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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> Proteolytic cleavage of hemagglutinin is required for cell entry and plays a role in influenza virus pathogenicity. Little is known about the role of the bacterial flora of birds on the persistence and replication of avian influenza virus. Previous studies identified 56 caseinolytic-secreting bacterial species capable of cleaving hemagglutinin in a "trypsin-like" manner. The goal of this study was to identify and characterize Aeromonas hydrophila secreted proteases. We separated secreted proteases ion via ion-exchange and size-exclusion chromatography cell-free supernatants and fractions exhibiting proteolytic activity were sequenced for protein identification. Seven caseinolytic proteins were selected for isolation and characterization to determine the role of these respective contributors in virus activation/inactivation. These proteolytic activities may reflect ecological and host environments in which avian influenza viruses persist and replicate. Complete understanding of proteases found in natural sites of infection is necessary to derive insight into mechanisms of pathogenicity, mechanisms of perpetuation, and ease of cross-species transmissibility.					
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## Objective

The project's objective is to isolate, identify, and characterize *Aeromonas caviae* secreted proteases responsible for cleavage of avian influenza virus hemagglutinin.

## Introduction

Proteolytic cleavage activation of influenza virus hemagglutinin (HA0) is required for cell entry via receptor-mediated endocytosis. Although numerous studies describe bacterial protease-mediated influenza A viral activation in humans, very little is known about the role of intestinal bacterial flora of birds (natural avian influenza reservoir) in HA cleavage or activation. Previous research has demonstrated the effects of bacterial microflora of the lower digestive tract of free-range waterfowl on influenza virus activation (Figure 1) (1). Characterization of proteases and lipase activities secreted from *Aeromonas* species are ongoing such that the role of these respective contributors in virus activation/inactivation can be firmly established. These activities may reflect ecological and host environments in which these viruses persist and may be a contributing factor in the ability of new viruses to transmit or adapt to new species, to include humans.

## Materials and Methods

Cell-free supernatants containing *Aeromonas caviae* secreted proteases were separated via ion-exchange chromatography and visualized via zymography (2,3).

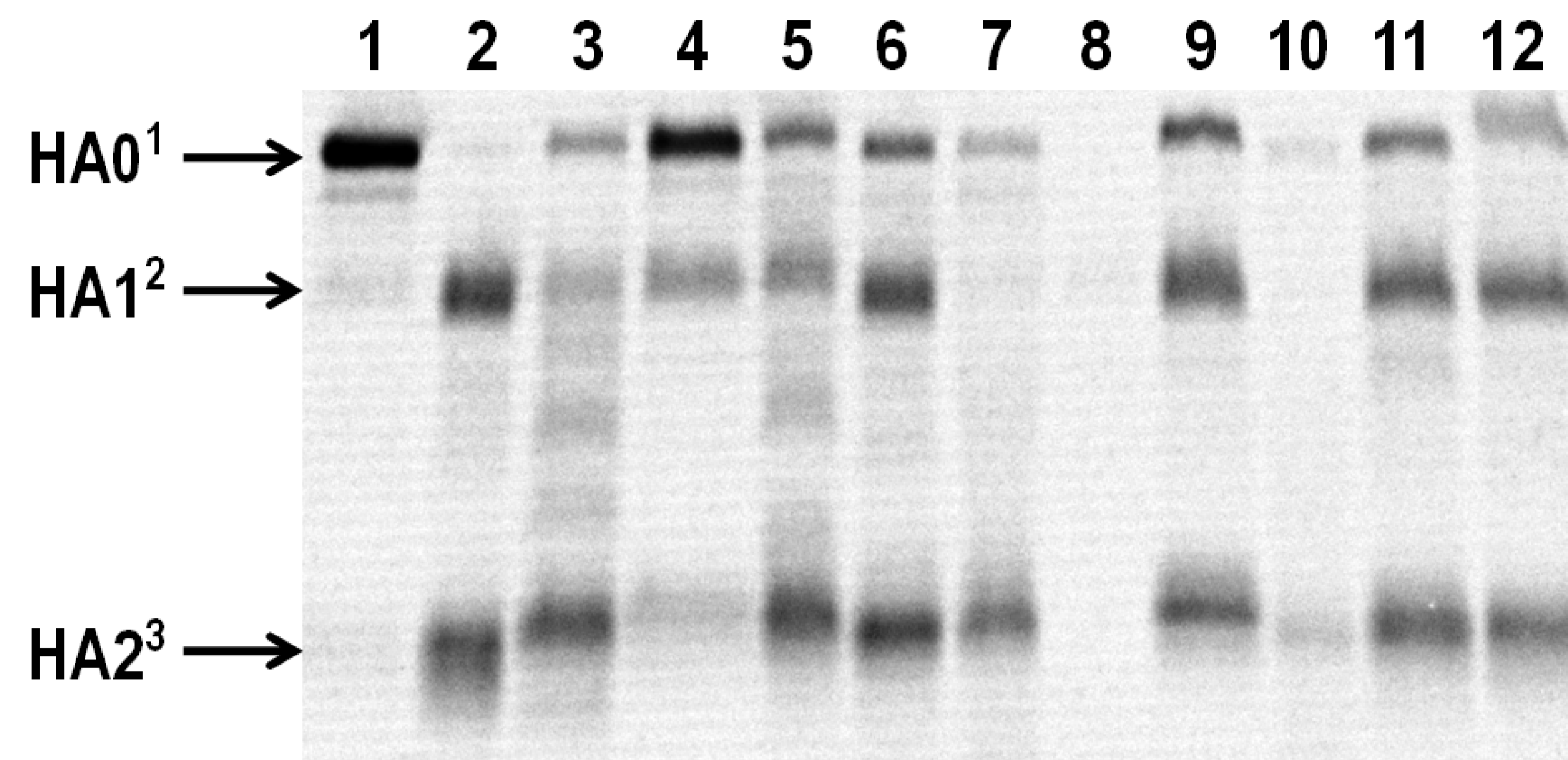


FIG. 1. SDS-PAGE analysis of polypeptide fragment patterns obtained following incubation of HA0 with supernatant material from protease-secreting bacterial isolates. Lanes: 1, PBS negative control; 2, trypsin, 10 mg/ml; 3, *Streptococcus hyointestinalis*; 4, *Aerococcus viridans*; 5, *Lysinibacillus sphaericus*; 6, *Bacillus amyloliquefaciens*; 7, *Kocuria kristinae*; 8, *B. pumilus*; 9, *Enterobacter cloacae*; 10, *Cellulosimicrobium cellulans*; 11, *A. sobria*; 12, *A. hydrophila*. The numbered arrows indicate the established molecular masses for HA0 and trypsin hydrolysis products HA1 and HA2 (80, 58, and 26 kDa, respectively) (1).

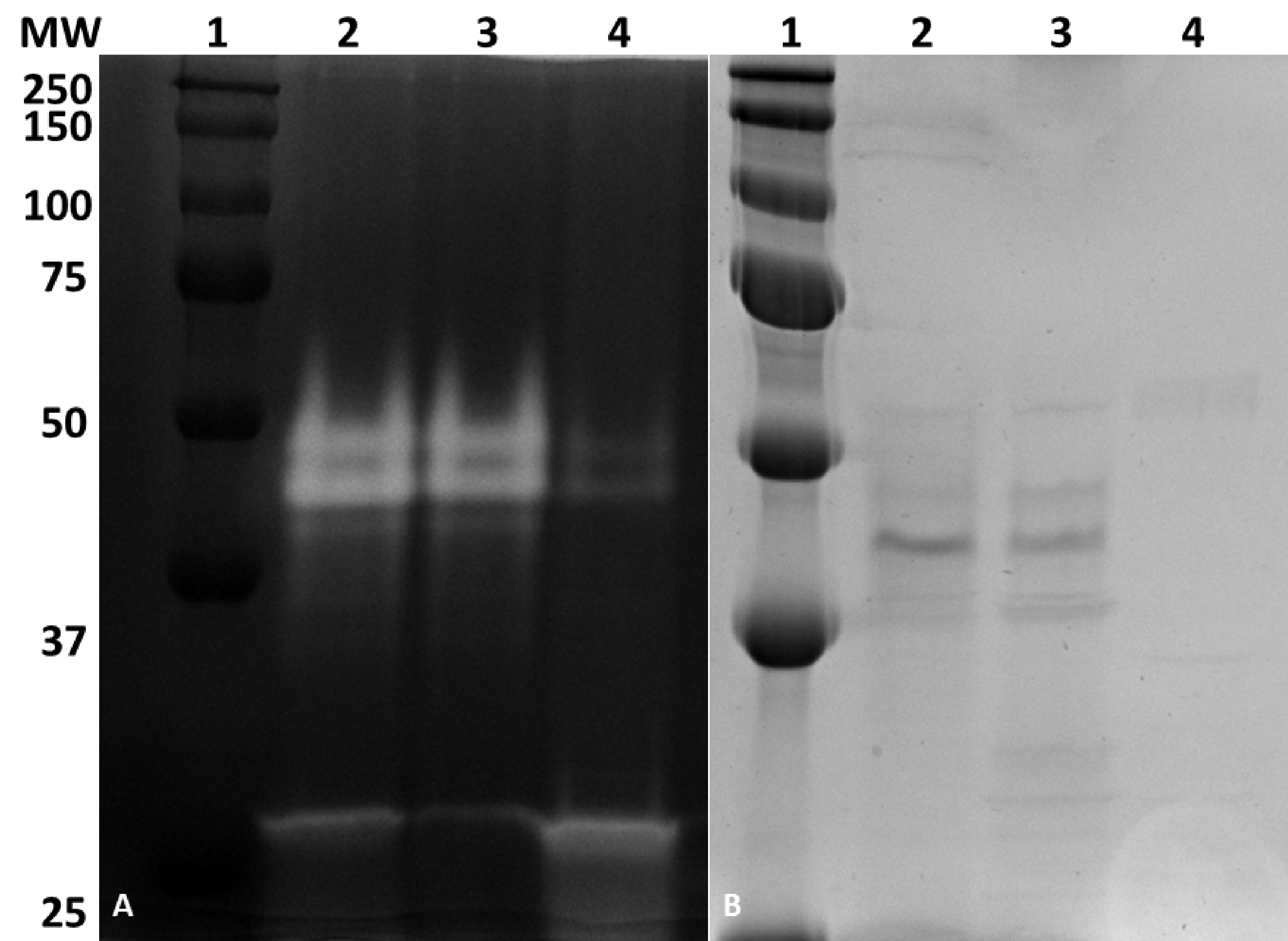


FIG. 2. Zymogram (A) and native gel (B) analysis of bacterial-secreted protease supernatants after CEX chromatography. Clear zones indicate proteolytic activity. Lanes: 1, molecular weight marker; 2, *A. caviae* sample control; 3, breakthrough; 4, 250mM NaCl fraction. Samples were run on cation exchange at 1 ml/min, 50mM acetate-NaOH, pH 5.

## Preliminary Results and Dis

Zymographic analysis (Figure 2) the presence of multiple *A. caviae* proteolytic enzymes at the binding utilizing cation-exchange chromatography (CEX) (lanes 3,4). Proteins at molecular masses approximately 45 to 50 kDa were submitted for amino acid sequence identification. Laboratory efforts are ongoing to determine the binding pH of the remaining proteases in order to facilitate protein identification. Ultimately, we will incubate purified proteases with avian influenza hemagglutinin cleavage (*in vitro*), as well as in waterfowl via *in vivo* plaque assays. This data will be useful for an understanding of avian influenza persistence mechanisms in reservoirs.

## References

1. King, M., et al., 2011. Effects of Intestinal Microflora of the Lower Digestive Tract of Free-Range Waterfowl on Influenza Virus Activation. *Appl. Environ. Microbiol.* 77: 41-48.
2. Vandooren J., et al. 2003. Zymography: A Method for Visualizing Hydrolytic Enzymes. *Methods.* 10:211-220.
3. Smith, J. 1998. Purification of Proteases by Conventional Chromatography. *Methods in Molecular Biology.*

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