

Short communication

### INHIBITION OF CALPAIN BUT NOT CASPASE ACTIVITY BY SPECTRIN FRAGMENTS

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**Abstract:** Calpains and caspases are ubiquitous cysteine proteases that are associated with a variety of cellular pathways. Calpains are involved in processes such as long term potentiation, cell motility and apoptosis, and have been shown to cleave non-erythroid (brain)  $\alpha$ - and  $\beta$ -spectrin and erythroid  $\beta$ -spectrin. The cleavage of erythroid  $\alpha$ -spectrin by calpain has not been reported. Caspases play an important role in the initiation and execution of apoptosis, and have been shown to cleave non-erythroid but not erythroid spectrin. We have studied the effect of spectrin fragments on calpain and caspase activities. The erythroid and non-erythroid spectrin fragments used were from the N-terminal region of  $\alpha$ -spectrin, and C-terminal region of  $\beta$ -spectrin, both consisting of regions involved in spectrin tetramer formation. We observed that the all spectrin fragments exhibited a concentration-dependent inhibitory effect on calpain, but not caspase activity. It is clear that additional studies are warranted to determine the physiological significance of calpain inhibition by

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Abbreviations used:  $\alpha$ I-N1 – first 156 amino acid residues of erythroid  $\alpha$ -spectrin;  $\alpha$ I-N3 – first 368 amino acid residues of erythroid  $\alpha$ -spectrin;  $\alpha$ II-N1 – first 147 amino acid residues of non-erythroid  $\alpha$ -spectrin;  $\alpha$ II-N3 – first 359 amino acid residues of non-erythroid  $\alpha$ -spectrin;  $\beta$ I-C1 – residues 1898 to 2083 of erythroid  $\beta$ -spectrin;  $\beta$ II-C1 – residues 1906 to 2093 of non-erythroid  $\beta$ -spectrin; Ac-DEVD-pNA – N-acetyl-Asp-Glu-Val-Asp-p-nitroaniline, a caspase substrate; BSA – bovine serum albumin; CHAPS – 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DMSO – dimethyl sulfoxide; DTT – dithiothreitol; EDTA – ethylenediamine tetraacetic acid; HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS – 5 mM phosphate buffer at pH 7.4 with 150 mM NaCl

spectrin fragments. Our findings suggest that calpain activity is modulated by the presence of spectrin partial domains at the tetramerization site. It is not clear whether the inhibitory effect is substrate specific or is a general effect. Further studies of this inhibitory effect may lead to the identification and development of new therapeutic agents specifically for calpains, but not for caspases. Proteins/peptides with a coiled coil helical conformation should be studied for potential inhibitory effects on calpain activity.

**Key words:** Spectrin, Calpain, Caspase, Calpain inhibition, Cysteine protease

## INTRODUCTION

Calpains and caspases are cysteine proteases that are ubiquitous in vertebrate cells. Degradation of proteins by these two proteases is associated with many cellular pathways, including long term potentiation in neurons [1], cell motility and apoptosis [2] amongst others. Over 100 proteins, including spectrin [3], are calpain substrates [2, 4, 5]. Calpains are activated by an elevation of the calcium concentration, with micromolar  $\text{Ca}^{2+}$  concentrations for calpain 1 ( $\mu$ -calpain) and millimolar  $\text{Ca}^{2+}$  concentrations for calpain 2 (m-calpain) [6]. These two calpains are most well-studied. Their activities are negatively regulated by a specific endogenous peptide inhibitor calpastatin [7], as well as several non-specific inhibitors [8]. The inhibitor Cerebrolysin has been shown to improve neurodegenerative dementia caused by Alzheimer's disease and to accelerate the overall restoration of function after acute brain ischemia [9]. Caspases are essential in the initiation and execution of apoptosis [10], and they account for the majority of cellular and morphological events that occur during cell death [11, 12]. Regulation of activities of both calpain and caspase with inhibitors may provide means to manipulate signal pathways and to protect neural cells from degeneration [8, 13, 14].

Spectrin is a major component of the cytoskeleton in a wide range of cells, including neurons. The  $\alpha$ - and  $\beta$ -spectrins associate to form an  $\alpha\beta$  dimer, and two dimers form a functional  $(\alpha\beta)_2$  tetramer [15]. In humans the known isoforms consist of erythroid  $\alpha$ -spectrin ( $\alpha$ I), non-erythroid (brain)  $\alpha$ -spectrin ( $\alpha$ II), erythroid  $\beta$ -spectrin ( $\beta$ I), non-erythroid  $\beta$ -spectrin ( $\beta$ II), and other  $\beta$ -spectrins.

The spectrin isoforms are potential targets for both calpain and caspase actions during cell morphology change and apoptosis. Different spectrin isoforms show different susceptibility toward these two enzymes. For example, calpain does not cleave  $\alpha$ I, but cleaves  $\beta$ I at a single site, W2061-A2062 [16],  $\alpha$ II at two sites, Y1176-G1177 and G1230-S1231 and  $\beta$ II at four sites, Q1440-S1441, S1447-Q1448, L1482-A1483 and A2066-2067 [17, 18]. Caspase does not cleave  $\alpha$ I and  $\beta$ I but cleaves  $\alpha$ II at two sites, D1185-S1186 and D1478-S1479 [19] and  $\beta$ II at two sites, D1457-S1458 and D2146-S2147 [20]. The cleavage of  $\alpha$ II spectrin produces two fragments (150 and 145 kDa), known as the spectrin break down products, which are used as markers of traumatic brain injury [21].

The tetramerization region on  $\alpha$ -spectrin is at the N-terminus while on  $\beta$ -spectrin is at the C-terminus. The solution NMR structure of an  $\alpha$ I fragment consisted of the first 156 amino acid residues ( $\alpha$ I-N1) shows a partial domain with first 20 residues unstructured followed by a single coiled coil helix (residues 21-45) [22]. A five-residue unstructured junction region (residues 46-51) connects the partial domain to the first triple helical structural domain (residues 52-156). Another  $\alpha$ I fragment that extends the  $\alpha$ I-N1 to consist of the second and third structural domains ( $\alpha$ I-N3) [23], as well as similar fragments of  $\alpha$ II,  $\alpha$ II-N3 [24] and  $\alpha$ II-N1 [23] have also been studied. The  $\beta$ -spectrin C-terminal fragment of  $\beta$ I residues 1898-2083 ( $\beta$ I-C1) [22], or  $\beta$ II residues 1906-2093 ( $\beta$ II-C1) [25], consists of the last structural domain of  $\beta$ -spectrin, followed by the two-helix partial domain, and have been used as model protein to study spectrin tetramer formation.

In this study, we report an inhibitory effect of these fragments ( $\alpha$ I-N1,  $\alpha$ I-N3,  $\alpha$ II-N1,  $\alpha$ II-N3,  $\beta$ I-C1 and  $\beta$ II-C1) on the activity of calpain, but not caspase.

## MATERIALS AND METHODS

### Chemicals

Calpain 1 ( $\mu$ -calpain, human erythrocytes), casein, calpastatin and 3-[(3-cholamido-propyl) dimethylammonio]-1-propanesulfonate (CHAPS) were from Calbiochem (La Jolla, CA); caspase-3 (human recombinant) and N-acetyl-Asp-Glu-Val-Asp-p-nitro-aniline (Ac-DEVD-pNA) were from Biomol (Plymouth Meeting, PA).

### Protein samples

Six recombinant spectrin proteins,  $\alpha$ I-N1,  $\alpha$ I-N3,  $\alpha$ II-N1,  $\alpha$ II-N3,  $\beta$ I-C1, and  $\beta$ II-C1 were expressed and purified following standard laboratory procedures [23]. Glutathione (GSH) is known to inhibit calpain activity [26] and was used in protein purification. The protein samples were applied to a gel filtration column to remove GSH, though GSH in some samples containing cysteine residues was difficult to remove [27]. Two of the spectrin fragments ( $\alpha$ I-N1 and  $\alpha$ II-N1) consisted of no cysteine residues and thus should have little residual GSH. The protein samples in 5 mM phosphate buffer at pH 7.4 with 150 mM NaCl (PBS) were concentrated to 200-300  $\mu$ M, and extensively dialyzed against PBS overnight prior to assay measurements. The samples of different spectrin fragments were dialyzed in the same physical buffer to ensure that all protein samples were in the same solution conditions. The PBS buffer used for dialysis was saved and used to prepare controls in the assays. Some protein samples, along with their PBS buffer used for dialysis, were frozen for storage, thawed and centrifuged to remove particulate, if any, before the assay.

Polyacrylamide gel electrophoresis, with 16% acrylamide in SDS, was used to assess protein purity [23].

### Calpain colorimetric assay

Published calpain assay procedures [9] were followed, except that the calpain concentration (0.11  $\mu\text{M}$ ) was two times lower, but 5 times higher than recommended by the manufacturer. Briefly, the calpain reaction solutions consisted of  $\mu$ -calpain (0.4  $\mu\text{l}$ ; 0.11  $\mu\text{M}$ ), casein (10 ml; 85  $\mu\text{M}$ ), imidazole (10  $\mu\text{l}$ ; 0.1 M),  $\text{CaCl}_2$  (10  $\mu\text{l}$ ; 5  $\mu\text{M}$  - 5 mM), cysteine (10  $\mu\text{l}$ ; 5 mM), water and PBS buffer (40  $\mu\text{l}$ ), spectrin in PBS (40  $\mu\text{l}$ ; 20 - 250  $\mu\text{M}$ ), or calpastatin in PBS (40  $\mu\text{l}$ ; 150 nM). The total reaction solution volume was 100  $\mu\text{l}$ .

The absorbance value at 750 nm ( $A_{750}$ ) of the assay solution was measured. The activity ratio of a sample with spectrin was calculated from its  $A_{750}$  value divided by the  $A_{750}$  value of a sample with equal volume of PBS, but no spectrin. A value of 1 was expected if no inhibition was observed. The reaction mixture was initially incubated at different temperatures and times to determine optimal conditions for spectrin inhibition.

### Caspase colorimetric assay

The hydrolysis of caspase-3 substrate Ac-DEVD-pNA produces pNA. The concentrations of pNA, with and without spectrin fragments, were monitored spectroscopically at 405 nm [28]. Quantitatively, caspase in HEPES buffer was diluted with HEPES buffer (50 mM, with 100 mM NaCl, 0.5% CHAPS, 1 mM EDTA, 10% glycerol and 10 mM DTT at pH 7.4) to give a solution of 10 U/ $\mu\text{l}$ , with 1U defined as the activity of turning over 1 pmol of Ac-DEVD-pNA to pNA in 1 min. The substrate, Ac-DEVD-pNA (100 mM), was dissolved in DMSO, and this solution (1 ml) was diluted with HEPES buffer to 400  $\mu\text{M}$ . The diluted caspase solution (5  $\mu\text{l}$ ), Ac-DEVD-pNA (51  $\mu\text{l}$ ) and HEPES buffer (144  $\mu\text{l}$ ) were mixed and divided into three equal portions. To each portion, HEPES, PBS, or spectrin fragments at different concentrations in PBS (30  $\mu\text{l}$ ) was added to give "HEPES sample", "PBS sample" or "spectrin sample", respectively. This practice of using triplet samples allowed us to monitor the effects of spectrin fragments directly since all three samples consisted of the exact same amounts of caspase (5U) and its substrate Ac-DEVD-pNA (68  $\mu\text{M}$ ). Three different spectrin fragments ( $\alpha\text{I-N3}$ ,  $\alpha\text{II-N1}$  and  $\alpha\text{II-N3}$ ) were used. When the hydrolysis reaction was completed (22 hrs), the absorbance value at 405 nm ( $A_{405}$ ) of each of triplet was obtained, and converted to pNA concentrations using an extinction coefficient of 10,500  $\text{M}^{-1}\text{cm}^{-1}$  [28].

## RESULTS AND DISCUSSION

To optimize spectrin inhibition conditions, we first varied the  $\text{Ca}^{2+}$  concentration in the assay solution from 2  $\mu\text{M}$  to 5 mM, and found, not surprisingly, that about 50  $\mu\text{M}$  of  $\text{Ca}^{2+}$  was sufficient for full  $\mu$ -calpain activity in the absence of spectrin (Fig. 1A). However, to be consistent with other published results [6], we continued to perform calpain assays with a  $\text{Ca}^{2+}$  final concentration of 5 mM. We then performed the calpain reactions in the presence of a spectrin fragment,

$\alpha$ II-N1, at two different concentrations (17 and 48  $\mu$ M), and at different temperatures (10-40°C). We found reduced calpain activities at both concentrations, with more reduction at a higher concentration. The activity ratio (activity in a sample with spectrin fragment/activity without spectrin fragment, see Methods) was about 0.7 for samples with 17  $\mu$ M  $\alpha$ II-N1 and only about 0.1 for samples with 48  $\mu$ M  $\alpha$ II-N1, at 10°C (Fig. 1B). Furthermore, the effect was

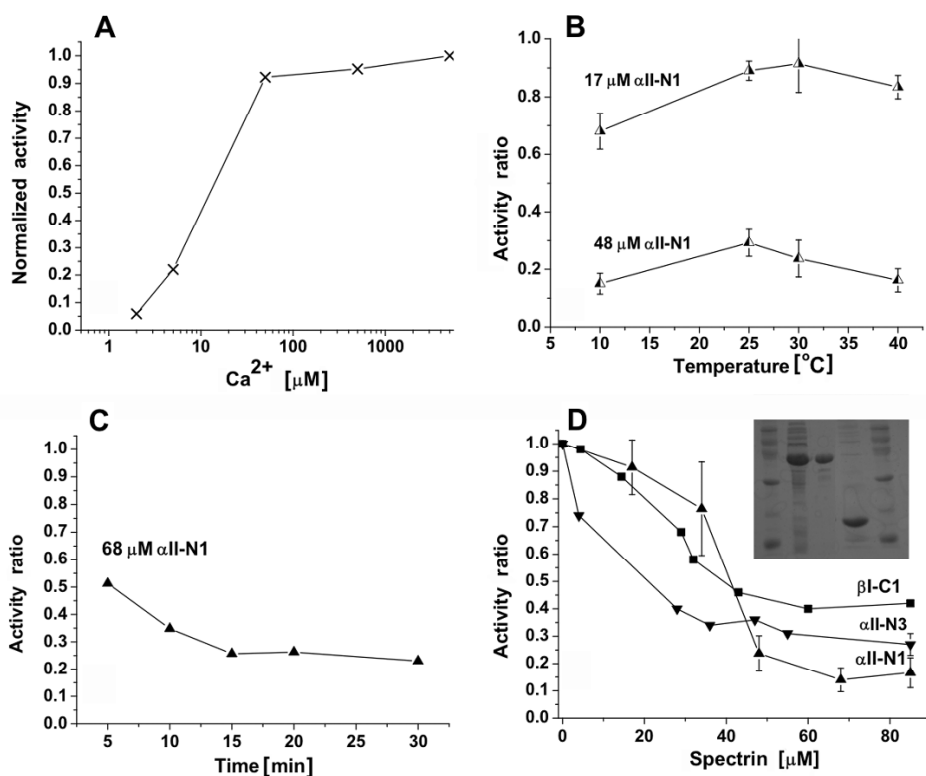


Fig. 1. Optimization of calpain assay conditions and calpain inhibition by spectrin fragments. A – The effect of Ca<sup>2+</sup> concentration on calpain activity, as reflected in A<sub>750</sub>. About 50  $\mu$ M Ca<sup>2+</sup> was sufficient for full calpain activity. B – The incubation temperature for the calpain reaction in the presence of  $\alpha$ II-N1 at 17  $\mu$ M or 48  $\mu$ M was varied. The inhibitory effect at each concentration was only slightly temperature dependent, with maximum activity ratio around 25-30°C. C – The inhibitory effect of  $\alpha$ II-N1 (68  $\mu$ M), measured by an activity ratio (see Methods), as a function of time. A value of 1 was expected if no inhibition was observed. The activity ratio leveled off after about 15 min. D – The concentration effect of  $\alpha$ II-N1 (up triangles),  $\alpha$ II-N3 (down triangles) and  $\beta$ I-C1 (squares) on calpain activity inhibition. Representative SDS-PAGE results are shown in inset, with the first lane (on the left) of the left panel being molecular mass markers (90, 66, 45, 30, 20 and 14 kDa from top to bottom), lane 2 being the cell lysate containing  $\alpha$ II-N3, and lane 3 being purified  $\alpha$ II-N3 (20  $\mu$ g) used in the experiments. The right panel lane 1 was size marker and lane 2 was  $\alpha$ II-N1. The concentration of Ca<sup>2+</sup> in B, C and D were 5 mM.

slightly temperature dependent at both concentrations, with a maximum activity ratio (least inhibition) around 25-30°C (Fig. 1B). All remaining assays were carried out on samples with calpain reaction at 30°C.

We also varied incubation time period of the calpain reaction in the presence of  $\alpha$ II-N1 (68  $\mu$ M) at 30°C. The activity ratio was about 0.5 after 5 min incubation and leveled off to about 0.25 after about 15 min (Fig. 1C). To ensure reaction completion, we used 30 min incubation time for all other assays.

Finally, we varied the concentration of spectrin fragments in the calpain reaction mixture. A clear concentration dependent effect was observed for  $\alpha$ II-N1 (Fig. 1D). Furthermore, a longer spectrin fragment,  $\alpha$ II-N3, and a  $\beta$ I-spectrin fragment of similar size to  $\alpha$ II-N1,  $\beta$ I-C1, appeared to exhibit similar concentration-dependent effect as  $\alpha$ II-N1. At saturating concentrations (50-80  $\mu$ M), the activity ratios were about 0.2-0.4. In samples with a spectrin fragment at 50  $\mu$ M, the spectrin to casein (substrate) ratio was about 0.6. Gel electrophoresis results of representative proteins showed that the proteins used were with high purity (inset of Fig. 1D).

Erythroid  $\alpha$ -spectrin fragments ( $\alpha$ I-N1 and  $\alpha$ I-N3) and non-erythroid  $\beta$ -spectrin fragment ( $\beta$ II-C1) exhibited similar inhibitory effects (Fig. 2). Activity ratio was about 0.25 at saturating concentrations (about 80  $\mu$ M) of  $\alpha$ -spectrin fragments, and about 0.4 for  $\beta$ -spectrin fragments.

We also tested the inhibition effect of calpastatin, a well known endogenous calpain inhibitor [29], under our assay conditions and found that the activity ratio was about 0.3 in the presence of 150 nM calpastatin. To test whether any protein will inhibit such inhibitory effect, we used BSA in same solution conditions and found no effect on calpain activity.

Results of protein gel electrophoresis performed on assay samples showed that the spectrin fragments remained intact (data not shown), indicating that the spectrin fragments were not cleaved by calpain. We observed no inhibitory effect on caspase activity by the same  $\alpha$ -spectrin fragments at the same concentration (about 80  $\mu$ M). It should be noted that the spectrin/caspase ratio was about 35 times higher than spectrin/calpain ratio. For  $\alpha$ I-N3 triplet samples (see Methods for the definition of the triplet samples), the average concentration of caspase cleavage product, pNA, was  $78.2 \pm 2.0$   $\mu$ M ( $n = 3$ ) for HEPES samples,  $69.7 \pm 5.1$   $\mu$ M for PBS samples, and  $81.0 \pm 4.5$   $\mu$ M for  $\alpha$ I-N3 in PBS. Similarly, for  $\alpha$ II-N1 triplet samples, the average concentration of pNA was  $79.4 \pm 10.7$   $\mu$ M for HEPES samples,  $68.6 \pm 15.1$   $\mu$ M for PBS samples, and  $80.4 \pm 12.6$   $\mu$ M for  $\alpha$ II-N1 in PBS samples. For  $\alpha$ II-N3 triplet samples, the average concentration of pNA was  $79.2 \pm 0.7$   $\mu$ M for HEPES samples,  $73.3 \pm 5.7$   $\mu$ M for PBS samples, and  $81.1 \pm 1.8$   $\mu$ M for  $\alpha$ II-N3 in PBS samples. These data indicate that there were no detectable differences in samples with and without spectrin fragments.

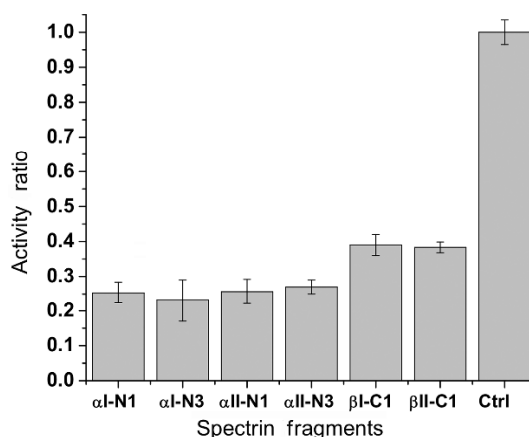


Fig. 2. The activity ratios of different spectrin fragments (about 68  $\mu\text{M}$ ) in PBS were about 0.25-0.35. The control sample consisted of PBS only. The concentrations of  $\text{Ca}^{2+}$  in these experiments were 5 mM.

In summary, with carefully controlled sample conditions, we showed a rather surprising, concentration dependent inhibitory effect on  $\mu$ -calpain activities of both erythroid and non-erythroid  $\alpha$ -spectrin N-terminal fragments, and  $\beta$ -spectrin C-terminal fragments. At saturating concentrations of spectrin fragments (about 50-80  $\mu\text{M}$ ), only about 30% calpain activity remained. However, no detectable spectrin effect was observed on caspase activity.

These spectrin fragments were found not to be substrates of calpain. Thus, the spectrin fragments bind to calpain, either at the active site to inhibit the action of calpain on the substrate casein, or to induce a conformational change rendering an inactive calpain molecule. The spectrin fragments that we used all consisted of triple helical bundle structural domain(s) and a partial domain that consists of "non-bundled" coiled coil helices, with one such helix in the  $\alpha$ -spectrin fragments and two in the  $\beta$ -spectrin fragments. Since the fragments consisted of three structural domains ( $\alpha\text{I-N3}$  and  $\alpha\text{II-N3}$ ) exhibited similar effects as the fragments consisted of only one structural domain ( $\alpha\text{I-N1}$  and  $\alpha\text{II-N1}$ ), we speculate that the partial domains in these fragments are responsible for the inhibitory effect. The structural requirements for substrate recognition by calpain are not well understood. A wide range of sequences have been found to flank known calpain cleavage sites, suggesting that calpain can accommodate a variety of side chains within its active site binding pocket [5]. It is generally considered that calpain probably recognizes global structural elements rather than specific primary sequences [5]. Analysis of published information on substrate proteins suggests that calpain cleaves within rather disordered segments of proteins, and interestingly, the segment C-terminal to the cleavage site resembles the consensus inhibitory region of calpastatin [5]. It appears that the binding of calpastatin to calpain induces an unstructured-to-helical conformational change

in calpastatin [30]. Thus, it is possible that the spectrin partial domain helix/helices may bind the calpain active site. Calpain has been reported to bind near to the C-terminal coiled-coil domain of SNAP-23 [31]. As mentioned above, spectrin itself is a substrate of calpain [16, 17], with the binding/cleavage site of non-erythroid  $\alpha$ -spectrin in the middle part of the molecule at an unstructured loop region [3, 16].

Recently we proposed a structure of  $\alpha$ II-N1 from spin-labeling electron paramagnetic resonance studies that is similar to  $\alpha$ I-N1, except in  $\alpha$ II-N1, the junction region is helical [24]. The  $\beta$ I-C1 and  $\beta$ II-C1 structures, though have not been reported, but through sequence homology analysis, is assumed to consist of two helices [25]. EPR studies support the helical conformation prediction in  $\beta$ I-C1 [32], and CD studies show that the two helices are unpaired [33]. The  $\alpha$ I-N1,  $\alpha$ I-N3,  $\alpha$ II-N1 and  $\alpha$ II-N3 proteins serve as model systems to study the spectrin tetramerization process since these proteins associate with  $\beta$ I-C1 or  $\beta$ II-C1 proteins with affinity similar to those of intact a and b association [23, 25].

It is not clear whether the inhibitory effect is substrate specific or is a general effect. In comparison with calpastatin, spectrin fragments used in this study were weak inhibitors. However, spectrin concentrations are relatively high in cells, *e.g.*, 10  $\mu$ M - 100  $\mu$ M in red blood cells [34]. Thus, high levels of spectrin fragments may potentially be found in cells. Physiological significance of spectrin fragments inhibition at micro-molar concentrations is not clear at this time but may warrant further studies. It is tempting to speculate the following. A frequently-encountered mechanism of enzymatic activity in neurons is the negative feedback regulation [35]. It is possible that spectrin breakdown products exert a negative feedback signal toward calpain activity, but not caspase activity. The appearance of non-erythroid spectrin breakdown products in cerebral fluid is considered as an early event in neural cell pathology [16, 20]. Accumulation of spectrin breakdown products may lead to inhibition of the calpain activity, thus preventing further disruption of the cytoskeleton. Future experimentation may provide insights on this potential mechanism of calpain on spectrin.

In addition to potential physiological significance of the inhibitory activity of specific spectrin fragments, our findings also allow us to suggest that, in future therapeutic agent research and development, proteins/peptides with coiled coil helical conformation should be tested for inhibitory effect on calpains. Such agents will be specific for calpains, but not for caspases. More detailed knowledge of molecular mechanism of this inhibitory effect will be needed to evaluate such possibility.

In conclusion, we have studied the effect of spectrin fragments on calpain and caspase activities. The erythroid and non-erythroid spectrin fragments were from the N-terminal region of  $\alpha$ -spectrin, and C-terminal region of  $\beta$ -spectrin, both consisting of regions involved in spectrin tetramer formation. We observed that all spectrin fragments exhibited a concentration-dependent inhibitory effect on



calpain, but not caspase activity. Further studies of this inhibitory effect may lead to the identification and development of new therapeutic agents specifically for calpains, but not for caspases.

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