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Drosophila Calpain B is Monomeric and Autolyzes Intramolecularly

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Drosophila **calpains, Calpain A and Calpain B, show typical calpain domain structures similar to mammalian calpains. However, the small subunit of mammalian calpains, shown to be essential in both genetic and biochemical aspects, is absent in *Drosophila* calpains and is not required for enzymatic activity. How they compensate for the lack of small subunit is mostly unknown. Here we conducted experiments using recombinant *Drosophila* Calpain B for further characterization of the enzyme with particular focuses on two issues: possibility of homodimerization and mode of autolysis. The native molecular weight of Calpain B indicates that the active enzyme is primarily monomeric. Co-expression of two recombinant Calpain B proteins each with a unique affinity tag and a subsequent single round of affinity tag purification resulted in isolation of only one recombinant calpain type, suggesting there is no homodimeric interaction. Also the C-termini of *Drosophila* calpains lack many of the key hydrophobic residues considered to be important in the dimerization of mammalian calpains. Further, initial autolysis of Calpain B seems to occur intramolecularly, which supports the monomeric nature of *Drosophila* calpains. These results strongly suggest that dimerization is not an essential requirement for *Drosophila* calpains.**

Key words: autolysis, calpain, Drosophila, intramolecular, monomer.

Abbreviations: 2-ME, 2-mercaptoethanol; AP, alkaline phosphatase; DAB, diaminobenzidine tetrahydrochloride; ECL, enhanced chemiluminescence; EDTA, ethylenediamine tetraacetic acid; HRP, horse radish peroxidase; IPTG, isopropyl β-D-1-thiogalactopyranoside; OD, optical density; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulphonylfluoride; PVDF, polyvinylidene difluoride; RT-PCR, reverse transcription polymerase chain reaction; SDS, sodium dodecyl sulphate; TBST, Tris buffered saline containing 0.05% Tween 20.

Calpain is a cysteine protease whose activity is triggered by binding of Ca^{2+} ions (*1*, *2*). Although it is known to interact with and modify various substrates *in vitro* including cytoskeletal elements and apoptotic proteins, it still remains unconfirmed whether or not these interactions have any relevance *in vivo*. The calpain family includes a large number of molecules from various species which vary greatly in terms of homology (*3*). Most calpain-like molecules of invertebrates, with the exception of the *Drosophila* calpains, significantly differ from the mammalian calpains in terms of amino acid sequence and domain structure (*4*) and hence cannot be assumed to have a similar set of functions. The typical four-domain structures of mammalian calpains (*3*) are rarely traceable in these invertebrates, with little more than the bare essential catalytic domain II intact. Considering that other domains significantly contribute to the known functions of calpain such as the site of autolytic cleavage in domain I and calcium binding of the EF hands in domains III and IV (*5*), the lack of some of these domains significantly decreases the usefulness of the invertebrate calpains in understanding the conventional calpains.

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In contrast, studies of calpains in *Drosophila melanogaster* have revealed that these in fact do have significant homology to the mammalian calpains (*6–8*). Much like mammals, *Drosophila* has two ubiquitously expressed and enzymatically active calpains named Calpain A and Calpain B, which are encoded by *CalpA* and *CalpB*, respectively (*9*). Though some features unique to *Drosophila* are present, the four domains and their essential structural components are intact in both enzymes. Enzymatic analyses of the two calpains also showed resemblance to the mammalian calpains, being activated *in vitro* by millimolar calcium concentration like m-calpain and their calcium sensitivity increased by addition of phospholipids (*10–12*). Considering the available information regarding *Drosophila* calpains combined with the ease of manoeuvrability and simplicity of this organism, *D. melanogaster* presents an attractive alternative to mammalian species for calpain studies.

Several key differences exist, however. Since the completion of the *Drosophila* genome, there have been efforts to locate genes for calpastatin and the 28 kDa calpain small subunit. Calpain inhibition has been detected in partially purified *Drosophila* homogenate (*13*), which indicates that there is an endogenous calpain inhibitor that differs significantly from the known calpastatins at least in its DNA sequence. On the other hand, there is no biochemical or genomic evidence

pointing towards existence of a small subunit homologue in *D. melanogaster*. Considering the importance and necessity of the calpain small subunit as illustrated by the embryonic lethality of knockout mice (*14*) and formation of insoluble inclusion body when the recombinant large subunits are expressed by themselves (*15*), it is rather puzzling to find out the apparent lack of a small subunit homologue in *D. melanogaster*.

The lack of the small subunit may be compensated by homodimerization, a phenomenon already reported in the lobster (*16*, *17*) which also lacks the small subunit. Further, dimers of mammalian calpain domain IV and p94 have been reported, which indicates that domain IV has an intrinsic property to dimerize (*18–20*). Hence there is a possibility that *Drosophila* calpains also form homodimers. This also raises an interesting possibility of *Drosophila* calpains being autolyzed intramolecularly between the two subunits.

Here we present results from experiments using Calpain B which suggest that dimerization does not take place and the enzyme is fully active in the monomeric form. Additionally, the initial phase of Calpain B autolysis is most likely an intramolecular event, which further supports the idea of monomeric *Drosophila* calpains.

MATERIALS AND METHODS

Expression Vectors—The cDNA sequence of *CalpB* with truncated N-terminus was obtained by reverse transcription polymerase chain reaction (RT-PCR) (*6*, *8*). PCR was performed using a forward primer designed to introduce an *Nde*I site and six residues of histidine at the 5′ end and a reverse primer incorporating an *Xho*I site at the 3′ end. The PCR product was then cloned into pET 23a and pET 24a vectors using the two restriction sites. Another forward primer was designed to add a strep tag to the N-terminus of the protein in place of six histidine residues. The sequences of primers are as shown subsequently.

Forward primer for His-Calpain B

```
                start
5′  aga cat ATG cac cac cac cac cac cac ccc tat cca act ggc atg cog 3′
      NdeI    His His His His His His
```

Forward primer for Strep-Calpain B

```
                start
5′  aga cat ATG tgg agc cac ccg cag ttc gaa aaa ggc gcg ccc tat cca act ggc atg cog c 3′
      NdeI                Strep tag
```

Common reverse primer

```
                    stop
5′  g cct cga gga TTA gat tag tagcca att g 3′
      XhoI
```

Site-Directed Mutagenesis—A mutation to the catalytic cysteine residue of Calpain B was introduced into pET 23A His Calpain B using GeneTailor Site-Directed Mutagenesis System (Invitrogen) according to the manufacturer's protocol. The sequences of primers used in the process were as follows:

```
Forward for cysteine 186 to serine    5′ - GCAACAAGGAGAACTTGGCGAATCCTGGCTACTGG  -3′
                                           ||||||||||||||||||||||
Reverse primer          3′  -AAGTGCTAAGCTACACGGTTGTTCCTCTTGAACCGCTT -5′
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where the underlined codon represents mutation to serine, and the two primers overlap by 22 residues.

Expression and Purification Of Proteins—Constructed plasmids were transformed into either JM83 or BL21 strains of competent *Escherichia coli* cells for plasmid and protein purification. Competent cells were electroporated at 1.5 kV for 5 ms in electroporation cuvette plus and Electrocell manipulator (BTX ECM 395), after which 960 μl of ice-cold LB was added immediately. Electroporated cells in LB were transferred to 1.5 ml tubes and incubated at 37°C for 1 h. Then they were spread on LB-agar plates containing antibiotics for selection.

Transformed *E. coli* colonies were picked and grown in 5 ml LB containing appropriate antibiotics until optical density (OD) 0.4. The 5 ml culture was inoculated into 50 ml LB with antibiotics and was grown at 37°C for approximately 3 h until desired density. Cells were cooled to room temperature (23°C) before induction by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and were vigorously shaken for additional 1–3 h.

Cells were pelleted and washed once in 2 ml lysis buffer [100 mM Tris–HCl pH 8.0, 150 mM NaCl, 10 mM ethylenediamine tetraacetic acid (EDTA), 0.1% 2-mercaptoethanol (2-ME), 1 μM pepstatin and 1 mM phenylmethanesulphonylfluoride (PMSF)] and resuspended in 2 ml lysis buffer prior to lysis by sonication. Cell suspension was lysed by sonicating twice for 20 s, with a 5 min break in between to keep the lysate ice-cold. Following sonication, the lysate was spun down at 14,000*g* for 10 min in order to pellet insoluble cellular components from soluble supernatant. Samples were taken of the total lysate, insoluble pellet and soluble supernatant.

Soluble supernatant was used for purification of recombinant proteins. His-tagged Calpain B was purified using Protino Ni-TED 1000 (Macherey-Nagel). Prior to loading soluble supernatant, the column was equilibrated using 2 ml of the afore-mentioned lysis buffer with 15 mM $MgCl_2$ to prevent chelation of Ni^{2+} by EDTA. The same concentration of $MgCl_2$ was also added to the soluble supernatant, which was subsequently passed through the column twice by gravity flow. The column was washed using 2×1 ml lysis buffer with $MgCl_2$, and His-tagged protein was eluted using lysis buffer containing 200 mM imidazole and 15 mM $MgCl_2$ in batches of 1 ml. EDTA was immediately added to the eluates to a final concentration of 10 mM. Strep-tagged Calpain B was purified using Strep-Tactin Sepharose column (IBA BioTAGnology). Two millilitres of lysis buffer were used for equilibration without adding $MgCl_2$. Binding and washing were carried out in the same manner. Strep-tagged protein was eluted using lysis buffer containing 1 mM desthiobiotin (Sigma-Aldrich) in batches of 100 μl. Obtained proteins were kept at –70°C with 30% glycerol until use. The added glycerol was removed by centrifuge filtration using Microcon Ultracel YM-30 (Millipore Corporation) for 30 min at 14,000*g* at 4°C.

The concentrated protein remaining on the filter is diluted to its original volume with lysis buffer.

Casein Assay and Autolysis—Purified calpains were tested for activity using casein as a substrate. In a mixture containing 0.4% casein in casein buffer (50 mM Tris–HCl pH 7.6, 200 mM NaCl with 0.1% 2-ME), purified calpains were added and activated by addition of $CaCl_2$ to a final concentration of 10 mM. The mixture was incubated at room temperature (25–30°C) for desired time lengths, and reaction was stopped by adding sodium dodecyl sulphate (SDS) sample buffer and heating at 95°C. The level of calpain activity is assessed by running these samples on SDS polyacrylamide gels and visualizing casein bands.

Autolysis was carried out using an appropriate amount of purified calpain diluted in 20 μl of the afore-mentioned lysis buffer without protease inhibitors and was activated by adding $CaCl_2$ to a final concentration of 20 mM. Autolysis was stopped by directly adding SDS sample buffer to the reaction mixture and heating at 95°C. The samples were run on SDS–PAGE and analyzed as indicated subsequently.

Polyacrylamide Gel Staining and Western Blot—Protein samples were separated using SDS–PAGE and native polyacrylamide gel electrophoresis (PAGE) which were analyzed by either gel staining or western blot.

After separation, gels were washed in H_2O for 3×10 min. Enough gel staining solution (Bio-Safe Coomassie, Biorad) was added to completely submerge the gels. After 30 min of staining, gels were washed again in H_2O. When needed, protein bands were quantified using Dolphin Band Tool (Wealtec).

Polyvinylidene difluoride (PVDF) membranes for western blots were activated by soaking in methanol for 5 min, then in western blot transfer buffer along with six pieces of filter paper for another 5 min. Semi-dry transfer device was used to transfer proteins from polyacrylamide gel to PVDF membrane, by running at 10 V for 45 min and then at 30 V for additional 45 min. The resulting membrane was blocked with 2% BSA in Tris buffered saline containing 0.05% Tween 20 (TBST) on a shaker for 1 h. For detecting His-tagged proteins, membranes were incubated for an hour in 1:1,000 dilution of rabbit anti 6 X His antibody (Bethyl Laboratories) in TBST with 1% BSA. Membranes were subsequently washed in TBST once for 15 min and then three times for 5 min, and incubated in 1:1,000 horse radish peroxidase (HRP) conjugated anti-rabbit donkey antibody (Amersham Life Science) in TBST with 1% BSA. The membranes were washed again in the same manner as mentioned earlier.

For detection of Strep-tagged proteins, membranes were incubated, following blocking in BSA as explained earlier, in 1:1,000 HRP conjugated Streptavidin polymer (Sigma Aldrich) for 1 h. Membranes were subsequently washed as mentioned before. Proteins of interest were detected using diaminobenzidine tetrahydrochloride, DAB/Metal Concentrate (Pierce) as the substrate for HRP according to the manufacturer's protocol. They were also detected using enhanced chemiluminescence (ECL) western blotting detection reagents and analysis system (GE Healthcare) and Lumino CCD camera detection device (Atto Corp.). Shortly, blots were allowed to react with the ECL substrate for 1 min and were subsequently taken for photography. Various time periods were used according to the strength of the signal. Photographed images were saved and analyzed by Line Analyzer 10H (Atto Corp.) provided with the camera device. This software allowed quantification of protein in each lane by measuring the luminescence intensities of the bands. Luminescence intensity was shown to be directly proportional to protein quantity in our test runs using serial dilutions of Strep-Calpain B (data not shown).

Size Exclusion Chromatography—A Sephacryl S-300 column (bed volume of 120 ml) was prepared by pre-equilibrating with minimum 2 × bed volume of chromatography buffer (50 mM Tris–HCl pH 7.0, 10 mM EDTA, 200 mM NaCl, 1 μM pepstatin, 1 mM PMSF and 0.1% 2-ME) prior to each run. Initial calibration was carried out in a manner shown subsequently by separating a mixture containing molecular weight standards. Elutions of Blue Dextran (200 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa) were centred around elution volumes of 35, 65, 80 and 104 ml, respectively. His-Calpain B was prepared from the frozen 30% glycerol stock using centrifuge filtration to a final volume of 2 ml.

The sample to be separated was prepared in a total volume of 2 ml and was fed into a pre-equilibrated Sephacryl S-300 column at a rate of 0.2 ml/min. Subsequently, chromatography buffer was pumped into the column at a rate of 0.2 ml/min and eluates are collected in fractions of 2 ml. The run is stopped after 120 ml of buffer is pumped into the column, resulting in 60 fractions. Eluted fractions were analyzed for proteolytic activity by casein assay and protein profile by SDS–PAGE and Commassie staining. For casein assay, 25 μl of selected fractions were concentrated to 2.5 μl and used in the reaction mixture. For protein profile, 200 μl of selected fractions were concentrated to 20 μl and run on SDS–PAGE.

Sequence Analysis of Calpain C-Termini—The C-terminal 40 residues (39 for m-calpain) of murine and *Drosophila* calpain proteins were aligned using Genetyx gene analysis software. They were also subjected to hydrophobicity evaluation by Kyte and Dolittle method using a window size of five with the same software.

RESULTS

Monomeric Calpain B is Fully Active—Recombinant Calpain B with a His-tag at the N-terminus (His-Calpain B) was successfully expressed and purified as shown in Fig. 1. The soluble supernatant contained an abundant amount of the recombinant Calpain B for use in purification and activity assay. Enzymatic activity was tested using casein as a substrate as described earlier (Fig. 1B). The purified protein cleaved casein in a dose-dependent manner, and its proteolytic activity was Ca^{2+} dependent which was effectively blocked by chelation of Ca^{2+} with EDTA.

The purification profile of His-Calpain B on SDS–PAGE shows that the molecular mass of this protein is ~83 kDa, which is slightly smaller than the theoretical molecular mass of 91 kDa predicted from the amino acid

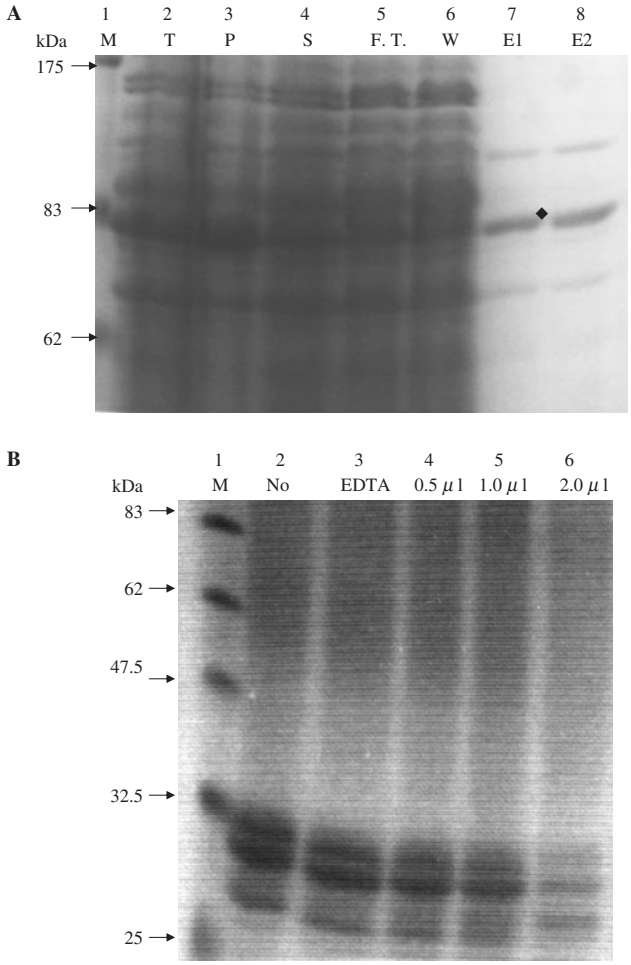

Fig. 1. **Purification and activity assay of His-Calpain B.** A: BL21 strain of *E. coli* expressing His-Calpain B was sonicated to obtain total cell lysate (lane 2, T), which was subsequently centrifuged to remove insoluble pellet (lane 3, P). The resulting soluble supernatant (lane 4, S) was passed through the purification column (lane 5, FT) and washed twice (lane 6, W). His-Calpain B (indicated with open square) was eluted from the column using 100 mM imidazole (lanes 8 and 9, E1 and E2). Lane 1 shows protein markers. B: Purified His-Calpain B was tested for activity using casein as a substrate. Casein was degraded in a dose-dependent manner as seen in lanes 4–6. Addition of EDTA in the reaction mixture resulted in inhibition of casein proteolysis (lane 3). Lane 1 (M): protein marker, lane 2 (No): no His-Calpain B in the reaction mixture, lane 3 (EDTA): EDTA in the reaction mixture with 2 μl of His-Calpain B, lane 4 (0.5 μl): 0.5 μl of His-Calpain B in the reaction mixture, lane 5 (1 μl): 1 μl of His-Calpain B in the reaction mixture, lane 6 (2 μl): 2 μl of His-Calpain B in the reaction mixture.

sequence. In order to determine whether the native form is monomeric, we subjected purified His-Calpain B to size exclusion chromatography using a Sephacryl-300 column. Analysis of eluted fractions using SDS–PAGE and activity assay are presented in Fig. 2A. The appearance of calpain, assessed by the band intensities on SDS–PAGE, first occurred at elution volume of 70 ml and ended at 80 ml, a range which typically includes proteins of 50–100 kDa such as BSA (Fig. 2A). On SDS–PAGE, the molecular mass of the eluted protein appears unchanged at ~83 kDa, showing no proteolytic event. To make sure that this protein is calpain, selected fractions were tested for proteolytic activity using casein assay. Activity assay revealed that the fractions containing the 83 kDa protein showed Ca^{2+} dependent proteolysis, confirming that the band corresponds to monomeric His-Calpain B. The lack of protein band on SDS–PAGE in the dimeric molecular weight range suggests that Calpain B is primarily monomeric in its native state and show proteolytic activity in this form.

If Calpain B is indeed monomeric, it should also appear as a monomer on native PAGE. The initial native PAGE showed a large proportion of His-Calpain B at ~70 kDa range (Fig. 2B). A much thinner band at ~170 kDa appeared on the gel, prompting the possibility of homodimeric Calpain B. Since subtle protein–protein interactions might get disrupted by the high salt concentration and reducing conditions of the sample buffer, it was possible that this high molecular weight band represented a small portion of dimeric Calpain B. To address this issue, samples of purified Calpain B were prepared in gel sample buffers containing either no NaCl (Fig. 2B, lanes 1 and 2) or 300 mM NaCl (lanes 3 and 4), and also containing either no (lanes 1 and 3) or 1 mg/l 2-ME (lanes 2 and 4). Under all tested conditions, the profiles of protein bands were identical, suggesting that the 170 kDa band might not be Calpain B but other protein species. To confirm that this high molecular weight band is not a dimeric form of Calpain, western blotting was employed. The use of anti 6 X His tag antibody should detect any forms of calpain. The result from native gel western blot shows exclusively monomeric calpain with no trace of the high molecular weight band (Fig. 2C). Therefore, it is most likely that the high molecular weight band is a contaminant which came with His-Calpain B during the purification process and that Calpain B exists in a monomeric form.

Calpain B Fails to Form Dimers—A different approach was taken to test whether or not Calpain B forms dimers. Our idea was to co-express two different recombinant calpains each with a unique affinity tag in the same *E. coli* cells and observe if purification using one affinity tag also pulls down the other. For this purpose, Strep-Calpain B was created by replacing His-tag of His-Calpain B with a Strep-tag, a modified segment of biotin which strongly binds to streptavidin (*21*). Since Strep-Calpain B binds to Streptavidin, it can be differentiated from His-Calpain B using western blots and streptavidin conjugated HRP or alkaline phosphatase (AP).

For the initial enzymatic assays, Strep-tag was expressed by itself and purified successfully as shown in Fig. 3A. Enzymatic activity was measured using casein assay. In order to roughly assess the specific activity of Strep-Calpain B in comparison with that of His-Calpain B, their concentrations were determined using SDS–PAGE. Instead of using Bradford assay which determines total protein concentration in a given sample regardless of protein identity, we opted to use the protein band on SDS–PAGE as a measure of enzyme concentration. As can be seen in Fig. 3C, the concentration of each enzyme was adjusted to be in a similar range.

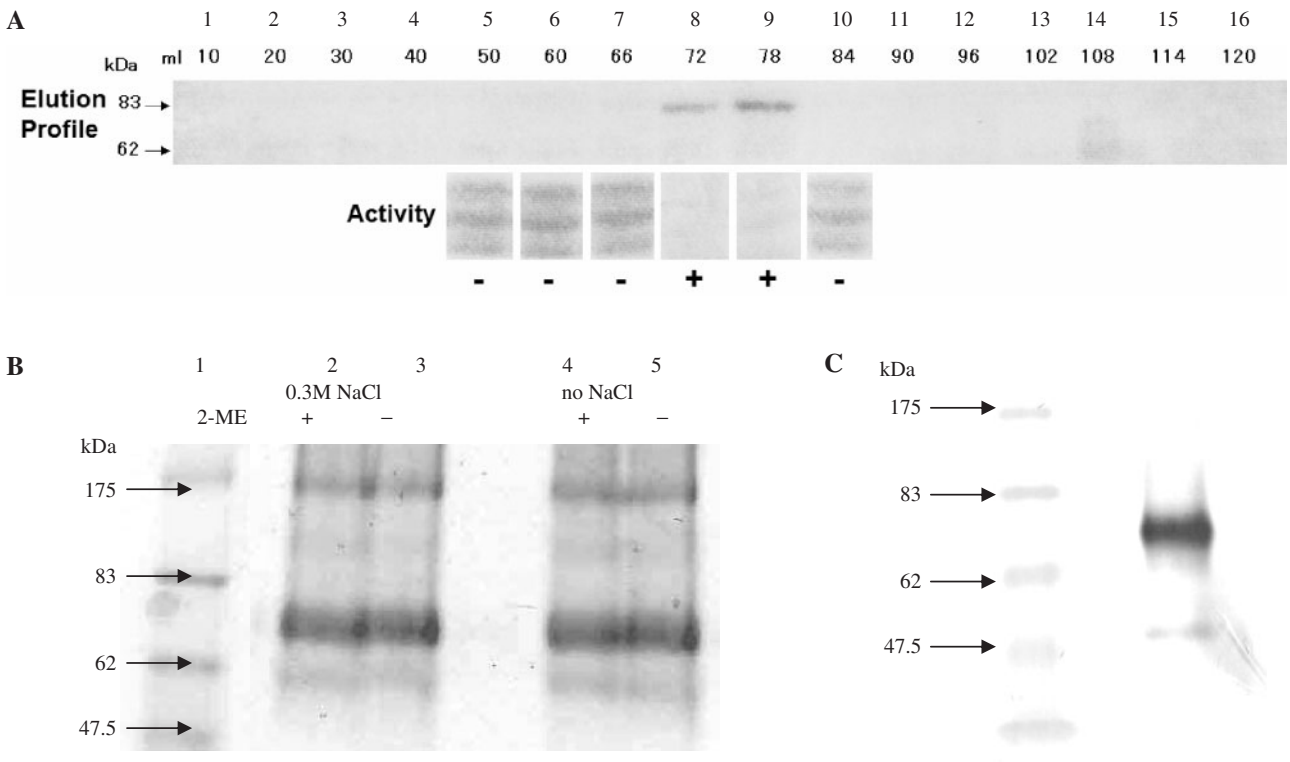

Fig. 2. **Calpain B is found in active monomeric form.** A: Purified His-calpain B was run through a Sephacryl-300 size exclusion chromatography column and the eluates were examined for their protein profiles using SDS–PAGE (upper) and their proteolytic activity by casein assay (lower). A protein band which appears to be ~83 kDa on SDS–PAGE is eluted in a range which typically includes proteins of less molecular mass possibly due to compact folding. Proteolytic activity measured by casein assay could be detected in the fractions containing monomeric calpains, indicating that Calpain B is active in monomeric form. Detection of proteolytic activity is indicated with plus (+), and absence of activity is indicated with minus (–). Marker proteins of BSA (66 kDa) and carbonic anhydrase (29 kDa) were eluted at elution volumes of 65 and 80 ml, roughly corresponding to lanes 7 and 10, respectively. B: The molecular form of purified His-Calpain B was examined using a native gel. Most His-Calpain B appeared as a monomer below the 83 kDa marker band. A much fainter band at ~170 kDa also appears. Possible dissociating factors of high salt concentration and reducing agent were added (indicated as either 0.3 M or no NaCl and 2-ME +/–) to the sample buffer to see if the profile could be changed. The addition of these factors did not result in any change between lanes 2 and 3 or lanes 4 and 5. Lane 1: protein marker, lane 2: His-Calpain B prepared with 0.3 M NaCl and 0.1% 2-ME, lane 3: His-Calpain B prepared with 0.3 M NaCl and no 2-ME, lane 4: His-Calpain B prepared with no NaCl and 0.1% 2-ME, lane 5: His-Calpain B prepared with no NaCl and no 2-ME. Native gels were made using the recipe for SDS–PAGE (*32*), but without adding SDS to the gel mixtures and sample buffer. C: Purified His-Calpain B was run on a native gel and tested by western blotting. While the thick band below the 83 kDa marker strongly reacted with anti-His antibody, the band at 170 kDa did not, indicating that it does not correspond to the dimeric form of Calpain B.

Activity assay of the new recombinant protein was carried out along with His-Calpain B as a reference (Fig. 3D left). Strep-Calpain B showed a comparable specific activity to that of His-Calpain B (Fig. 3D, right), as shown by the similar degree of casein degradation for each amount of enzyme input.

Following the initial characterization, Strep-Calpain B was paired up with His-Calpain B for co-expression in *E. coli*. Using the soluble supernatant of the *E. coli* lysate, Calpain B was purified using either His-tag affinity column or Strep-tag affinity column. Samples during the purification process were collected and analyzed using SDS–PAGE and western blots. Both purification processes successfully purified calpains, as shown by the bands at 83 kDa range in the elution samples (Fig. 4A upper lanes 7 and 14). With western blot, purified calpains obtained by each method could only be detected by the affinity tag used in the purification process. In other words, in the batch of calpain purified using His-tag, there was no Strep-Calpain B to be found (Fig. 4A middle). The opposite was also true for Strep-tag purified calpain (Fig. 4A lower).

To further confirm lack of interaction, we created a third recombinant Calpain B to be used in co-expression. This time, an inactive enzyme was created by mutating the active site cysteine of His-Calpain B at position 186 (314 in full length Calpain B) to serine by site-directed mutagenesis. Substitution of His-Calpain B with an inactive mutant allows distinction from Strep-Calpain B not only by immunological means but also by enzymatic activity. The resulting enzyme (His-Calpain B C186S) was expressed in *E. coli* and purified (Fig. 3B) for an initial enzymatic assay along with His-Calpain B and Strep-Calpain B (Fig. 3D). Despite having a similar concentration to those of His-Calpain B and

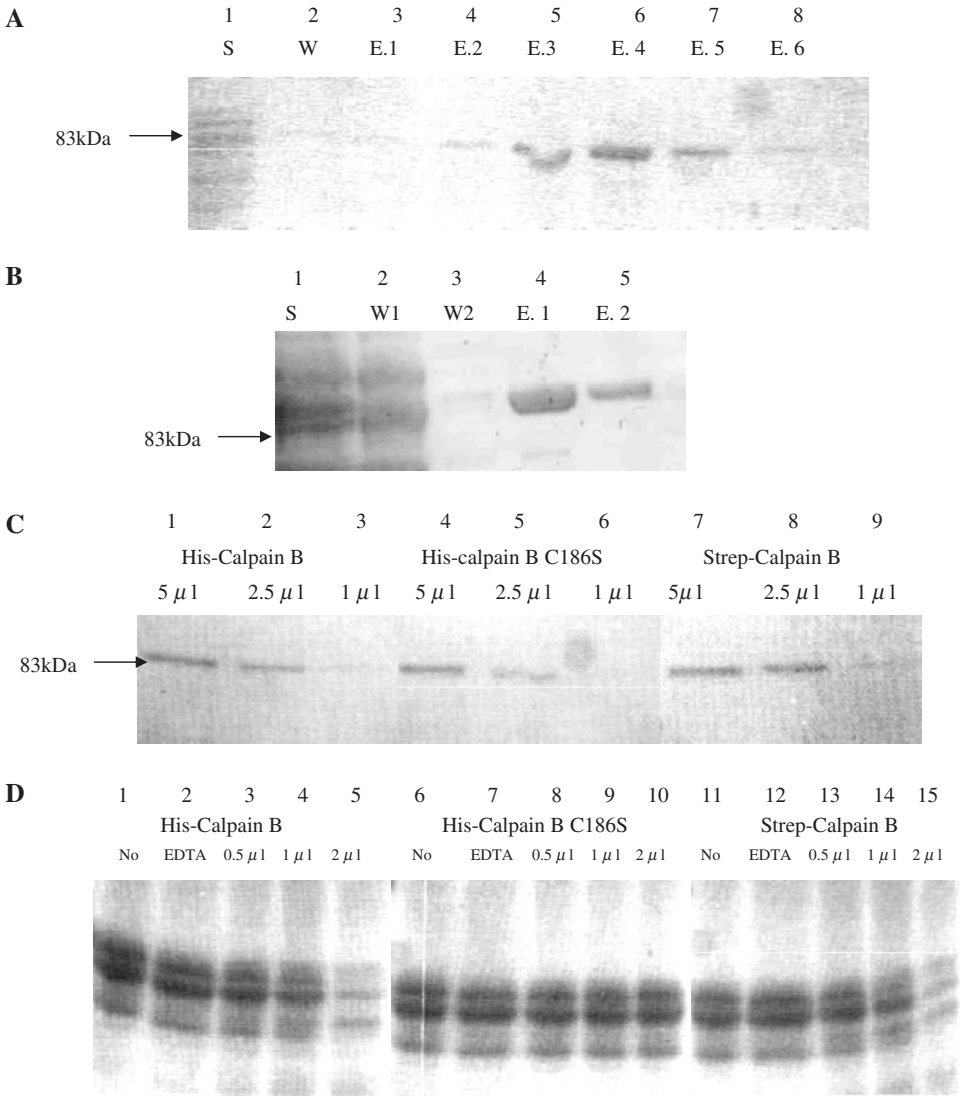

Fig. 3. **Purification and activity assay of Strep-Calpain B and His-Calpain B C186S.** A: Strep-Calpain B was expressed and purified successfully in BL21 strain of *E. coli*. Elution fractions 3 and 4 (lanes 5 and 6) show highest concentrations of the purified enzyme. Lane 1 (S): soluble supernatant, lane 2 (W): Strep-tag purification column wash, lanes 3-8 (E.1–E.6): eluted fractions 1–6 from Strep-tag purification column. B: His-Calpain B C186S was expressed and purified successfully in BL21 strain of *E. coli*. Elution fraction 1 (lane 4) has the highest concentration of the purified enzyme. Lane 1 (S): soluble supernatant, lane 2 (W1): His-tag purification column wash 1, lane 3 (W2): His-tag Strep purification column wash 2, lanes 4 and 5 (E.1 and E.2): eluted fractions His-tag purification column. C: Enzyme quantification using SDS–PAGE and Coomassie staining. Since the affinity purification processes resulted in contaminating proteins, equalization of enzyme content was carried out by visualizing the protein band of each enzyme on a gel for the subsequent activity assay. As can be seen in the dilutions of each enzyme type, there are approximately equal amounts of protein per volume. D: Activity levels of the three recombinant calpains were compared using casein assay. All three were inactive when EDTA was put into the reaction mixture (lanes 2, 7 and 12). His-Calpain B and Strep-Calpain B showed comparable levels of proteolytic activity, whereas His-Calpain B C186S did not show any signs of activity. Lanes 1, 6 and 11 (No): no enzyme in the reaction mixture, lanes 2, 7 and 9 (EDTA): EDTA in the reaction mixture with 2 μl of Calpain B, lanes 3, 8 and 13 (0.5 μl): 0.5 μl of Calpain B in reaction mixture, lanes 4, 9 and 14 (1 μl): 1 μl of Calpain B in reaction mixture, lanes 5, 10 and 15 (2 μl): 2 μl of Calpain B in reaction mixture.

Strep-Calpain B (Fig. 3C), His-Calpain B C186S did not show any hint of proteolytic activity (Fig. 3D, middle lanes). This clearly demonstrates that the mutation of active site cysteine has made His-Calpain B C186S an inactive enzyme.

His-Calpain B C186S was then co-expressed in *E. coli* with Strep-Calpain B for purification by either method. Unless there is interaction between His-Calpain B C186S and Strep-Calpain B, only inactive enzyme should be obtained by His-tag purification from these cells. As a comparison, we tested whether calpain purified from cells expressing His-Calpain B and Strep-Calpain B showed proteolytic activity. As expected, calpains obtained by both methods showed activity which degrades casein (Fig. 4B). On the other hand, when His-Calpain B C186S and Strep-Calpain B were

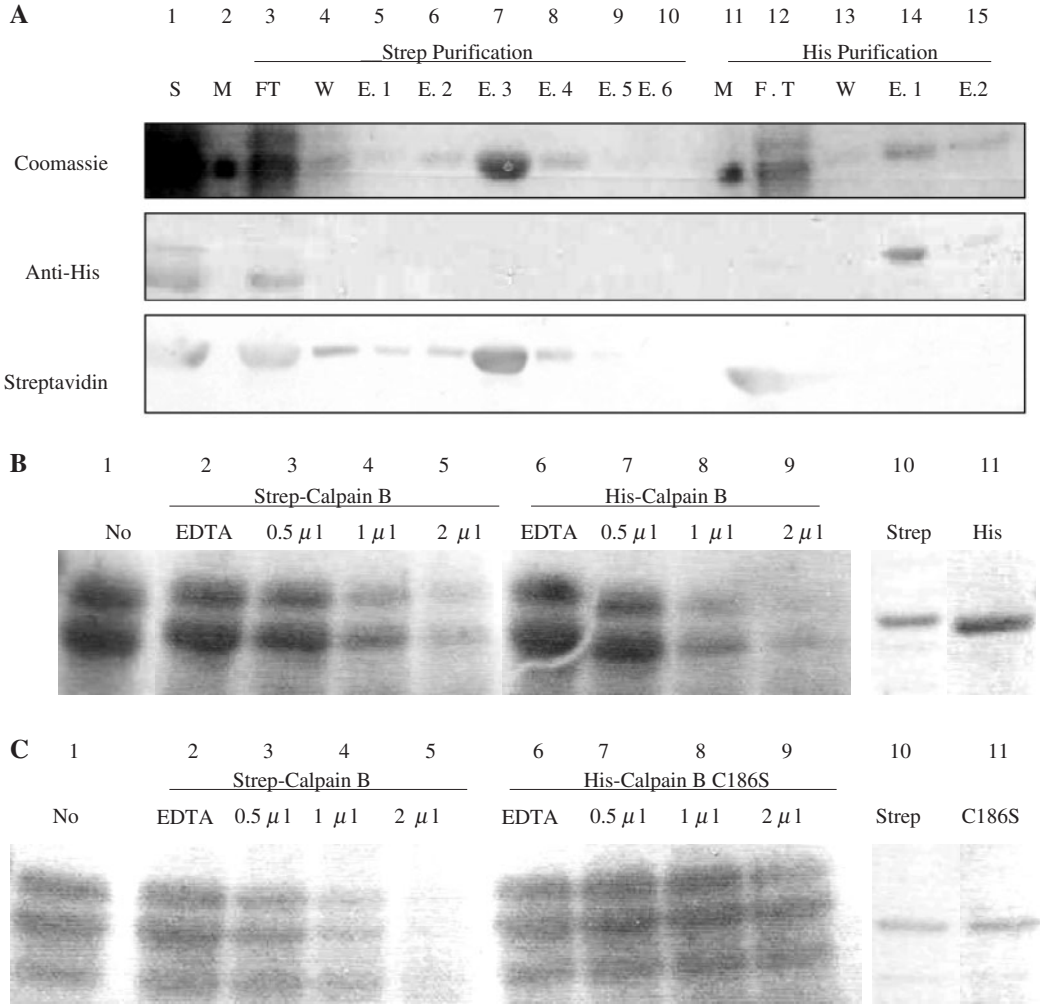

Fig. 4. **Calpain B does not form homodimers.** A: Strep-Calpain B was co-expressed with His-Calpain B in *E. coli* and its soluble supernatant was used for purification using His-tag affinity purification and Strep-tag affinity purification. Samples obtained from both purification processes were analyzed using Coomassie staining (upper), anti-His X 6 antibody western blot (middle) and Streptavidin western blot (lower). Both purification methods yielded visible amounts of expressed proteins on SDS–PAGE stained with Coomassie (lane 7 for Strep-Calpain B, lane 14 for His-Calpain B), indicating that both recombinant calpains were expressed successfully and abundantly. However, when the same samples were reacted with anti- His X 6 antibody (middle), only the soluble supernatant (lane 1), column flow-throughs (lanes 3 and 12) and elution fractions 1 and 2 of His-Tag purification (lanes 14 and 15) could be detected. Conversely, when Streptavidin was used (lower) instead of anti-His x 6 antibody, the soluble supernatant (lane 1), column flow-throughs (lanes 3 and 12) and strep purification elutions (lanes 4–8) were detected. The two western blots indicate that there is no interaction between the two differently tagged recombinant Calpain B proteins. Lane 1 (S): soluble supernatant, lanes 2 and 11 (M): protein marker, lane 3 (FT): Strep-tag purification column flow through of the soluble supernatant, lane 4 (W): Strep-tag purification column wash, lanes 5–10 (E.1–E.6): eluted fractions 1–6 from Strep-tag purification column, lane 12 (FT): His-tag purification column flow through of the soluble supernatant, lane 13 (W): His-tag purification column wash, lanes 14 and 15 (E.1 and E.2): eluted fractions 1 and 2 from His-tag purification column. Shown here is an experiment using *E. coli* expressing Strep-Calpain B and His-Calpain B. Results obtained from Strep-Calpain B and His-Calpain B C186S expressing *E. coli* are essentially identical and are omitted. B: Enzymes purified from *E. coli* expressing Strep-Calpain B and His-Calpain B were tested for proteolytic activities using casein assay. The concentrations of each enzyme were examined by running 10 μl of purified enzyme (lane 6 for Strep-Calpain B, lane 11 for His-Calpain B). Both Strep-Calpain B and His-Calpain B showed easily detectable proteolytic activity. Lane 1 (No): reaction mixture with no Calpain, lanes 2 and 6 (EDTA): reaction mixture with EDTA with 2 μl of Calpain B, lanes 3 and 7 (0.5 μl): 0.5 μl of Calpain B in reaction mixture, lanes 4 and 8 (1 μl): 1 μl of Calpain B in reaction mixture, lanes 5 and 9 (2 μl): 2 μl of Calpain B in reaction mixture, lane 10 (Strep): 10 μl of purified Strep-Calpain B for comparison of enzyme quantity, lane 11 (His): 10 μl of purified His-Calpain B for comparison of enzyme quantity. C: Enzymes purified from *E. coli* expressing Strep-Calpain B and His-Calpain B C186S were tested for proteolytic activity using casein assay. The concentrations of each enzyme were examined by running 10 μl of purified enzyme (lane 6 for Strep-Calpain B, lane 11 for His-Calpain B). While Strep-Calpain B showed easily detectable proteolytic activity (lanes 4 and 5), His-Calpain B C186S did not (lanes 7–9). This indicates that His-tag purification only isolated inactive His-Calpain B C186S and that there is no Strep-Calpain B being co-purified with His-Calpain B C186S, strongly suggesting lack of association between the two. Lanes are the same as in C, but with His-Calpain B C186S in place of His-Calpain B (lane 11).

expressed and purified using His-tag purification column and the resulting enzyme did not show any detectable activity (Fig. 4C, His-Calpain C186S), Strep-Calpain B from these cells showed activity comparable to those obtained from the cells expressing His-Calpain B and Strep-Calpain B. Taken together, there seems to be no detectable interaction between the two different recombinant calpains even when they were co-expressed in the same *E. coli* cells.

Calpain B Shows Rapid Intramolecular Autolysis—Autolysis of calpain at the N-terminus is believed to be an important trigger of enzymatic activity by significantly lowering the Ca^{2+} requirement (*22*, *23*). Opinions of both intramolecular (*24*) and intermolecular (*25*) autolysis have been reported, although a recent study of calpain crystal structure suggests that intramolecular cleavage of the large subunit N-terminus is practically unattainable due to the short distance between the site of autolysis and the active site (*19*).

Since dimerization of Calpain B is an unlikely event, we hypothesized that the mechanism of autolysis is an intermolecular event. To examine this idea, the following tests were performed. First, each type of recombinant calpain was allowed to autolyze in the presence of Ca^{2+} (Fig. 5A–C). Five minutes after the initiation of autolysis, most of the full-length His-Calpain B and Strep-Calpain B disappeared. On the other hand, the full-length His-Calpain C186S did not decrease as can be seen in the equal band intensity throughout all the time points (0–30 min).

Subsequently, a mixture of active and inactive calpains in 1:1 ratio was subjected to autolysis. If the autolysis reaction is an intramolecular event, active calpains should not cleave the inactive mutants, resulting in persistence of the full-length calpain band corresponding to the mutant calpain. Western blot luminescence quantification (Fig. 5D and E) was used to measure the rate of autolysis for both Strep-Calpain B and His-Calpain B, which showed that there was a significant delay in the autolysis of His-Calpain B C186S compared to Strep-Calpain B and His-Calpain B. In contrast to immediate decrease in the level of unautolyzed Strep-Calpain B, His-Calpain B C186S persisted for 5 min without noticeable decrease. After this apparent lag phase, His-Calpain B C186S was eventually degraded by Strep-Calpain B. Therefore the mechanism of cleavage in this secondary phase is intermolecular. From these observations it can be said that there are two phases of autolysis in the mixture of Strep-Calpain B and His-Calpain B C186S: initial intramolecular autolysis of Strep-Calpain B which rapidly takes place and then subsequent degradation of His-Calpain B C186S.

DISCUSSION

In our present article, we used *Drosophila* Calpain B for the study of dimerization and autolysis based on the following reasons. First, domain structures of the two *Drosophila* calpains (Calpain A and Calpain B) resemble those of mammalian calpains. Both enzymes have the typical four calpain domain structures with the characteristic features of each intact. Of the two, Calpain B is considered to be closer to mammalian calpains because it does not have the hydrophobic insert found in domain IV of Calpain A. This hydrophobic segment in domain IV is one of the major factors causing the recombinant Calpain A to be rather insoluble, as deletion of this segment led to a significant increase in solubility (*8*). This deletion, however, did not make Calpain A better than Calpain B in terms of yield. Considering the length of the deleted segment, this modification represents a significant departure from the natural form of Calpain A and is thus undesirable. Because nearly all expressed Calpain A forms insoluble aggregates, renaturing of the aggregate is required which then yields an enzyme with much weaker specific activity when compared to Calpain B. This indicates that the renatured enzyme may not fully have regained its native structure, which may in turn result in disruption of possible intra- and intermolecular interactions. Secondly, Calpain A was identified from *Drosophila* extract using the classical biochemical methods, during which the molecular weight of the active enzyme was determined to be approximately 84 kDa by size-exclusion chromatography (*7*). This result suggests that the enzyme is active as a monomer. In contrast, Calpain B was never purified from *Drosophila* extract in this manner but only obtained as a recombinant protein expressed in *E. coli* and isolated using an affinity tag.

As reported previously by Friedrich (*8*), His-Calpain B was successfully expressed and purified using *E. coli*. Further, we created two modified versions of the recombinant Calpain B, which are Strep-Calpain B and His-Calpain B C186S. The two active recombinant proteins demonstrate that *Drosophila* Calpain B does not require a small subunit homologue for proper functioning, despite the similarities in terms of domain structures to the mammalian counterpart. Absence of the small subunit causes mammalian recombinant calpains to be insoluble and inactive, which may be the reason behind the embryonic lethality of the small subunit knockout mice (*14*). Therefore, it was somewhat surprising that when recombinant Calpain B of *Drosophila* is expressed in *E. coli*, a significant proportion of soluble and active enzyme is obtained (*8*). This, along with the fact that domain IV of the mammalian calpain form a homodimer, has led to speculations that Calpain B might also be forming a similar homodimeric complex (*19*, *20*). However, the results presented in this article strongly suggest that dimerization neither is a requirement for activity nor is readily observed. Our conclusion is based on the following results. On a native gel, most of the purified Calpain B appears to be monomeric. A much fainter protein band at 170 kDa appears (Fig. 2B), but shows no immunoreactivity with the antibody directed against the recombinant enzyme (Fig. 2C). The monomeric calpain was also isolated using gel filtration and tested for proteolytic activity, which showed an activity level indistinguishable from the enzyme stock not subjected to purification in the same manner (Fig. 2A). This again shows that monomeric calpain is fully active, and accounts for most of the total activity. The results from co-expression also clearly indicates that homodimerization of Calpain B does not take place (Fig. 4).

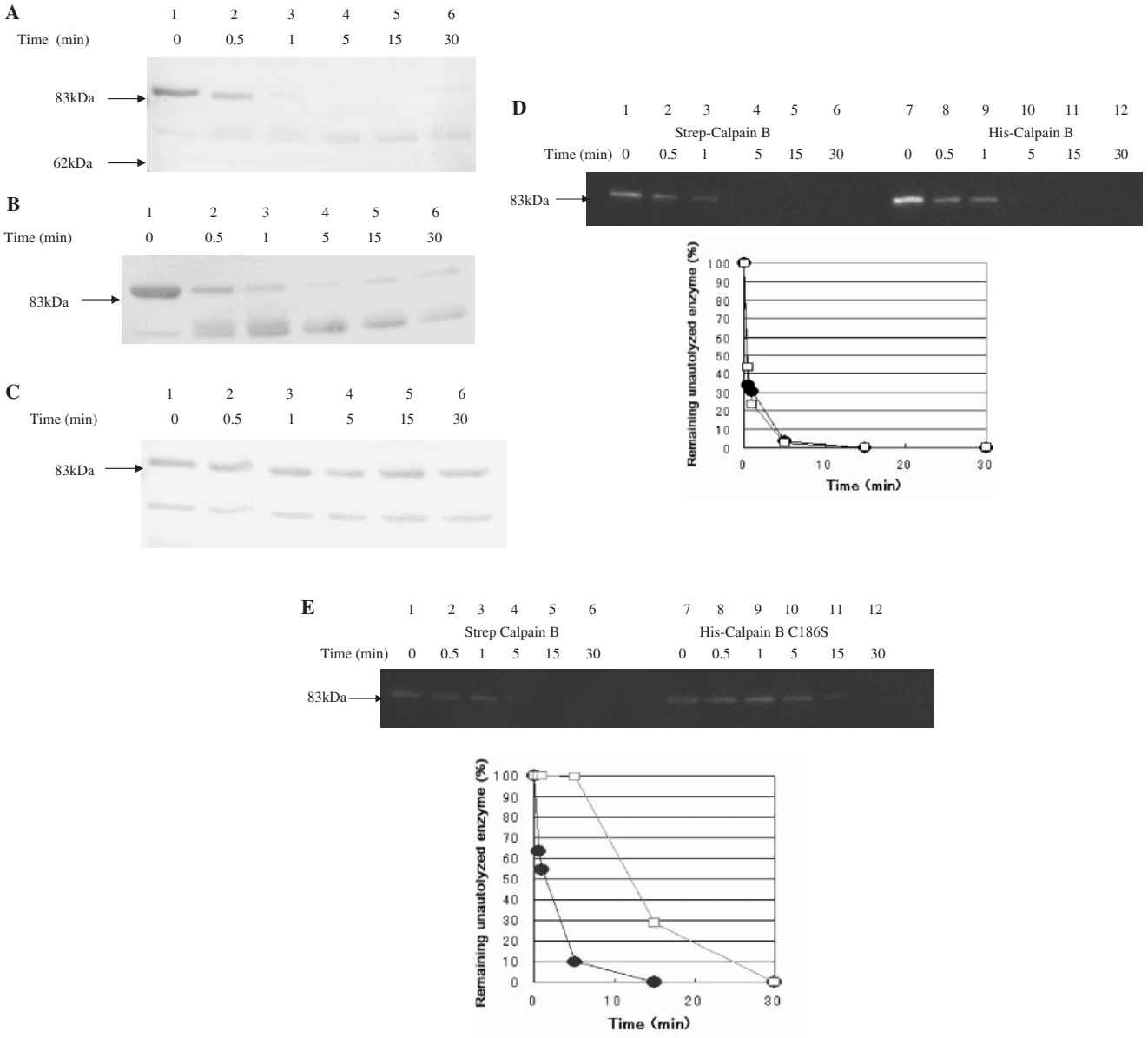

Fig. 5. **Autolysis of Calpain B.** A, B and C: Autolysis of Strep-Calpain B, His-Calpain B and His-Calpain B C186S was carried out as described in MATERIALS AND METHODS. While the amount of full-length Strep-Calpain B (A) and His-Calpain B (B) gradually decreased with time, His-Calpain B C186S did not (C). D: Strep-Calpain B and His-Calpain B were mixed in 1:1 ratio and allowed to autolyze for the indicated time periods. Autolysis was analyzed using western blot and luminescence quantification for each calpain (Strep-Calpain B: filled circle, His-calpain B: open square). More than 90% of the enzyme was similarly autolyzed within 5 min. E: Strep-Calpain B and His-Calpain B C186S were mixed in 1:1 ratio and allowed to autolyze for the indicated time periods. Autolysis was analyzed in the same manner as in (D). Compared to Strep-Calpain B (left, filled circle), His-Calpain B C186S persisted in its unautolyzed state for nearly 5 min, and then was gradually cleaved until becoming undetectable after 30 min (right, open square).

Dimerization was tested in two different ways. First, the lysate and purified proteins were tested using western blots and detection methods which detect the affinity tags used in purification (Fig. 4A). While both Strep-tagged and His-tagged proteins could be found in the supernatant prior to purification, only one type was detected after purification steps. Further, His-Calpain C186S obtained by His-tag purification from cell lysate also containing active Strep-Calpain B, failed to show any proteolytic activity, indicating that there was no association between the two recombinant enzyme types (Fig. 4C). The notion of the monomeric form of Calpain B is also supported from the lag period in the N-terminal cleavage of the inactive His-Calpain B C186S by active Strep-Calpain B. Formation of the hypothetical dimeric interaction of calpain from the mixed pool of Strep-Calpain B and His-Calpain B C186S monomers would result in 50% Strep-Calpain B and His-Calpain B C186S

heterophilic interaction and 25% each of Strep-Calpain B and His-Calpain B C186S homophilic interaction. Cleavage of His-Calpain B C186S should take place immediately after addition of Ca^{2+} in such a case. The apparent lag in cleavage observed in Fig. 5F suggests that such dimeric interaction is unlikely to occur, although the possibility cannot be completely ignored. It is interesting to note that a mammalian stomach-specific calpain (nCL-2) is found as a monomer and a possible dimer, both in soluble form without the small subunit, despite being highly resemblant of m-calpain in terms of amino acid sequence and biochemical properties (*26*). This result supports our idea that Calpain B can function as a monomer without a small subunit. They also report detection of a calpain oligomer which is formed via interaction of domain III, an unprecedented observation which did not occur in our experiments.

One possible explanation for the difference in regard to the small subunit between mammalian calpains and *Drosophila* calpains is that despite their similarity in domain structures, amino acid sequences of the two differ significantly. Sequence homology between m-calpain and Calpain B without the N-terminus is ~45%, enough to retain the essential features but not necessarily well conserved. The least conserved domain is domain IV, which is involved in dimerization with the small subunit in mammals (*27–29*). In fact, when the nine residues involved in 80–28k interaction (*1*) were compared, only three turned out to be common between Calpain B of *Drosophila* and murine m-calpain (Fig. 6A). The differences in the C-termini result in a significant increase in hydrophilicity of *Drosophila* calpains as judged by Kyte and Doolittle method (Fig. 6B and C). This increased hydrophilicity along with the absence of six or seven out of the nine residues in the C-termini of both *Drosophila* calpains represent a significant difference in the dimerization domain, which could explain the functional *Drosophila* calpain monomers.

We had initially hypothesized that Calpain B was intermolecularly autolyzed since it was thought that a monomeric calpain molecule, based on the crystal structure of mammalian m-calpain, was not likely to cleave its own N-terminus (*29*) and there was no detectable dimerization taking place. Much to our surprise, Calpain B initially appears to be autolyzed exclusively via intramolecular mechanism. This is explained by the persistence of His-Calpain B C186S (Fig. 5E), which should not occur in the case of intermolecular autolysis where active Strep-Calpain B should cleave both itself and the inactive His-Calpain B C186S indiscriminately. We also observed eventual cleavage of His-Calpain B C186S, which occurred after most of Strep-Calpain B had been autolyzed. This is because the concentration of calpain in our *in vitro* experimental condition was very high and the autolytic cleavage site is a good substrate for calpain proteolysis. Thus, intramolecular autolysis observed *in vitro* is also expected to be the predominant mechanism *in vivo* where calpain molecules are more sparsely distributed, unless a yet unknown mechanism of localization gathers calpain molecules in distinct subcellular spaces, organelles and/or structures.

A

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m-calpain      652  DDELIIDFDN FVRCLVRLET LFKIFKQLDP ENTGTIQLNL ASWLSFSVL-
μ-calpain      664  EPDLAVDFDN FVCCLVRLET MFRFFKLLDT DLDGVVTFDL FKWLQLTMFA
Calpain A      779  SRDGKIAFDD FIMCAVKIKT YIDIFKERDT EKNETATFTL EEWIERTIYS
Calpain B      876  SREGQIPFDD FLMCAIKVRT FIEMFRERDT DNSDTAFFNL DDWLERTIYS
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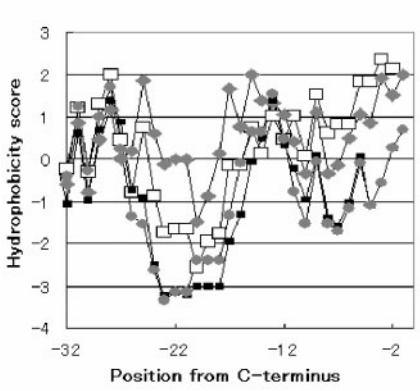
B



C

calpain species	Score
μ-calpain	0.467
m-calpain	0.0906
Calpain B	−0.825
Calpain A	−0.935

Fig. 6. **Differences in the properties of C-terminal sequences of mammalin and *Drosophila* calpains.** A: Alignment of the C-termini of murine m- and μ-calpains and *Drosophila* Calpains A and B. Underlined residues represent putative sequences involved in 80/28 kDa interaction (*18*). Out of the nine residues, *Drosophila* calpains only have two or three residues in common. B: Hydrophobicity of the C-termini of m-calpain (open square), μ-calpain (open square), Calpain A (filled square), and Calpain B (filled circle) were evaluated using Kyte and Dolittle method. Compared to the two mammalian calpains, the *Drosophila* calpains are less hydrophobic. B: Average hydrophobicity scores of the N-terminal 33 residues of m-calpain, μ-calpain, Calpain A and Calpain B were calculated using Kyte and Dolittle method. In line with b), the C-termini of the two *Drosophila* calpains have much lower hydrophobic scores than those of the mammalian calpains.

The 5 min lag phase in the autolysis of His-Calpain B C186S also indicates that unautolyzed Calpain B may be an inactive proenzyme which is not capable of cleaving anything other than itself. Such a lag phase have been observed in other calpain studies, where calpain autolysis took place before proteolysis of substrates (*24*, *30*). On the other hand, proteolytic activity of unautolyzed calpain has also been reported (*23*, *31*). The topic of proenzyme is much debated, and there is no unanimously agreeable theory which can explain all the observations made thus far. However, our results clearly indicate that the autolytic cleavage site of the active Calpain B molecule is preferentially cleaved intramolecularly prior to cleaving the mutant calpain molecules (Fig. 5E). It may be the case where an unautolyzed calpain molecule gains some proteolytic activity upon binding Ca^{2+}, at which point its own autolytic cleavage

site is a better substrate than any of the known substrates due to its proximity to the active site. Additionally, autolysis may induce a further conformational change which increases its proteolytic activity, which can be interpreted as an activation step.

Resemblance of *Drosophila* calpains to the mammalian counterpart offers a valuable tool for understanding the calpain system in an organism which is simpler and easier to manipulate. The full potential of *Drosophila* calpains is yet to be exploited, with many aspects such as genetic analyses, three-dimensional structures and substrate identification still remaining to be determined. Additional characterization of the enzymes themselves as well as their physiological implications using biochemical and genetic means will offer further insights into not only the *Drosophila* calpain system, but possibly those of higher organisms as well.

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