博士（水産科学）　THITIMA JANTAKOSON

学位論文題名

Characteristic denaturation of myosin and actin in whiteleg shrimp (*Litopenaeus vannamei*) abdominal muscle upon heating and freezing

（タイ国産エビの加熱、凍結によるミオシンとアクチンの特徴的な変性）

学位論文内容の要旨

Shrimps are important internationally traded fishery products. Major species of cultured shrimp are black tiger shrimp and whiteleg shrimp. The latter is the most recently abundantly cultured shrimp in Thailand because of its merits of quick growth, high tolerance to change in salinities, easy domestication, and ability to spawn in captivity. Shrimp and shrimp products are distributed in frozen state to global markets. In spite of its importance, there is little information on the properties of the edible meat parts; muscle protein, myosin and actin. The purpose of the project was to clarify the properties of shrimp muscle proteins, myosin and actin, and to characterize their denaturation profiles upon heating and frozen storage. To study these, various types of materials were employed from the simplest material of myosin isolated, myofibrils and shrimp meat itself.

In chapter 1, the fundamental properties of shrimp myosin were investigated using whiteleg shrimp, black tiger shrimp, and kuruma prawn as shrimp samples. Myofibrils of these showed similar striation on the surface by microscopic observation indicating the typical structure of striated muscle. Shrimp myosin was isolated from these myofibrils by a direct application of the established method for fish myosin isolation, which is the ammonium sulfate fractionation method in the presence of Mg-ATP. The Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) pattern of purified shrimp myosin showed two types of Light Chain component (LC) that were similar to the ones found with crayfish myosin. Three types of ATPase activities (Ca^{2+}-, K^{+}-, and Mg^{2+}-ATPase) of shrimp myosin were also similar to those of fish myosin showing high activities of Ca^{2+}- and K^{+}-ATPase and negligible Mg^{2+}-ATPase activity.

In chapter 2, the internal structure of shrimp myosin was studied using chymotryptic digestion to determine whether shrimp myosin is cleavable at two distinct regions, namely at Subfragment-1 (S-1)/rod and Heavy MeroMyosin (HMM)/Light MeroMyosin (LMM) junctions dependent on the conditions. Shrimp myosin in myofibrils (filamentous form) was cleaved at the S-1/rod junction under a low salt medium in the presence of EDTA. Cleavage of myosin was accompanied by the degradation of a type of myosin light chain. However, the cleavage at the site was not specific for shrimp myosin and the cleavage at several sites on S-2 were also found. Such cleavage was never observed with fish myosin. The most striking difference between shrimp and fish myosin was that shrimp myosin was not cleaved into HMM and LMM even though the digestion was carried out at high concentrations of NaCl. Instead, shrimp myosin contained a unique cleavage site that was not affected by filament formation or

by Ca^{2+} addition. The cleavage at the site was prominent when digested in a medium containing 0.3 M NaCl and Ca^{2+}. These results indicate the internal structure of shrimp myosin differs from those of fish myosin. The cleavage sites in shrimp myosin Heavy Chain component (HC) were identified by determining the several N-terminal sequences of the fragments produced. For the identification of the cleavage sites, myosin from kuruma prawn instead of whiteleg shrimp was used because kuruma prawn myosin was similarly cleaved as whiteleg shrimp myosin and its primary structure of myosin HC has been determined. Two types of kuruma prawn myosin HC were reported, type A and B. All of the chymotryptic fragments produced were only from type B HC. Thus, types B would be the myosin mainly expressed in the abdominal muscle tissues.

Comparison of amino acid sequences around the three regions (S-1/rod junction, HMM/LMM junction, and newly found cleavage site on shrimp myosin) among myosin from several species showed the similar amino acid sequences at S-1/rod junction. The amino acid sequence around HMM/LMM junction for kuruma prawn was practically the same as those for other species of myosin containing cleavable hydrophobic amino acid (W). Nevertheless, shrimp myosin was not cleaved at the site. Amino acid sequence around the new cleaved site for kuruma prawn was different from others, and hydrophobic amino acid (F) was detected only with kuruma prawn. The secondary structure at the new cleavage site found with shrimp myosin in S-2 region was in the helical region. Accordingly, the secondary structure does not always determine the cleavage. Shrimp myosin as well as carp myosin showed a random coil structure at HMM/LMM junction area. However, chymotrypsin failed to cut shrimp myosin at the region. It was concluded that a much higher structure (tertiary structure) determined the cleavage.

In chapter 3, thermal denaturation of shrimp myosin in isolated form and in myofibrils was studied by using whiteleg shrimp mainly. As whiteleg shrimp is cultured in warm (23 to 32°C) culture ponds in Thailand, its high stability was expected. Thermal stability of myosin has been discussed using thermal inactivation rate of Ca^{2+}-ATPase quantitatively. The inactivation rate is usually calculated from the slope of the straight line produced by plotting logarithmic remaining activity against heating time by assuming a first order reaction mechanism. However, whiteleg shrimp myofibrils showed a curved inactivation, whereas myofibrils from fish showed a straight line as expected. As myosin isolated from whiteleg shrimp gave a straight line, whiteleg shrimp abdominal muscle contained practically a single type of myosin in terms of the stability. Several possible mechanisms to produce the curved profile were examined. The first possibility was less amounts of actin in the myofibrils. Relative amounts of actin to myosin were indistinguishable between tilapia and whiteleg shrimp myofibrils. The next possibility was the low affinity of shrimp myosin to F-actin showing insufficient protection in an early phase of the heating. The method used was to study NaCl concentration dependent thermal inactivation rates because increased NaCl concentration reduces the affinity. A complete loss of protection was achieved at 0.75-0.8 M with whiteleg shrimp myofibrils, which was roughly half that required for fish myofibrils (1.5 – 2.0 M). This suggests a weak binding of myosin to F-actin. It has been reported that binding of myosin to actin prevents salt-induced actin denaturation. Low affinity of shrimp myosin and actin at increased NaCl concentration was studied by measuring actin denaturation upon

treatment of shrimp myofibrils with NaCl on ice. Treatment above 0.75 M NaCl denatured actin. Another possibility was that F-actin in shrimp myofibrils protects myosin weakly. The strategy to answer the question was that F-actin in shrimp myofibrils was replaced by carp F-actin after denaturation of shrimp actin in myofibrils by NaCl-treatment. Such reconstituted actin/myosin complex also gave a curved profile as original shrimp myofibrils when proper amounts of actin (approximately 50%) were mixed with the treated myofibrils. When the amount of actin added was increased to 100%, inactivation became linear and slope became small. The fact indicated that myosin in shrimp myofibrils was not protected fully by F-actin present. It was concluded that curved inactivation for shrimp myofibrils was formed not by actin but by myosin. Black tiger shrimp and kuruma prawn myofibrils also gave a curved inactivation profile. A commonly observed property with kuruma prawn myofibrils was the loss of the protection by F-actin at lower concentration of NaCl (0.8 M).

In chapter 4, freeze denaturation of myosin and actin when stored in myofibrils and in meat was investigated at -20°C. As fresh whiteleg shrimp was not available, kuruma prawn was used. A strong protective effect of F-actin on myosin during the frozen storage of myofibrils was expected. However, Ca^{2+}-ATPase inactivation for myofibrils and isolated myosin was identical, indicating no protective effect of F-actin. The phenomenon was explained by a preceded and quick denaturation of actin in myofibrils taking only one day. In contrast, such actin denaturation was not observed when shrimp meat itself was frozen and stored. Actin denaturation was much slower than myosin denaturation. A half inactivation of Ca^{2+}-ATPase occurred after 300 days with meat, while a few day with myofibrils. It was proven that myosin is kept very stable in frozen meat probably by a strong protection by F-actin binding. The above results obtained with myofibrils and meat showed that myofibril cannot be used as a model material for meat even though a basic arrangement of myosin and actin is the same as in meat.

The extraordinarily high stability of myosin in frozen shrimp meat proved a strong protective effect of F-actin. Another possible factor involved in the high stability of myosin was stabilization by organic compounds in shrimp meat. The stabilizing effect of the compounds was suggested by the decreased stability of myosin in dialyzed muscle homogenate than homogenate itself. Moreover, myosin in the dialyzed meat was less stable than that in the original meat, but more stable than in myofibrils and was similar to one in dialyzed muscle homogenate. This indicates that unknown compounds in the shrimp meat also contributed to the increased stability of myosin in meat. Possible compounds are organic acid salts and amino acids.

The results obtained in these chapters provide evidence that frozen storage of shrimp meat does not denature myosin due to two factors: protection by F-actin and organic compounds in meat for a long period. The results suggest that high-quality shrimp meat can maintain its quality after 1 year of storage at -20°C. This will benefit shrimp manufacturers and consumers.

学位論文審査の要旨

主　査　　教　授　　川　合　祐　史
副　査　　教　授　　今　野　久仁彦
副　査　　准教授　　埜　澤　尚　範

学位論文題名

Characteristic denaturation of myosin and actin in whiteleg shrimp (*Litopenaeus vannamei*) abdominal muscle upon heating and freezing

(タイ国産エビの加熱、凍結によるミオシンとアクチンの特徴的な変性)

エビ類は世界中いたるところで最も好まれる水産物の一つである。また、単価の高い水産物であり、経済的な重要性も高い。天然エビの供給が限られる中、養殖エビの占める割合がどんどん大きくなっている。日本ではクルマエビが九州地方で養殖されているが、世界的に見た場合、東南アジア各国がその主要生産国である。かつてはブラックタイガーが養殖エビの大部分を占めていたが、現在は中南米が原産国であるバナメイエビに取って代わられている。その理由として、遊泳性であり水深が最大限に利用できること、性質が穏やかで共食いなどをしないこと、塩濃度変化、病原菌に対する耐性が高いこと、成長が早いことなどである。タイ国もその主要生産国である。養殖は主として輸出目的で行われている。養殖されたエビはさまざまな形で流通されるが、いずれもが凍結品である。それゆえ、その品質の維持は非常に重要である。このように重要な水産物でありながら、鮮度維持などn関する研究の蓄積は多いが、可食部である筋肉タンパク質に関する研究は日本を含めほとんどなされてこなかった。本論文では、主としてバナメイを用い、その筋肉タンパク質、ミオシンの単離、酵素的特性、内部構造などから魚類ミオシンとの相違を検討した。さらに貯蔵性に大きな影響を与えるエビミオシンの安定性について、筋原線維(Mf)およびエビ尾肉そのものを用い加熱、凍結によるミオシンおよびアクチン変性について魚類ミオシンと比較しながら検討した。

エビミオシンの三種の ATPase の酵素的特徴は魚類の良く似ており、低い Mg-ATPase 活性と高い Ca-, K-ATPase 活性が認められた。魚類ミオシンは哺乳類のものと同じような内部構造を持ち、キモトリプシンで切断を受ける部位が二箇所存在する。それらは低塩濃度で Ca 非存在下で S-1 と Rod に切断される部位と、高濃度の塩が存在し、ミオシンが溶解した条件で HMM と LMM に切断される部位であ

る。これら魚類ミオシンで確立されている切断はエビでは起こらなかった。すなわち、S-1/Rod に相当する切断は認められたが、Rod 内部の S-2 の複数の部位での切断が起きた。また、HMM/LMM に相当する部位では全く切断は認められなかった。さらに、魚類では切断されない新たな切断部位が S-2 に存在した。これらの結論は生きたクルマエビから調製した標品でも同じであったので、エビ類固有の内部構造であると結論した。この切断位置の道程は生成断片の N 端のアミノ酸配列分析と既知のクルマエビミオシン重鎖の配列の比較から得られた。

筋原線維(Mf)の熱変性を ATPase 失活から検討したところ、魚類 Mf では残存活性の対数値を加熱時間に対してプロットすると直線になる一次反応に従うが、エビの場合は曲線となった。その理由はアクチンが不足しているわけでなく、アクチンとの親和力が小さく安定化作用が弱いことであると推定した。アクチンをコイのものに取り換えても同じ結果を得たので、ミオシンの性質が異なると結論した。さらに、Mf を NaCl 濃度を変えて加熱した実験から、アクチンによる安定化作用が 0.75 M で失われることから、結合が弱いことが支持された。

M を-20℃で凍結貯蔵すると、アクチンは凍結一日でほぼ完全に変性し、その後 1 週間でミオシンの変性が続くことを見出した。このような変性は加熱では認められず、アクチンはミオシンの完全変性後も未変性のまま維持された。一方、エビそのものを凍結保存した場合にはアクチン変性はほとんど検出できないほど緩やかに進んだ。それより先にミオシン変性が認めらたが、Mf と比べると著しく遅かった。この理由として、アクチンによる安定化が維持されていること、さらに、エビ筋肉中に含まれている低分子成分の関与が示唆された。この成分の同定は出来なかったが、可能性のある成分として有機酸塩やアミノ酸が考えられた。加熱変性と凍結変性に対する基礎研究から凍結では変性抑制に必要な濃度が著しく小さくなり、全ての成分の作用は和となるので十分可能性は高い。

これらの研究成果は、アジアで重要な資源であるエビ類の凍結保蔵、品質維持のために大きな貢献をする可能性を示している。よって、審査員一同は申請者が博士（水産科学）の学位を授与される資格があるものと判定した。