論文内容の要旨

論文題目：Biochemical analyses of *Drosophila* Calpain B and endogenous calpain inhibitor
(ショウジョウバエのカルパイン B 及び内在性阻害因子の生化学的解析)

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The term calpain refers to a family of Ca^{2+} activated cysteine proteases. Among many different isoforms from various organisms, the mammalian μ- and m-calpains are considered as "typical" calpains and thus define the characteristics of what a calpain is. Despite many years of research, the activation mechanism and physiological functions of calpains are poorly understood. The Ca^{2+} requirement for activation of calpains is much higher than those observed in normal cells, and it is not well understood how these proteases can be activated in their natural habitat. Although many protein molecules have been identified as calpain substrates, the *in vivo* relevance of their interaction with calpain is unknown given that some of the fundamental properties of the enzyme remain unsolved. Knockout mice yielded limited information, either showing little to no defect or dying prematurely during the embryonic stages.

While most invertebrate calpains show significant deviations from the typical profile of calpains, those found in *Drosophila melanogaster* show significant homology and similarity to the typical mammalian calpains. Of the 4 calpain and calpain-related molecules identified, *Drosophila* Calpain A and Calpain B are highly

resemblant of mammalian m-calpain, both in terms of structure and enzymatic behavior. Thus, they show promising signs that could make *D. melanogaster* an alternative organism for use in the calpain research.

While the striking similarities are undeniable, several differences also exist in what we currently know about the *Drosophila* calpain system. First, the two typical calpains of *Drosophila* lack a small subunit, which is known to be essential in mammals. Secondly, the endogenous calpain inhibitor, termed calpastatin in mammals, remains to be identified in *Drosophila*. Added to this, characterization of the enzymes themselves have not progressed enough to safely assume that the mammalian and *Drosophila* calpain systems are true parallels.

I have conducted studies to draw further parallels between the mammalian and *Drosophila* calpain systems. My efforts were concentrated on:

1) Characterization of Calpain B and interaction with *Drosophila* homologs of known calpain substrates,
2) Investigation of Calpain B in regard to formation of dimer/polymers and the mechanism of autolysis,
3) Identification of the *Drosophila* calpain inhibitor.

1) Characterization of Calpain B and interaction with *Drosophila* homologs of known calpain substrates

Calpain B was expressed recombinantly and enzymatic properties were investigated. As reported previously, Calpain B was activated by millimolar Ca^{2+} concentration. It was also autolyzed much like m-calpain. The active cysteine residue, predicted based solely on m-calpain sequence to be at position 314 in the full length enzyme, was mutated to serine and alanine and activity was completely abolished. Thus, structural and behavioral similarities between Calpain B and m-calpain is apparent from these results.

In order to find out whether Calpain B is similar to m-calpain in terms of substrate specificity, *Drosophila* homologs of well-known calpain substrates were recombinantly expressed. The degradation patterns of these proteins by both Calpain B and m-calpain were examined. The two patterns were nearly identical to each other for each of the tested substrates, and also agreed with the predicted degradation

pattern from the known calpain cleavage sites in the mammalian substrates. Therefore, not only is Calpain B itself similar to m-calpain, but also shows similar substrate specificity, suggesting further parallels in their *in vivo* function. In addition, these results show that modification of the studied substrates by calpain cleavage has been conserved during evolution, adding much significance to the role of calpain in regulation of the substrates.

2) Investigation of Calpain B in regard to formation of dimer/polymers and the mechanism of autolysis

How Calpain B overcomes lack of a small subunit is examined in several ways. First, its natural molecular mass was examined using size exclusion chromatography and native polyacrylamide gel electrophoresis. Both methods suggested that the native molecular mass of active Calpain B is approximately 83 kDa, identical to that of monomeric Calpain B. Secondly, co-expression of 2 recombinant calpains having different affinity tags and subsequent purification methods targeting only one affinity tag at a time were used to see whether the 2 different recombinant calpains could be pulled down together. In case of dimeric interaction, purification by one affinity tag must pull down the other affinity-tagged calpain as well. However, this was not observed, and each purification method exclusively pulled down the intended affinity tags. The experiment was repeated using a mutant enzyme and an active enzyme having different tags, which allowed identification by activity. The result was identical. Therefore, Calpain B was found to be monomeric.

Mechanism of autolysis was studied using the inactive mutant and active Calpain B. In the mixture of the two, there was a significant delay in autolysis of the inactive mutant molecule, whereas the active enzyme was swiftly autolyzed. This suggests that the mechanism of autolysis is intramolecular, i.e.-self cleaving. Namely, only the active Calpain B underwent self cleavage (autolysis) in the early phase, and subsequently cleaved other molecules including the inactive mutant Calpain B.

3) Identification of the *Drosophila* calpain inhibitor

The *Drosophila* calpain inhibitor remains unidentified after 20 years of the first report describing its activity. There is no sequence in the *Drosophila* genome which resembles mammalian calpastatin, thus indicating the inhibitor may be

significantly different in the amino acid sequence from the mammalian inhibitor. Purification of *Drosophila* calpain inhibitor was attempted using some of the known properties of the mammalian calpastatin. As reported previously the substance was heat-resistant, allowing removal of heat labile proteins by boiling. Further purification was done using Q-sepharose and size exclusion chromatography, results of which suggested that the molecule may be 50 to 80 kDa in molecular mass. Further purification was unsuccessful due to fragility of the inhibitor. From our results, however, the inhibitor appears to be a protein.

Taken together, the results shown in the present thesis show several new aspects of the *Drosophila* calpain system that parallel the mammalian counterpart. First, the properties of Calpain B resemble those of mammalian m-calpain. Second, Calpain B show similar substrate specificity to m-calpain. Third, Calpain B does not form homomeric complexes. Fourth, there are strong indications of existence of a proteinacious endogenous calpain inhibitor in *Drosophila*. Further, I have characterized autolysis of Calpain B to be an intramoleular event. With these promising signs, the *D. melanogaster* could be an ideal organism for conducting experiments for calpains, and may contribute to expanding our understanding of these enigmatic proteases.