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**Topic Category:** 4035-ASIP Extracellular matrix - proteases and remodeling**First Author:** Akia Parks

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**First Author is a:** Graduate Student**First Author is a member of:** Not a Member of a Host EB Society**First Author Degree:** BA, BS, or equivalent**Presentation Preference:** Oral**Sponsor:** Manu Platt**Sponsor Phone:** 404.385.8531

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**Sponsor's Society:** Biomedical Engineering Society (BMES) - APS Guest Society**Keywords:** 1. cathepsin 2. collagen 3. proteolysis

## Consecutive, But Not Concurrent, Cathepsin Incubation with Type I Collagen Results in Extended Proteolysis

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Many tissue-destructive diseases, such as atherosclerosis, osteoporosis, and tendinopathy, are characterized by a loss of the extracellular matrix protein type I collagen, in part mediated by proteases. Cysteine cathepsins are a family of powerful proteases implicated in collagen degradation. We have previously shown in a rat model of early tendon injury that cathepsin K (catK) and cathepsin L (catL) were upregulated at 4 weeks of injury, but only catL remained active at 8 weeks, with resultant tendon degeneration at 10 weeks. Given this temporal activity pattern, our objective was to elucidate how these proteases act in concert to degrade type I collagen by testing the hypothesis that catK, the most potent mammalian collagenase able to cleave it at multiple sites, initiates collagen degradation exposing sites susceptible to subsequent catL hydrolysis. To test this, we prepared collagen gels and incubated with concurrent recombinant catK with catL for 8 hrs, or consecutively, where the primary enzyme was incubated with collagen first, then the secondary enzyme was introduced after 4 hrs and continued to a total of 8 hrs.

Type I collagen gels (n=3) were incubated for 8 hrs at 37°C at pH 6 with recombinant catK alone (K), catL alone (L), concurrently (K&L), or consecutively (K-L, L-K, K-K, or L-L). Collected supernatant, referred to as the soluble fraction, contained released collagen fragments and soluble proteases, and the remaining collagen pellet was collected and contained any proteases still associated with the insoluble collagen gel. Cleavage of  $\alpha 1$  and  $\alpha 2$  chains was determined by SDS-PAGE and densitometry, and indicated that the consecutive protease incubations (K-L) resulted in at least four times greater degradation than K, L, or K&L (n=3, p<0.02).

Consecutive protease pair analysis also revealed that cat K and cat L (K-L, L-K), regardless of which was the primary enzyme, degraded as much collagen as consecutive K-K. L-L was significantly different (n=3, p<0.001) and only able to degrade 8% of the collagen. Western blot was used to verify that catK and catL were both still present after 8 hrs, and the amount of active protease was measured by zymography after 8 hours. In K&L and K-L studies, only catK was found in the collagen pellet, suggesting that either catL remained in the soluble fraction and did not remain bound to the collagen, or that catL may be free to cleave catK. Furthermore, catK was more present by western and active by zymo when consecutive with L (K-L) than when concurrent (K&L), suggesting that catL may have degraded catK in the system in addition to or instead of the collagen. This phenomenon has been coined cathepsin cannibalism by our group.

Taken together, these results showed catK & catL exhibited greater collagen degradation when incubated consecutively. The behaviors of multiple proteases on a collagen I substrate do suggest that ordered protease regulation may be important for enhanced pathological collagen disruption. This work begins to elucidate potential cathepsin behaviors in pathobiology and will inform protease inhibition strategies to prevent further degeneration of collagen I-rich tissues.

### Support or Funding Information

NIH T32 Cell and Tissue Engineering Training Grant