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Replacement of Potassium Chloride by Potassium Glutamate Enhances the *In Vitro* Proteolytic Activity of Lon Protease

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Abstract of the Thesis

Replacement of Potassium Chloride by Potassium Glutamate Enhances the *In Vitro* Proteolytic Activity of Lon Protease

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Critical for cell survival, protein quality control and protein turnover are orchestrated by AAA+ (ATPases associated with diverse cellular activities) proteases. In bacteria, Lon protease is one such ATP-dependent enzyme whose function, among others, includes degradation of misfolded proteins (1). In *Yersinia pestis* Lon protease is central to host invasion and pathogenicity. Among several substrates of Lon is the regulator of virulence A (RovA), a temperature-sensing protein in *Yersinia* (2). In this study, I have employed an *in-vitro* system to gain a better understanding of the proteolytic activity of *Yersinia* Lon. I show that while Lon is able to degrade RovA in the presence of potassium chloride, its proteolytic activity increases significantly when a more physiologically relevant salt, potassium glutamate, is used instead.

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LIST OF ABBREVIATIONS

Yp	*Yersinia pestis*
IPTG	Isopropyl β-D-1-thiogalactopyranoside
DTT	Dithiothreitol
UI	Uninduced
W	Whole Cell
P	Pellet/ Insoluble
S	Soluble
W1	Wash 1
W2	Wash 2
FT	Flow through
BC	Before Column
E1, 2, 3, 4	Elution 1, 2, 3, 4
KCl	Potassium Chloride
KGlu	Potassium Glutamate
CK	Creatine Kinase
MW	Molecular Weight

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INTRODUCTION

An important requirement for maintaining cellular homeostasis is the adequate synthesis of necessary proteins as well as their timely removal. Several integral cellular processes such as cell division, DNA replication, and gene expression rely on efficient ATP-dependent machines called proteases that belong to the AAA+ family (ATPases associated with diverse cellular activities) of proteases. These versatile enzymes consisting of an N-terminal domain that is, often, responsible for substrate recognition and an ATPase domain that uses the energy derived from ATP binding and hydrolysis to unfold and translocate the substrate into the final peptidase domain, which irreversibly degrades the protein into small peptides. These proteases not only degrade misfolded/defective proteins, accumulation of which would otherwise have deleterious effects, but also enable the removal of regulatory proteins that influence the expression of genes required for bacterial interaction with and invasion of host cells. In bacteria, five types of AAA+ proteases work in concert to promote cell survival: Lon, ClpXP, ClpAP, HslUV, and FtsH (1).

Lon was the first ATP-dependent protease to be identified in bacteria and is found in Archaea as well as the mitochondria and chloroplasts of eukaryotic cells (3). This means that knowledge gained through studies on bacterial Lon can be applied to other organisms as well. Structurally, Lon forms a homohexamer, with each monomer containing an N-terminal domain, an ATPase domain, and a peptidase domain, all encoded in a single gene (3).

Figure 1. Diagrammatic representation of Lon domains

In *Yersinia pestis,* the causative agent of plague, Lon activity facilitates host invasion and pathogenicity. This is achieved by regulating the expression of Type III secretion system factors via Lon-mediated proteolysis of *Yersinia* modulator A protein (YmoA) and regulator of virulence A protein (RovA), an intrinsic temperature-sensing protein. Previous studies addressing the temperature-dependent changes in *Yersinia* revealed that a shift from moderate temperatures (20-25 °C) to 37 °C results in a rapid transition of gene expression, including stress adaptations genes and genes encoding for virulence factors (2). In *Yersinia pestis*, RovA regulates transcription of invasion, which leads to a faster progression of infection and promotes colonization of the Peyer's patches. At 25 °C, RovA strongly binds DNA and is stable, but loses its DNA-binding ability via conformational changes that are induced at 37 °C, the host temperature. Loss of DNA-binding facilitates rapid degradation by Lon protease *in vivo*. However, *in vitro* degradation of RovA by Lon is very inefficent and requires the presence of an unknown cofactor (2). It was suggested that such rapid removal of RovA might be required to prevent sequestration of regulatory components through association with RovA (2). Recent data from our lab demonstrates that robust proteolysis by Lon is bolstered by HspQ (heat shock protein Q), a heat-shock induced, specificity-enhancing factor of Lon (Neha Puri and Wali Karzai, unpublished data).

In this study, I determine the most suitable buffer conditions for *in vitro* proteolysis of RovA by Lon in the presence of HspQ. Salts are required components of all biological buffers, and this important requirement is most commonly satisfied by sodium chloride (NaCl) or potassium chloride (KCl) salts. However, potassium glutamate (KGlu) is a more physiologically relevant but infrequently used salt. Since most protein interactions are salt sensitive, I examined the effects of replacing chloride by glutamate on the *in vitro* degradation of RovA by Lon protease in

the presence of HspQ in order to mimic more closely the physiological solute environment in which these enzymatic reactions occur in bacteria. My results indicate that the degradation rate of RovA by Lon protease in the presence of HspQ is significantly faster with KGlu as compared to KCl.

MATERIALS & METHODS

Bacterial strains and plasmids

E. coli strain BL21 (DE3)/pLysS and BL21 Star (DE3) were used to express *Yp*-RovA and *Yp*-Lon, respectively. The gene encoding RovA was cloned into 2BT vector. The gene encoding Lon was cloned into pET28b vector (*lon* cloning was done by a previous graduate student, Ge Z).

Protein expression and purification

To purify RovA, *E. coli* strain BL21 (DE3)/pLysS harboring 2BT-RovA-His6 was grown in 2 liters of LB supplemented with 50 µg/mL ampicillin and 25 µg/mL chloramphenicol. RovA expression was induced at OD_{600} ~0.7 by addition of 1 mM IPTG and continued for 90 minutes at 37 °C. Cells were harvested by centrifugation, and the resulting pellets were stored at -80 °C. The frozen cell pellets were re-suspended in 30 mL of lysis buffer (50 mM Tris pH 7.5, 1 M NH_4Cl, 2 µM β-Mercaptoethanol) and lysed by sonication at 4 °C. Cellular debris was removed by centrifugation at 15,000 x g for 30 minutes. The clarified cell lysate was mixed with 320 µL of 80% Ni-NTA slurry equilibrated in the lysis buffer (with 10 mM Imidazole) and the bound

protein was eluted with 10 x 0.5 mL of elution buffer (50 mM Tris pH 7.5, 1 M NH_4Cl, 2 µM β-Mercaptoethanol and 250 mM Imidazole). The eluted protein was loaded on Superdex 75 gel filtration column and eluted with gel filtration buffer (50 mM HEPES pH 7, 150 mM KCl, 1 mM DTT, 2% Glycerol). Protein aliquots containing RovA were flash frozen and stored at -80 °C.

Yp-Lon was expressed in *E. coli* strain BL21 Star (DE3) and grown in 2 liters of LB supplemented with 50 µg/mL of kanamycin. Expression was induced at OD_{600} ~0.7 by addition of 1 mM IPTG and continued for 2 hours at 37 °C. Cells were harvested by centrifugation and the resulting pellets were stored at -80 °C. The frozen cell pellets were re-suspended in 30 mL of lysis buffer (50 mM $KHPO_4$ pH 7, 1 mM EDTA, 1 mM DTT and 10% Glycerol) and lysed by sonication at 4 °C. The clarified cell lysate was loaded by gravity flow onto 20 mL of pre-equilibrated with P11 resin and washed with 100mL of lysis buffer. Bound proteins were eluted with 10mL of elution buffer (400 mM $KHPO_4$ pH 7, 1 mM EDTA, 1 mM DTT and 10% Glycerol). The eluted proteins were concentrated to 5 mL, buffer exchanged into Q-Sepharose buffer A (50 mM Tris pH 7.5, 50 mM KCl, 1 mM DTT, 10% glycerol) and loaded onto a pre-equilibrated Source 15Q anion exchanger column. The column was washed with 5 column volumes of buffer A and the bound protein was eluted by the application of a linear KCl gradient from 0% Buffer B to 80% Buffer B (50 mM Tris pH 7.5, 1 M KCl, 1 mM DTT, 10% glycerol). The eluted proteins were concentrated to 2 mL and loaded onto Sephacryl S300 column, pre-equilibrated in Lon storage buffer (50 mM Tris pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1mM DTT and 20% glycerol). Fractions containing purified Lon were pooled, concentrated, and flash frozen in liquid nitrogen and stored at -80 °C.

In vitro proteolysis assay

Each *in vitro* proteolysis assay was carried out in a reaction mixture containing Lon activity buffer (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, and 10% glycerol), ATP regeneration system (50 mM creatine phosphate, 80 μg/mL creatine kinase, and 4 mM ATP), 10 μM RovA, 10 μM HspQ and Lon protease (200 nM hexamer). The Lon activity buffer used had 3 variations: No salt, 100 mM KCl and 100 mM KGlu. The reaction mixture was assembled and incubated at 37 °C. Aliquots were taken at designated time points and the reaction stopped by adding SDS-PAGE sample buffer. The reaction products were resolved by electrophoresis using a 15% Tris-tricine gel, stained with Coomassie Brilliant Blue, and quantified by Image Studio software. The signal of RovA was normalized to creatine kinase signal to account for loading errors.

RESULTS

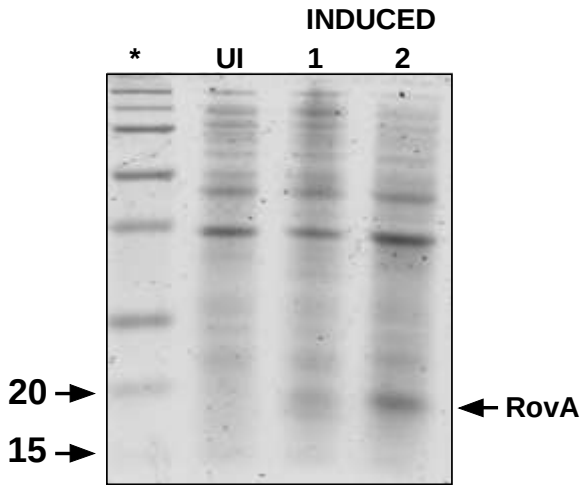
To calculate the degradation rate of RovA in the presence of HspQ by Lon protease, I first purified RovA by Ni-NTA chromatography and size-exclusion chromatography (Figure 1), followed by purification of Lon by P11 phosphocellulose, ion exchange, and size-exclusion chromatography (Figure 2). Neha Puri, a graduate student in the lab, provided purified HspQ protein. The *in vitro* proteolytic activity was tested by degradation assays, which were carried out in a buffer containing no salt, 100 mM KGlu, or 100 mM KCl (Figure 3). After staining with Coomassie Brilliant Blue, the gels reveal the robust degradation of RovA in the buffer containing no salt. The degradation rate of RovA in the buffer with 100 mM KGlu was comparable to the no

salt buffer, whereas its degradation rate in the presence of buffer with 100 mM KCl was significantly reduced (Table 1).

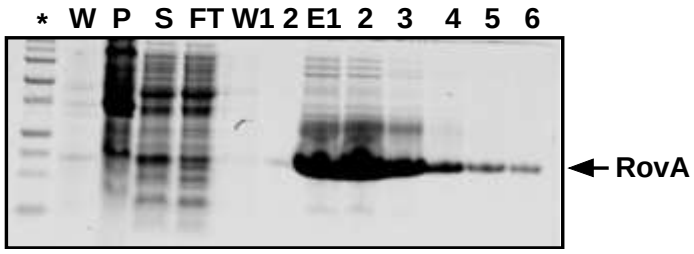
Figure 2. RovA purification

a) RovA expression: 2BT-RovA was induced at OD_{600} 0.6 with 1mM IPTG for 90 minutes at 37 °C. Total cell lysates from an uninduced control and two induced samples were resolved by electrophoresis on a 15% Tris-tricine gel. The position of induced RovA (18 KDa) is indicated on the right.

*The left most column of the gel shows the molecular weight standards.

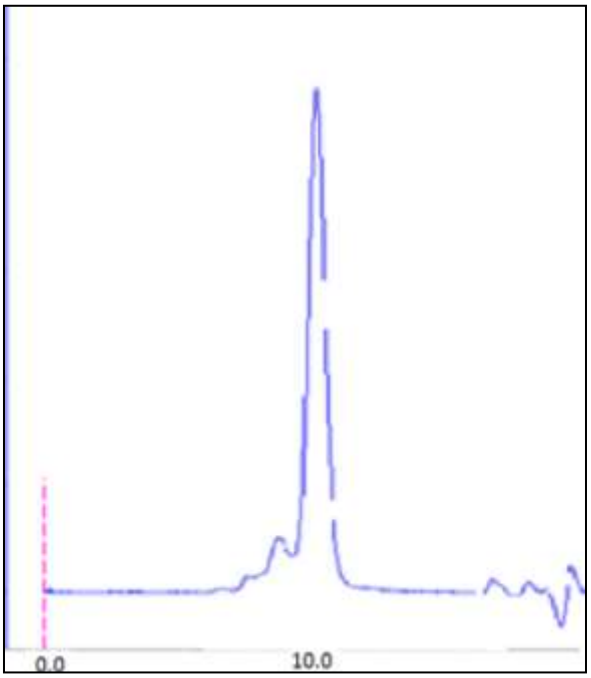


b) RovA Ni-NTA chromatography purification: Clarified cell lysate was incubated with 250 µL Ni-NTA beads for 1 hour and washed with buffer containing 10 mM Imidazole before eluting with buffer containing 250 mM Imidazole



c) Top panel: RovA elution profile- Two injections at 0 and 20 mL, elutes at 10 mL and 30 mL respectively.

Bottom panel: RovA gel filtration purification: Size-exclusion chromatography was performed using Superdex 75 column. The fractions containing the protein were resolved by using a 15% Tris-tricine gel.



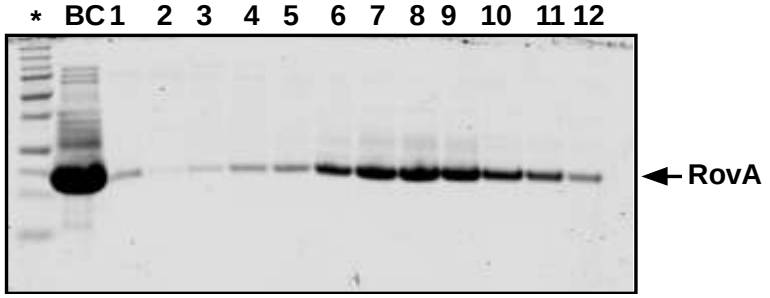
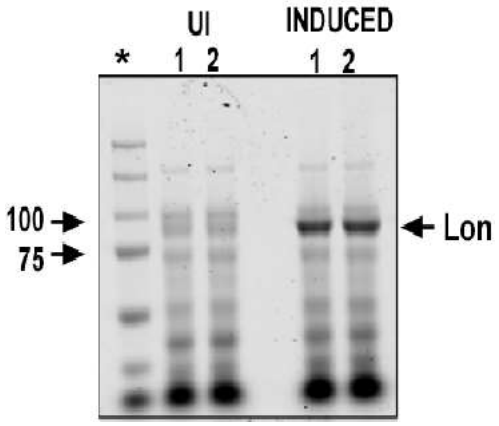


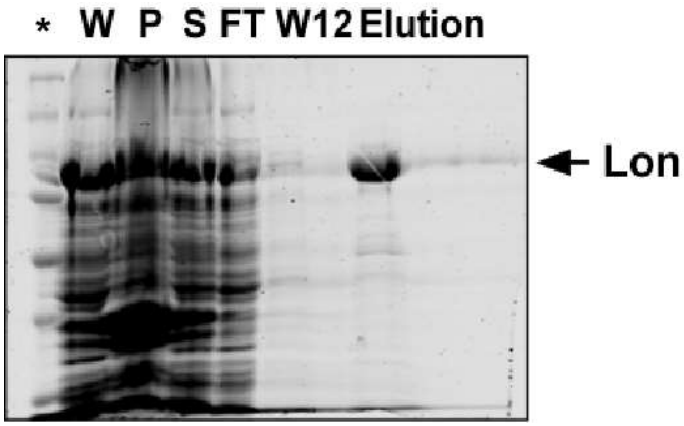
Figure 3. *Yersinia pestis* Lon purification

a) Lon expression: pET28b-Lon was induced at OD_{600} 0.6 with 1mM IPTG for 2 hours at 37 °C. Total cell lysates from two uninduced controls and induced samples were resolved on a 15% Laemmli SDS-PAGE. The expected size of Lon seen on the gel is 89KDa.

*The left most column of the gel shows the molecular weight standards



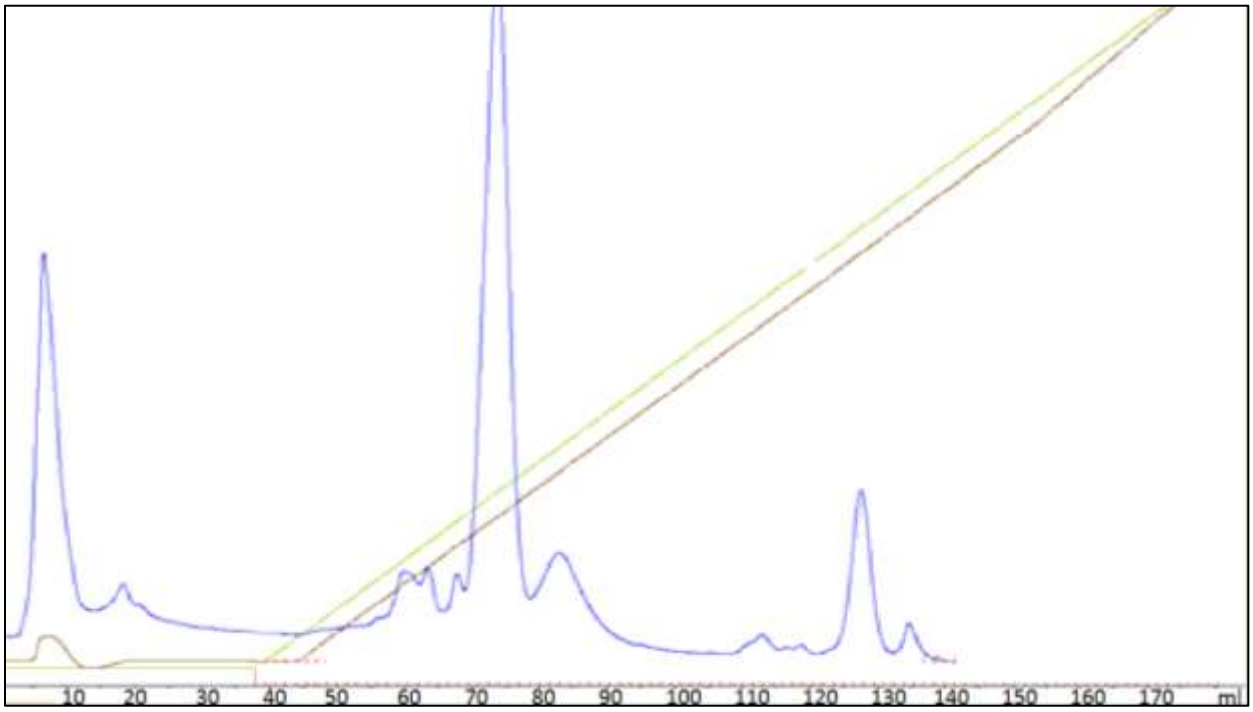
b) P11 phosphocellulose chromatography: Clarified cell lysate was incubated with 20 mL of P11 resin for two hours and washed with 100 mL lysis buffer before eluting with 10 mL of elution buffer, 400 mM $KHPO_4$.

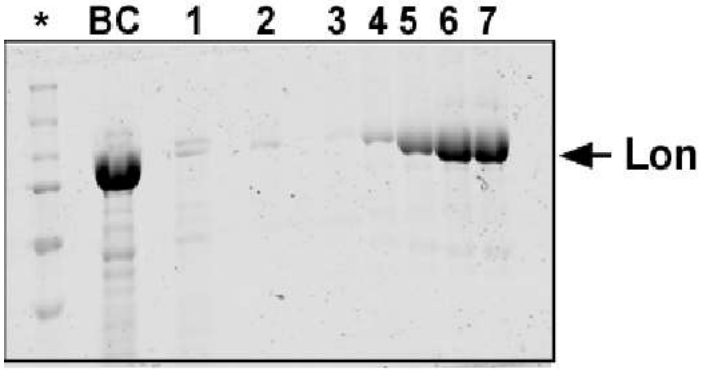


c) Ion exchange chromatography purification: was performed using S15Q (anion) column

Top: Elution profile of Lon from S15Q column where it elutes at 300 mM KCl.

Bottom: The fractions containing Lon were resolved on 15% Laemmli SDS-PAGE.

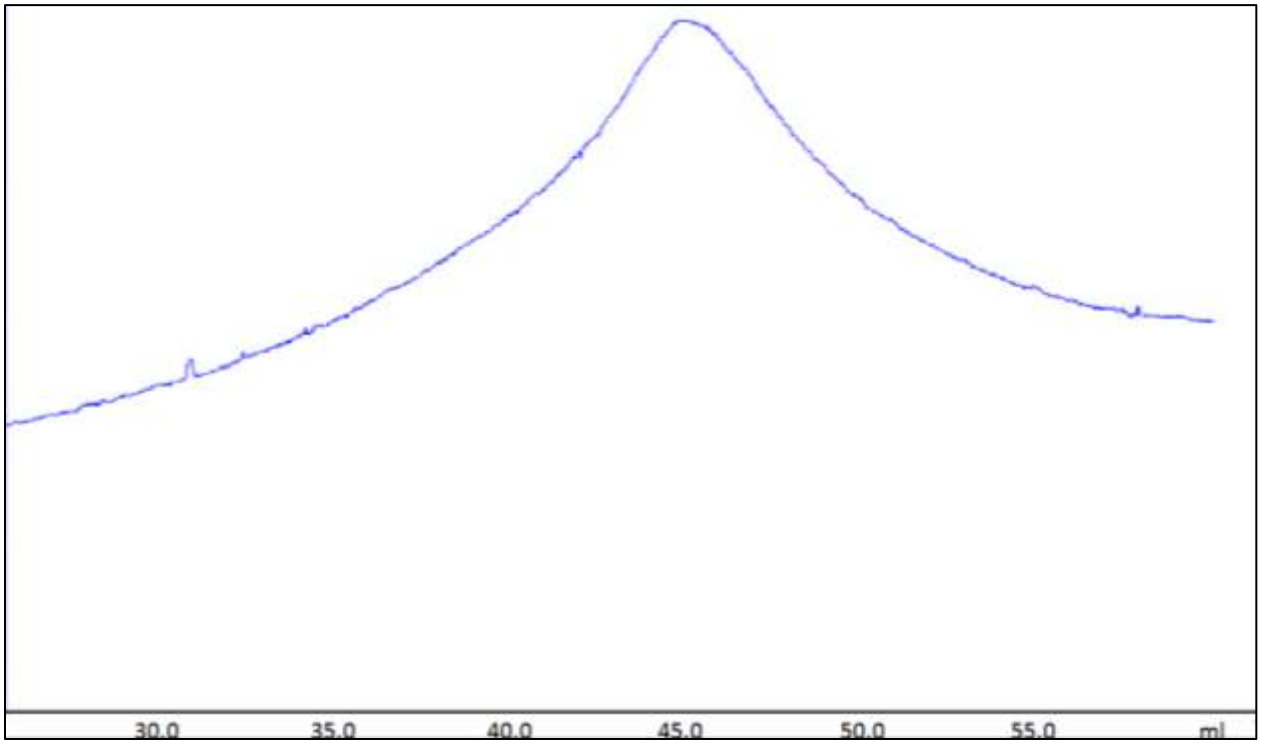




d) Gel filtration purification was performed using Sephacryl S300 sizing column

Top panel: Elution profile of Lon from S300 column, where it elutes ~ 45 ml.

Bottom panel: The fractions containing Lon were resolved on 15% Laemmli SDS-PAGE.



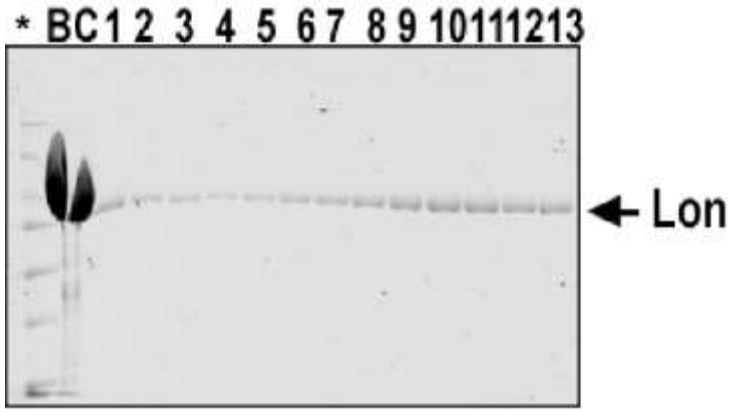
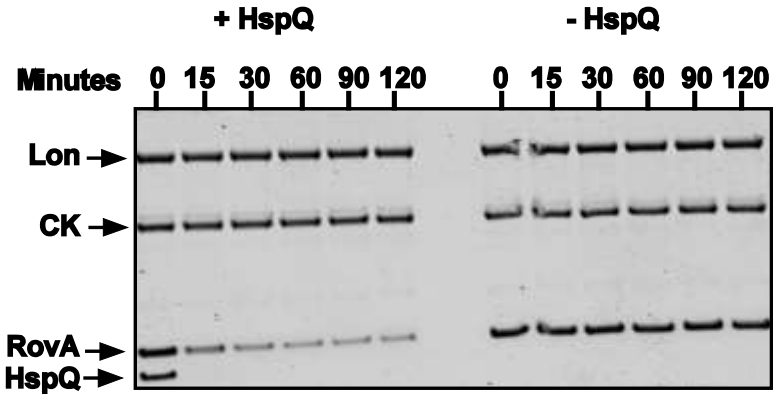


Figure 4. ***In vitro*** **RovA proteolysis assay**s

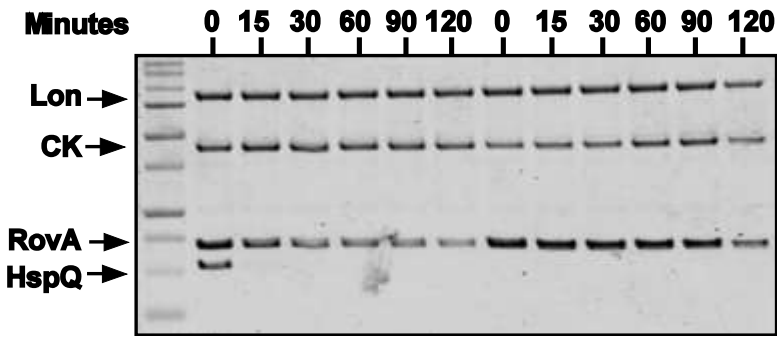
Purified RovA (10 µM) was subjected to proteolysis by Lon (200 nM hexamer) in the presence of an ATP regeneration system, and 10 µM HspQ when present. The buffers and salts used in the proteolysis assays were as follows:

a) **No salt buffer**: 50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, and 10% glycerol
b) **100 mM KGlu salt buffer**: 50 mM Tris-HCl pH 8.0, 100 mM KGlu, 10 mM $MgCl_2$, 1 mM DTT, and 10% glycerol
c) **100 mM KCl salt buffer**: 50 mM Tris-HCl pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, and 10% glycerol

a) No salt



b) 100 mM KGlu



c) 100 mM KCl

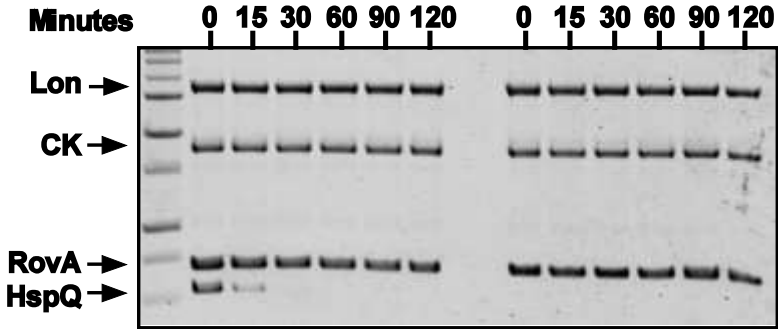
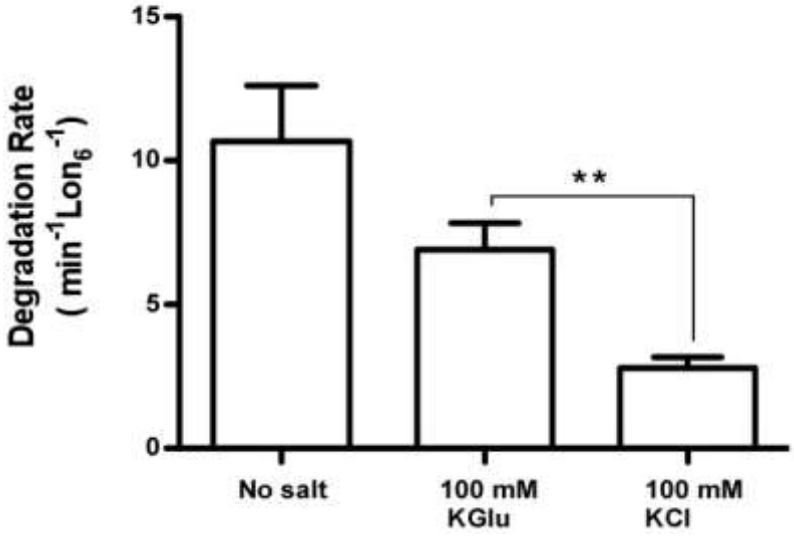


Table 1. Degradation Rate of RovA

Buffer	Degradation Rate (min^{-1} Lon_6^{-1})
No salt	10.67 ± 1.944
100 mM KGlu	6.9 ± 0.9274
100 mM KCl	2.8 ± 0.3742

Figure 5. Degradation kinetics of RovA: The degradation rate of RovA in the presence of HspQ by Lon protease. The rates were calculated with a minimum of 3 repeats for each condition and a graph for these rates was plotted using Prism. It shows fastest degradation in a buffer that contains no salt, followed by a buffer containing 100 mM KGlu and then 100 mM KCl. A minimum of 3 independent repeats was carried out for each buffer condition. Two-tailed unpaired t-test was carried out on KGlu versus KCl and the p value was 0.0034.



DISCUSSION

Here, I examined the effect of potassium glutamate and potassium chloride salts on the proteolytic activity of Lon. In this particular study, I used RovA, a DNA-binding protein, as a substrate to study this proteolytic activity. RovA belongs to SlyA/Hor/Rap family, whose members control a wide range of physiological processes ranging from environment adaptation and pathogenesis (4). One of the important functions of RovA in of *Yersinia pestis* is the regulation of internalization factor *invasin* expression as well as genes encoding other adhesins (2). This prepares the bacteria for host cell invasion and facilitates pathogenesis. While RovA expression is promoted via a positive auto-regulatory feedback loop at 25 °C via binding of RovA to promoter sequences to activate transcription, there is a drastic change following a shift in temperature to 37 °C. It has been shown that a conformational change in RovA significantly decreases its ability to bind DNA at 37 °C (5). This allows Lon and Clp proteases to recognize and degrade RovA. In the case of Lon protease, this degradation is dependent on HspQ, a recently discovered specificity-enhancing factor of Lon (Neha Puri and Wali Karzai, unpublished data).

The aim of this study was to test the effect of salt on the proteolytic activity of Lon. Initial experiments revealed that RovA degradation rate in a buffer lacking salt was significantly higher compared to a buffer containing 100 mM KCl. In *E. coli*, it is known that glutamate is the predominant ion as compared to chloride ion, which is present in very low concentrations inside cells (6). Thus, we reasoned that substituting KGlu for KCl might alter the proteolytic activity of Lon. We found that the presence of 100 mM KGlu increased RovA degradation rate by Lon by more than 2-fold, and was comparable to the degradation rate in absence of salt. This is an important finding since it suggests that KGlu may be a more appropriate salt for the study of Lon

as well as other proteases. Future studies using a similar approach, wherein a physiologically relevant salt is used, could reveal previously unknown properties of important bacterial proteases, knowledge of which could then be used for development of novel drugs and therapeutics to inhibit pathogens.

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