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Non-binding property of cathepsin L to myosin

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Abstract

Disodium pyrophosphate at 10 mM concentration, was effective in dissociating myosin and actin from actomyosin in walleye pollock (*Theragra chalcogramma*) surimi and red bulleye (*Priacanthus macracanthus*) surimi. After Sepharose 2B gel filtration, cathepsin L contained in the actomyosin was obviously non-binding to myosin. Actomyosin from carp (*Cyprinus carpio*) muscle was not dissociated in pyrophosphate solution in the absence of $MgCl_2$ and it was successfully dissociated by 10 mM pyrophosphate in the presence of 2 mM $MgCl_2$. Cathepsin L in carp actomyosin was shown to be much more complicated than that in the above two surimis. After Sepharose 2B gel filtration, there were two activity peaks of cathepsin L in carp, one almost corresponding with actomyosin, the other obviously separated from actomyosin. Both of the peaks were non-binding to myosin.

Keywords: Cathepsin L; Pyropshosphate; Walleye pollock surimi; Red bulleye surimi; Carp; Dissociation; Myosin

1. Introduction

Cathepsins are usually characterized as members of the lysosomal cysteine protease (active site) family and the name of cathepsin family had been synonymous with lysosomal proteolytic enzymes. Among these cathepsins, cathepsin B and L has proteolytic activity for a variety of protein substrates (Jiang, Lee, & Chen, 1996; Okitani, Matsukura, Kato, & Fujimaki, 1980; Yamashita & Konagaya, 1991). Cathepsin L is very unstable at neutral and alkaline pH (Dufour, Dive, & Toma, 1988), despite the fact that most of the other cathepsins are stable over a wide range of pH values. Since most of the lysosomal proteinases are active at acidic pH, they are considered to be involved in the postmortem tenderization of terrestrial animal muscles (Etherington, Taylor, Wakefield, Cousins, & Dransfield, 1990) and muscle softening of marine fish during spawning migration (Yamashita & Konagaya, 1990a, 1990b, 1990c, 1991). However, in none of the above studies, were cathepsins considered to cause surimi gel softening. An, Weerasinghe, Seymour, and Morrissey (1994) found that cathepsin B and H in Pacific whiting could be removed by washing treatments, but cathepsin L could not. Jiang, Lee, Tsao, and Lee (1997) reported that cathepsin L was stable and difficult to remove, and the residual cathepsin L, as well as cathepsin B, had MHC-degrading activity and consequently cause gel softening. Until now, few studies exist on the binding properties of cathepsin L to myofibrils, despite the fact that it is difficult to remove from myofibrils.

In the previous study (Hu et al., in press-a), we endeavoured to reveal the non-binding property of cathepsin L to actomyosin. In walleye pollock surimi and red bulleye surimi, cathepsin L showed one main activity peak separated from the AM peak, indicating it was non-binding to AM (Hu, Morioka and Itoh, 2007a, in press-a). In carp, cathepsin L was much more complicated than that in many other species, as there were two main activity peaks, one almost corresponding to the AM peak, the other, separated from the AM peak. The separated peak was about 81% of the total activity (Hu et al., in press-b). These two surimis and carp muscle were used as materials in this study because cathepsin L contained in them may be different, considering their different gel filtration profiles. The objective of this

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study was to further elucidate the non-binding property of cathepsin L to myosin.

2. Materials and methods

2.1. Chemicals and surimi material

Z-Phe-Arg-MCA was purchased from Peptide Institute Inc. (Osaka, Japan). All the other chemicals were of analytical grade. Frozen walleye pollock (*Theragra chalcogramma*) surimi (SS1 grade) was provided by Maruha Ltd. Co., Japan. Frozen red bulleye (*Priacanthus macracanthus*) surimi was provided by Southern Cross, Indonesia. The frozen surimi was immediately used or kept at −80 °C for future use. A live carp (*Cyprinus carpio*) with fork length of 21.5 cm and body weight of 207.88 g was obtained from a local fish vender and was sacrificed immediately in ice. After its death, actomyosin was extracted from the dorsal muscle.

2.2. Actomyosin extraction

Actomyosin was prepared using the previous method (Hu, Morioka, & Itoh, 2007b). Surimi was washed three times with low ionic strength buffer and was then treated with one cycle of dilution–precipitation. The actomyosin obtained was dispersed in 0.6 M NaCl–50 mM phosphate buffer containing 10 mM pyrophosphate (buffer D, pH 7.0) or buffer D containing 2 mM $MgCl_2$ (buffer E, pH 7.0) and was ready for gel filtration.

2.3. Determination of protein concentration

Protein concentration was assayed by the Biuret method (Robinson & Hodgen, 1940) with bovine albumin as standard.

2.4. Assay of enzyme activity

Enzyme activity was measured by the previous method (Hu et al., 2007b) using a specific substrate of Z-Phe-Arg-MCA (Barrett & Kirschke, 1981). One unit was defined as 1 nmol AMC liberated within 30 min at 25 °C.

2.5. Sepharose 2B gel filtration of actomyosin

The sample solution was loaded onto an Excell SD450 column (2.6 × 40 cm) which was packed with Sepharose 2B (Pharmacia Fine Chemicals). The column was eluted with buffer D or buffer E at 0.35 ml/min. Fractions (5 ml) were collected by a fraction-collector (Gilson 202, France). Cathepsin L activity was monitored for each fraction.

2.6. Sepharose 6B gel filtration of carp actomyosin

Sepharose 6B gel filtration of carp actomyosin was performed as previously reported (Hu et al., 2007b). The actomyosin solution was loaded onto an Excell SD450 column (2.6 × 40 cm) packed with Sepharose 6B (Pharmacia Fine Chemicals). The column was eluted at 0.5 ml/min. Fractions were collected 10 ml/tube by a fraction-collector (Gilson 202, France). Cathepsin L activity was also monitored in each fraction.

3. Results and discussion

The myosin–actin interaction is very important, not only for muscle contraction but also for the stability of the two proteins (Torigai & Konno, 1996). Myosin from fish as well as from rabbit, is significantly stabilized by forming actomyosin complexes. Yamashita, Arai, and Nishita (1978) reported that actin from rabbit, carp and scallop equally stabilized myosin of these three species in the same manner.

ATP is well known to dissociate actin–myosin complexes into each protein in a high-salt medium in addition, pyrophosphate could be substituted for ATP. Pyrophosphate could dissociate the actomyosin and therefore, enhance the thermal denaturation of Alaska pollock myosin at high-salt concentration (Konno, 1992). A greater efficacy of pyrophosphate in the dissociation of kuruma prawn actomyosin was observed when compared with ATP (Benjakul, Visessanguan, Aewsiri, & Tanaka, 2007). This result was in contrast to the report of Torigai and Konno (1996) that ATP was more effective in dissociating carp myofibrils than was pyrophosphate, and the reason was proposed to be due to the differences in the structural arrangement of muscle proteins between fish and prawn.

Sepharose is a bead-formed agarose-based gel filtration matrix. Both Sepharose 2B and Sepharose 6B have broad fractionation ranges, 7×10^4–4×10^7 and 1×10^4–4×10^6 (molecular weight of globular proteins) respectively, which makes them suitable for separating or fractionating samples containing components of diverse molecular weight. Previously, we found that after Sepharose 6B gel filtration, cathepsin L in walleye pollock surimi and red bulleye surimi, was obviously separated from actomyosin (Hu et al., in press-a, 2007a) and the main activity peak of cathepsin L was more than 90% of the total activity (Hu et al., in press-b). The results strongly suggested that cathepsin L, in these two surimis, was possibly non-binding to actomyosin. In this study, in the presence of pyrophosphate, actomyosin from walleye pollock surimi and red bulleye surimi was easily dissociated into myosin and actin. Sepharose 2B medium could separate peaks of actin and myosin from that of actomyosin (Figs. 1 and 2) better than Sepharose 6B (data not shown). After gel filtration by Sepharose 2B, three peaks were visible. Peaks a, b and c were actomyosin, myosin and actin, respectively (Figs. 1 and 2). It was obvious that the cathepsin L activity peak was separated from that of the myosin peak in both the figures, suggesting it was non-binding to myosin.

It may be due to the difference of the actomyosin structure between surimi and fresh fish muscle that under the same filtration conditions, carp myosin (peak b) was not

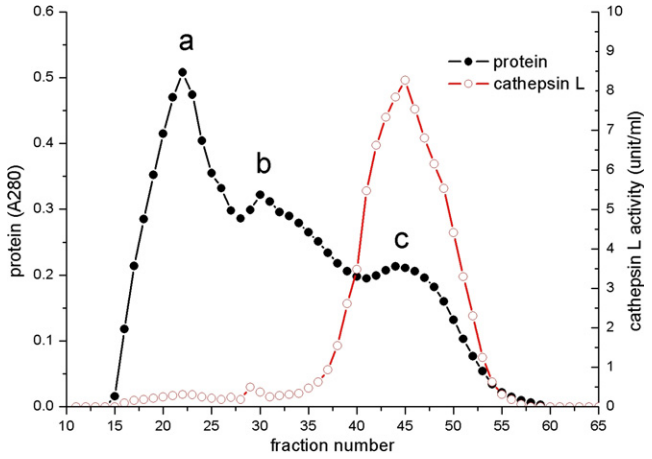

Fig. 1. Sepharose 2B gel filtration of AM from walleye pollock surimi. AM (75 mg) was loaded, eluted with 10 mM pyrophosphate–0.6 M NaCl–50 mM phosphate buffer (pH 7.0), 0.35 ml/min. Fractions of 5 ml/tube were collected.

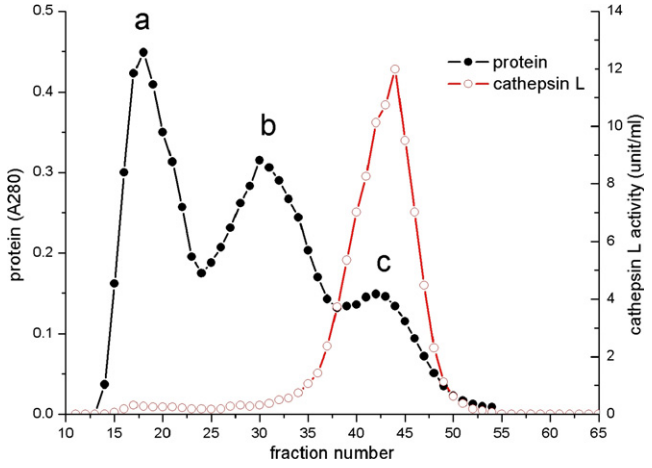

Fig. 2. Sepharose 2B gel filtration of AM from red bulleye surimi. AM (65 mg) was loaded, eluted with 10 mM pyrophosphate–0.6 M NaCl–50 mM phosphate buffer (pH 7.0), 0.35 ml/min. Fractions of 5 ml/tube were scollected.

successfully dissociated from actomyosin (peak a), despite a small amount of actin (peak c) being observed (Fig. 3). Cathepsin L was shown to have two activity peaks, one closely corresponding to the AM peak (P1), the other separated from that of AM (P2). In addition, P2 was about 53% of the total activity.

Actomyosin is salt soluble, and as a result, the concentration of the salty solvent would affect the stability of the protein. Dissociation of actin from myosin by the addition of pyrophosphate was restricted to salt concentrations between 0.3 and 1.0 M (Torigai & Konno, 1996). In this experiment, 0.6 M NaCl solution was sufficient for the dissociation by pyrophosphate. On the other hand, the addition of pyrophosphate in combination with $MgCl_2$ was reported to be more effective than was ATP in dissociating the actomyosin complex (Benjakul et al., 2007). To dissociate carp actomyosin into myosin and actin, $MgCl_2$ was added to the AM sample to a final concentration of 2 mM.

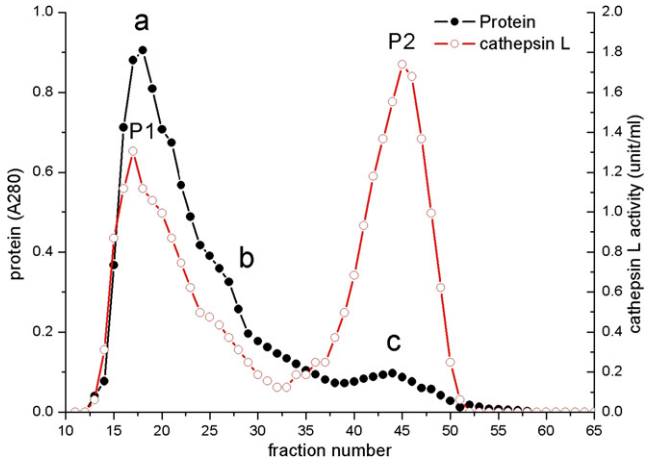

Fig. 3. Sepharose 2B gel filtration of AM from carp in the absence of $MgCl_2$. AM (65 mg) was loaded, eluted with 10 mM pyrophosphate-0.6 M NaCl–50 mM phosphate buffer (pH 7.0), 0.35 ml/min. Fractions of 5 ml/tube were collected.

The resulting AM sample was loaded onto a Sepharose 2B column and eluted with buffer E (buffer D containing 2 mM $MgCl_2$, pH 7.0). After gel filtration, myosin (peak b) and actin (peak c) were successfully dissociated from actomyosin (peak a). Cathepsin L was shown to have two main activity peaks, one almost corresponding with that of actomyosin (P1), the other obviously separated from that of AM (P2). P1 and P2 were about 32% and 54% of the total activity, respectively (Fig. 4).

Previously, carp AM was subjected to Sepahrose 6B gel filtration, eluted with 0.6 M NaCl–50 mM phosphate buffer (buffer C, pH 7.0) by the same method as described before (Hu, Morioka, & Itoh, 2007a, in press-a). In the absence of pyrophosphate, actomyosin kept intact. Cathepsin L was also shown to have two main peaks (Fig. 5), where P2 was about 81% of the total activity (Hu et al., in press-b). In this study, we found that after Sepharose 2B gel filtration,

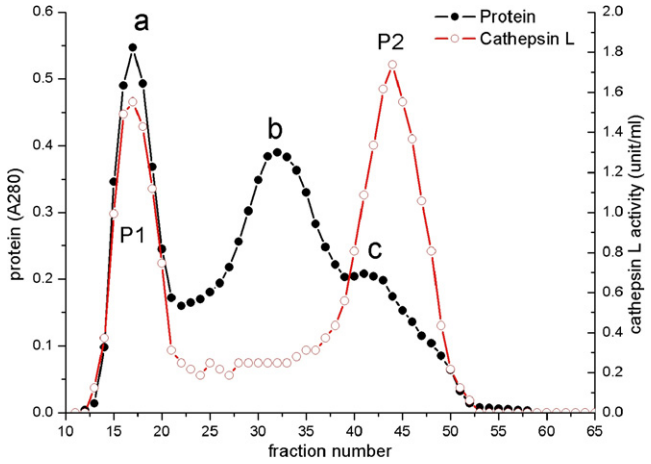

Fig. 4. Sepharose 2B gel filtration of AM from carp in the presence of $MgCl_2$. AM (65 mg) was loaded, eluted with 2 mM $MgCl_2$–10 mM pyrophosphate–0.6 M NaCl–50 mM phosphate buffer (pH 7.0), 0.35 ml/min. Fractions of 5 ml/tube were collected.

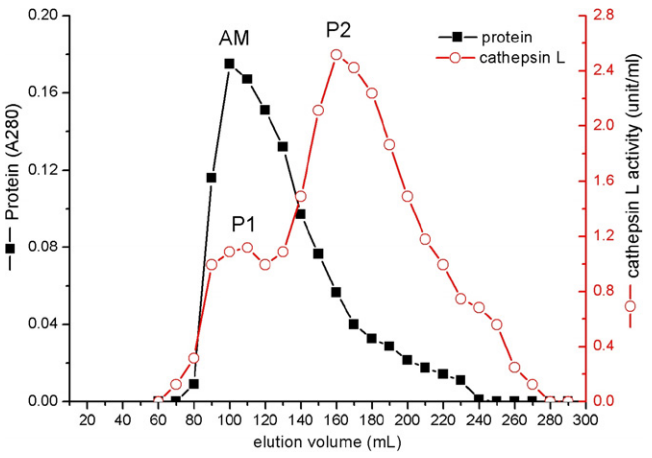

Fig. 5. Sepharose 6B gel filtration of AM from carp. AM suspended in 0.6 M NaCl–50 mM phosphate buffer (buffer C, pH 7.0) was loaded to Sepharose 6B column, eluted with buffer C, 0.5 ml/min. Fractions of 10 ml/tube were collected.

P1 and P2 of cathepsin L separated from each other much better (Figs. 3 and 5). Considering P1 and P2 in Figs. 3–5, it was possible that P1 was associated with actomyosin. Whether the characteristics of P1 and P2 are similar or not remains unknown in this experiment. But we could conclude that both P1 and P2 are non-binding to myosin. The results suggested that there are two kinds of cathepsins L in carp actomyosin, one closely bound to AM, the other non-binding to AM or myosin.

Although the reason was not clear, the differences of cathepsin L contained in surimi and carp are very interesting. Many aspects should be considered including the differences between fresh water fishes and marine water fishes, variation of fish species, effects of the living environments of fishes as well as the differences between fresh fish muscle and surimi.

4. Conclusion

In conclusion, cathepsin L contained in walleye pollock surimi and red bulleye surimi is myosin non-binding. In carp, although there are two kinds of cathepsin L, both of them are myosin non-binding.

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