomyosin) and might undergo aggregation. That may be why the fresh and heat denatured actomyosin also yielded similar plots.

Fig. 1-A showed that the increase in trypsin concentration limits the initial activation and inactivation phases to shorter durations. Obviously by increasing trypsin concentration a stage of complete omission of the activation could be achieved. Such a critical concentration of trypsin for carp, yellowtail and other unstable fish species seems to be very low in comparison with that for tilapia and tunas. The results of the work in progress and to be published latter on, show that the initial activation can be achieved in case of unstable actomyosins also, if the concentration of trypsin as well as the temperature of incubation are low.

Due to obvious differences between tryptic modification of ATPase, and its inactivation by

heat and urea, the extent of interspecies variations is not of the same order and magnitude. Even in case of myosin Ca^{2+} -ATPase of tilapia and skipjack tuna, the differences are not so distinct. These discrepancies may be due to two reasons: 1) the difference is related to the red (skipjack tuna) and white (tilapia) musculature of respective species; or 2) some aggregation during frozen storage occurred and this aggregate was digested at the slow rate to give the stability effect to skipjack tuna actomyosin or myosin Ca^{2+} -ATPase. However, the latter assumption requires a comparison between the fresh muscles of tilapia and skipjack tuna. As far as the former suggestion is concerned, there is considerable information showing the slower tryptic digestion of red muscle than white⁶⁻⁸⁾. Even in case of carp, red muscle myosin had been found to be digested at 6 times slower rate than its white muscle myosin⁹⁾.

The present findings suggest that if actomyosin is to be chosen as the interspecies criterion of tryptic susceptibility, then the assay at higher KCl concentration is desirable, otherwise, the myosin should be chosen to compare the susceptibility of ATPases. The order of stability of myosin ATPase is in good agreement with that established on the basis of heat or urea inactivation. This adds to the general belief that a protein susceptible to a particular denaturant is susceptible to others also. Therefore, the relative stability of tilapia and tuna myofibrillar proteins, in comparison with other fish species studied here, can be considered

reliable. This will in turn mean that these myofibrillar proteins may withstand commercial processing such as dehydration or chemical modification to fish protein concentrates (FPC) best.

The above findings further suggest that the favourable conditions to prepare heavy-meromyosin or subfragment-1 from fish muscle would be substantially different from those of the rabbit HMM or S-1.

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