

Purification of the Metalloprotease of *Listeria monocytogenes*

Honors Thesis

Presented to the College of Agriculture and Life Sciences
Department of Animal Science of Cornell University

Research Conducted in the Department of Microbiology and Immunology
of Cornell University
in Partial Fulfilment of the Requirements for the Research Honors Program

By
Gabriela R. Wagner
May 2011
Research Advisor: H el ene Marquis

ABSTRACT:

Listeria monocytogenes is a gram-positive bacterium and the causative agent of listeriosis, a food-borne infection. During infection, the bacterium invades host cells and lyses phagocytic vacuoles to gain access to the cytosol where it multiplies. One of the virulence factors involved in escape from vacuoles is a secreted zinc-dependent metalloprotease (Mpl). Mpl is made as a zymogen of ~55 kDa and is composed of a propeptide and a catalytic domain. Mpl maturation occurs by autocatalysis in a pH-dependent manner. To better understand the mechanism of Mpl autocatalysis, we aimed to purify Mpl for structure/function analysis. Mpl was purified in its natural host, *L. monocytogenes*, grown under conditions that favor the expression of Mpl, and the supernatant was recovered by centrifugation and filtration. Secreted proteins, including Mpl, were concentrated by ammonium sulfate precipitation, recovered by centrifugation, dissolved in water, and passed through a buffer exchange column to eliminate the salt. Mpl was purified by ion-exchange chromatography at pH 7.0, and eluted from the column at 500 mM NaCl. It was further purified via Flag-tag affinity chromatography. Fractions containing Mpl were identified by Western immunoblot with an anti-Mpl antibody, and Coomassie Blue staining of protein gels was used to verify purity. At the conclusion of these experiments, 4 nonspecific proteins in very small concentrations were found to co-purify with Mpl.

ACKNOWLEDGEMENTS

Brian Forster, PhD Candidate
Alan Bitar, Lab Technician
Russell and Bynoe labs
Dextra Undergraduate Research Endowment Fund
Hughes Scholars Program

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INTRODUCTION:

Listeria monocytogenes is a gram-positive bacterial rod found ubiquitously in various locations such as food, water, soil, humans, and animals. *L. monocytogenes* is the etiologic agent of listeriosis, one of the most deadly food-borne diseases (Vazquez-Boland et al., 2001). Though the incidence is low, the mortality rate is ~20%, the highest rate of any food-borne diseases (Center for Disease Control and Prevention). The high frequency is due to meningitis and/or septicemia that characterize severe listeriosis. *L. monocytogenes* is capable of growing within a wide range of temperatures including refrigeration temperature (2-4°C) and body temperature (37°C). The bacteria are inactivated after subjection to pasteurization temperatures, and thus the predominant source of infection comes from food that was not properly pasteurized or that was contaminated during processing. *L. monocytogenes* is found most often in soft cheeses, cold cuts, smoked fish, vegetables, and milk products. Pregnant women, elderly persons, immunocompromised individuals, and small ruminants are those that are most at risk of developing listeriosis. Of those affected by listeriosis, 30% are pregnant women who often undergo abortion and/or neonatal meningitis of their developing fetus.

There are two main modes by which *L. monocytogenes* can enter into the host through the intestinal mucosa: 1) direct invasion of the enterocytes lining the absorptive epithelium of microvilli, leading to infection of the intestinal cells; 2) translocation across the M-cells of Peyer's patches. The first mechanism is the most efficient at direct invasion in humans (Jensen et al., 1998).

To first invade the gastrointestinal epithelium, the bacteria adhere to the surface of epithelial cells. Unlike most bacteria that produce mucinases in order to invade the

underlying mucus layer, *L. monocytogenes* produces surface proteins that interact with human mucin (Linden et al., 2008) and host cell heat-shock protein 60 (Wampler et al., 2004). Through an interaction between internalin A and human E-cadherin, a protein expressed at the basolateral surface in the tight junctions of enterocytes, *L. monocytogenes* are then able to invade the enterocytes (Pentecost et al., 2006). Upon uptake into the enterocyte, the pathogen becomes entrapped in a primary vacuole.

In order for *L. monocytogenes* to replicate, the bacterium must escape from the vacuole and enter the host cytosol (Tilney and Portnoy, 1989). *L. monocytogenes* is capable of escaping the host cell vacuole with the assistance of a cholesterol-dependent, pore-forming cytolysin known as Listeriolysin O (LLO) and two secreted phospholipases C (PLC): a broad-range phospholipase C (PC-PLC) and a phosphatidylinositol specific PLC (PI-PLC) (Portnoy et al., 1992; Smith et al., 1995). Upon escape from the primary vacuole, the bacteria replicate in the cytosol of host cells and synthesize a surface protein called ActA. ActA mediates the polymerization of actin filaments, which is used for *L. monocytogenes* intracellular movement, enabling the bacteria to spread from cell-to-cell without exiting into the extracellular milieu (Kocks et al., 1992). Formation of a secondary vacuole occurs during this cell-to-cell spread, and again, the method of escape requires LLO, PI-PLC, and PC-PLC.

PC-PLC initially is secreted in an inactive state containing a propeptide (pro-PC-PLC, ~33 kDa). Activation of the proenzyme requires cleavage of the propeptide, which is mediated by a metalloprotease (Mpl) and a decrease in pH (Marquis and Hager, 2000). The importance of Mpl in the pathogenesis of *Listeria monocytogenes* initiated our

efforts to purify it in an effort to better understand mechanisms of virulence factors to develop treatments.

Mpl is a metalloprotease and member of the zincin superfamily (Miyoshi and Shinoda, 2000). More specifically, it is a member of the thermolysin family. A homology model was constructed using the model protease of the thermolysin protein family from *Bacillus thermoproteolyticus* (Holmes and Matthews, 1982). From this, the catalytic domain of Mpl was determined to contain two structural subdomains within the functional catalytic domain: an α -helical predominant domain and a β -strand predominant domain.

Common to all in the zincins superfamily of metalloproteases is a characteristic HEXXH motif (Miyoshi and Shinoda, 2000). The active site zinc ion of metalloproteases is coordinated by a water molecule and three amino acid residues, including two histidines within the HEXXH motif and a glutamic acid present 20 residues downstream of the motif (Banbula et al., 1998; Holmes and Matthews, 1982). Furthermore, a histidine residue 83 residues downstream of the HEXXH motif in conjunction with a glutamic acid residue located within the HEXXH motif interact together at the active site. This second interaction is required for catalysis (Banbula et al., 1998; Beaumont et al., 1995).

Mpl is produced initially as a zymogen (~55 kDa) but undergoes intramolecular autocatalysis due to a decrease in vacuolar pH to render a catalytically active domain (~35 kDa) (Bitar et al., 2008). Then, mature Mpl secretion into the host cell occurs upon a decrease in pH (Forster and Marquis, unpublished data). Mature Mpl proteolytically cleaves of PC-PLC's 24-amino-acid N-terminal propeptide, converting pro-PC-PLC to active PC-PLC. *L. monocytogenes* is then capable of degrading its vacuolar membrane

and being released into the host cell. This efficacy of escape from host cell vacuoles utilizing first Mpl's ability to autocatalyze followed by the activation of PC-PLC is attributed to its success as a pathogen.

Several other proteins have been attributed with the ability to autocatalyze. One such protein is myelin basic protein-component 1 (MBP-C1) from multiple sclerosis tissue and has been shown to undergo autocatalytic cleavage at a slightly alkaline pH (D'Souza et al., 2005). Also, prions have been shown to replicate in an autocatalytic manner converting cellular prion to the misfolded infectious form (Bieschke et al., 2004). Structural analysis of Mpl may potentially lead to a model for simulating mechanisms of autocatalysis.

Thus the main objective of this study was to purify Mpl in hope that its purification would allow for better understanding of the mechanisms that regulate virulence factors, which may then lead to the development of *L. monocytogenes* infection treatments.

MATERIALS AND METHODS

Bacteria Strain.

The *L. monocytogenes* strain used for these experiments expresses a catalytic mutant of Mpl with a C-terminal Flag tag (Mpl E350Q-Flag_{C-cat}) (Bitar et al., 2008). Additionally, we deleted the genes coding for listeriolysin O, the broad-range phospholipase C, Internalins A and B because these proteins were found to co-purify with Mpl. Lastly, the gene coding for Mpl E350Q-Flag_{C-cat} was expressed from its own promoter on pAM401, a plasmid that reaches high copy number in *Listeria*.

Culture Growth:

Bacteria were grown overnight at 37°C, non-shaking, in Luria-Bertani (LB) media supplemented with 50 mM MOPS (pH 7.3)-25 mM glucose-1-phosphate-50, 0.2% activated charcoal, and 10 µg/ml chloramphenicol. The initial OD₆₀₀ from the overnight culture was measured to determine the inoculum required to start a new culture at an OD₆₀₀ of 0.12 in a larger volume of fresh medium. The culture was incubated at 37°C, non-shaking until it reached an OD₆₀₀ of ≈1. The culture was cooled on ice, and protease inhibitors were added: PMSF (1.0 mM), leupeptin (0.001 mM), and pepstatin A (0.001 mM). The bacteria were pelleted by centrifugation at 10,000 rpm for 15 minutes at 4°C, and the supernatant was decanted and filtered through a 0.22µm polyvinylidene difluoride membrane.

Protein Precipitation: Secreted proteins were precipitated by 70% saturation of the supernatant with ammonium sulfate salt. Over a 40 minute time period, ammonium sulfate (NH₃SO₄) was incrementally dissolved into the supernatant maintained at 4°C while stirring the supernatant, which remained stirred at 4°C overnight. The precipitate was recovered by centrifugation at 10k rpm for 20 minutes at 4°C. The precipitate was dissolved in water.

Buffer exchange. Buffer exchange was used to eliminate most ammonium sulfate salt by replacing the highly salt-concentrated solution in which the total protein was dissolved with a different buffer. The column was equilibrated with 25 mL of ion-exchange washing/binding buffer (Table 1). A 3 mL aliquot of the protein solution was added to the equilibrated resin, and flow thru was collected. Proteins were eluted with 4 mL of the

same binding buffer. Buffer exchange columns were recycled by running 60 mL of Cleaning Buffer through the resin, followed by equilibration in Storage Buffer (Table 1).

Purification.

1. Ion Exchange Chromatography: The first purification process of Mpl made use of a 20 ml hand-poured anion-exchange sepharose column and a BioLogic Low Pressure chromatography system (Bio-Rad). This system is controlled by a computer and is equipped with a UV (OD_{280}) detector and a conductivity flow cell to monitor protein concentration and conductivity of the solution, respectively. The system is also equipped with a mixer module and a fraction collector. For purification, the resin was initially equilibrated with binding/washing buffer at a rate of 2.00 mL/min for 10 minutes.

Thereafter, the protein sample was manually added to the upper surface of the column and allowed to gravity filtrate. The column was then reconnected to the system, and the resin was washed with the same buffer until the OD_{280} reading returned to basal level, indicating that all unbound proteins had been eluted. At that point, a linear NaCl gradient of zero to 1 M, manually programmed into the system, was initiated to elute bound proteins. The gradient progressed over a period of ~4.10 hours at a rate of 2ml/min until the conductivity of the eluted solution reached a plateau. The remaining bound proteins were eluted with buffer containing 2M NaCl. Five mL fractions were collected throughout the process and immediately stored on ice. The resin was regenerated by purging it with dH_2O and storing the column in 20% ethanol at a constant pressure.

Fractions containing Mpl were identified by Western immunoblot. Samples were selected based on the presence of protein absorbance peaks. In addition, to ensure that Mpl elution would not be missed, approximately one of every 5 samples were selected for analysis.

2. Affinity Purification:

The second step of Mpl purification made use of a 1 mL M2 FLAG-Affinity Column. Steps included washing the resin with 5 mL of 0.1M glycine HCl, pH 3.5 followed by equilibration of the resin with 5 mL of neutralizing buffer at 4°C (Table 3). The protein sample was passed through the column three times utilizing gravity flow at 4°C and the flowthru was collected. The resin was washed four times with 5 mL of Binding Buffer (+) CHAPS and for each wash the resin was mixed with the buffer by putting the sealed column on a nutator for five min at 4°C. The wash fraction was then eluted by gravity. One last 5 mL wash was done as previously described but with Binding Buffer without CHAPS. Protein bound to anti-Flag M2 antibodies on the resin were eluted as described for the wash by mixing the resin with 1 mL of binding buffer without CHAPS supplemented with 50µg/ml of Flag peptide for 10 minutes at 4°C on a nutator. Five 1 mL elutions were collected. The resin was regenerated by washing the resin stuck with 8 mL binding buffer (+) CHAPS. Three consecutive 1 mL washes of 0.1 M glycine at pH 3.5 followed by two consecutive 8 mL washes with Neutralizing Buffer. The column was then stored in resin storage buffer at - 20°C.

Buffers. Several buffers were used in this experiment, as listed in Tables 1-3.

Table 1. Buffer Exchange Buffers:

Buffer Exchange Column Cleaning Buffer	Buffer Exchange Column Storage Buffer	Buffer Exchange Column Binding Buffer
5M NaCl, 4% NaN ₃	25mM Hepes pH 8.0, 50mM NaCl, 0.02%NaN ₃	20mM Tris-HCl, pH 7.0, 5mM CaCl ₂ , 0.02%NaN ₃

Table 2. Ion Exchange Buffers:

Binding Buffer	Washing Buffer	Elution Buffer
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20mM Tris-HCl, pH 7.0, 5mM CaCl ₂ , 0.02%NaN ₃	20mM Tris-HCl, pH 7.0, 5mM CaCl ₂ , 0.02%NaN ₃	20mM Tris-HCl, pH 7.0, 5mM CaCl ₂ , 1M NaCl, 0.02%NaN ₃
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Table 3. M2 FLAG-Tag Affinity Chromatography Column Buffers:

Binding Buffer	0.1M Glycine, pH 3.5	Neutralizing Buffer	Resin Storage Buffer	Elution Buffer
20 mM Tris- HCl pH 7, 150 mM NaCl, 0.1% CHAPS (w/v), 10 uM ZnSO ₄	N.A.	10 mM Tris- HCl pH7 → Add 250 ul of 1 mM Tris-HCl pH 8	10 mM Na ₂ PO ₄ pH 7.4, 150 mM NaCl, 0.02% (w/v) Na Azide, 50% glycerol	1X Flag peptide solution (50ug/mL), 20 mM Tris-HCl pH 7, 150 mM NaCl, 10 uM ZnSO ₄

Protein Concentration:

Purified proteins in each fraction were precipitated on ice for 1 hour with 5% trichloroacetic acid. The precipitates were recovered by centrifugation at 4°C, washed with acetone, and dissolved in sample buffer (10% SDS, 0.5M Tris pH 6.8, Glycerol, Biomedical Phenol Blue, dH₂O).

Western immunoblotting

Proteins were resolved on 12% SDS- PAGE gel, and transferred to polyvinylidene difluoride membrane using a semidry electroblotting apparatus. The membrane was blocked in 2% bovine serum albumin and was then reacted with rabbit immune serum to *L. monocytogenes* Mpl at a dilution of 1:1,000 followed by goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (24 ng/ml) (Jackson ImmunoResearch Laboratories, Inc.). Enzymatic reactivity was detected with nitroblue tetrazolium (0.33 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.17 mg/ml).

Coomassie Blue Stain

Throughout the experiment sample ranging from 250 μ l- 1 mL volumes were taken after each purification step. The samples were incubated on ice and at 4°C for 1 hour in 5% trichloroacetic acid (TCA). The samples were then spun down at 14 K rpm for 20 minutes at 4°C and then were decanted. Samples were washed with 1 mL of ice cold 100% acetone and spun down at 14 K rpm for 14 min at 4°C. Following decantation, 2X sample buffer was added to resuspend the samples. They were then boiled at 100°C for 10 minutes, pulse spun, and then resolved by SDS-PAGE. The gel then was stained with Coomassie Blue Stain overnight. The Coomassie Blue Stain was then removed and replaced with De-Staining Buffer.

Bicinchoninic Acid (BCA) Assay

The same samples that had been collected for the western immunoblotting and for the coomassie blue staining analysis were collected for use in a BCA Assay to determine total protein concentrations. Samples and a standard (bovine serum albumin) were prepared in a 1:2 dilution using the FLAG buffer without CHAPS as the diluent. The samples were loaded onto a 96-well plate and were incubated with a BCA Reagent (Pierce) for 30 minutes at 37°C. The plate was then read on a plate reader at 562 nm.

RESULTS:

In order to purify Mpl zymogen, a catalytic mutant (HEL-1098) was used so that the protein would be unable to undergo autocatalysis to have its entire form purified. HEL-1098 expresses Mpl E350Q-Flag_{C-cat} on both the bacterial chromosome and on a multi-copy plasmid in order to increase the yield of Mpl. (Bitar et al., 2008). The Flag-tag is a biochemical tool used to sequester proteins due to the tag's affinity for its resin-conjugated antibody. Despite this high affinity, which would seemingly provide a direct method to directly target and purify Mpl, previous experiments showed that Mpl was not exclusively purified in this process. Despite deletions of genes coding for proteins previously found to co-purify with Mpl, additional accessory proteins in later experiments co-purified with Mpl. Thus we used two methods, ion-exchange chromatography and affinity-purification chromatography, to attempt purifying Mpl.

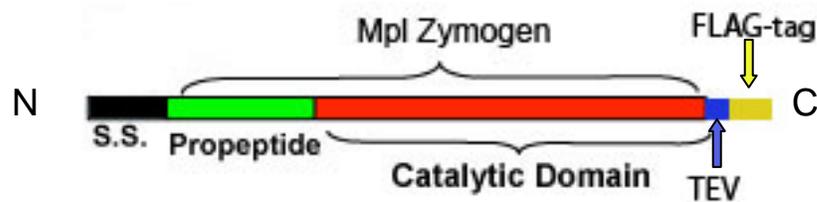


Fig 1. Cartoon Depiction of Mpl. Black= Signal Sequence (0.5 kDa); Green= Propeptide (20 kDa); Red= Catalytic Domain (35 kDa); Blue= TEV linker sequence (7 amino acids); Yellow= FLAG-tag (2.7 kDa); Green + Red= Zymogen (~55 kDa)

Ion-exchange chromatography separates biomolecules on the basis of charge characteristics, as specifically determined by the unique charge properties of each surface exposed amino acid within the tertiary structure of the protein of interest. When in the proper environment through specific buffers such as those described in the methods,

proteins will bind to the ion-exchange resin by forming ionic bonds. The different species of proteins can then be eluted sequentially by increasing the concentration of salt. Weakly charged proteins will bind weakly to the resin and will be competed out at a low salt concentration, whereas highly charged proteins will form stronger ionic bounds and will require a higher salt concentration for elution. Salts affect protein elution by either altering the binding affinity of proteins for the resin, changing the conformation of the protein or aggregating the proteins bound to the column, or modifying non-specific protein binding to sites that are not bound to the resin that change the overall conformation of the protein (Tsumoto et al., 2007).

The charge of a protein is dependent upon the environmental pH. At the isoelectric point (pI), the protein has neutral charge. When the environmental pH is greater than the pI of the protein of interest, the protein will have a net negative charge and will only bind to anion-exchange resins. Reciprocally when the pH of the protein of interest is lower than that of the pI, the protein will have a net positive charge and will only bind to cation-exchange resins. The estimated pI of the Mpl zymogen, based on sequencing data from the ExPASy Proteomics Assay website, is 6.0. Because the buffers in which used to purify Mpl were above pH 6.0, Mpl was predicted to have a negative charge. Thus an anion-exchange chromatography was conducted.

To elute Mpl two different conditions were modified separately and then conjunctly: increasing the concentration of salt and decreasing the pH of the solutions. First experiments on linearly increasing concentrations of salt were conducted.

The BioLogic machine reads out the conductivity of the sample in milliSiemens per cm (mS/cm). In order to correlate conductivity with salt concentration, four solutions

were made with varying salt molarity (250 mM NaCl, 500 mM NaCl, 750 mM NaCl, and 1 M NaCl). Each of these solutions was tested for conductivity. The system was rinsed with distilled water between each solution in order to return conductivity levels to baseline and adequately judge the correlation between salt concentration of each standard solution and conductivity in mS/cm. The results from this experiment are presented in figure 2.

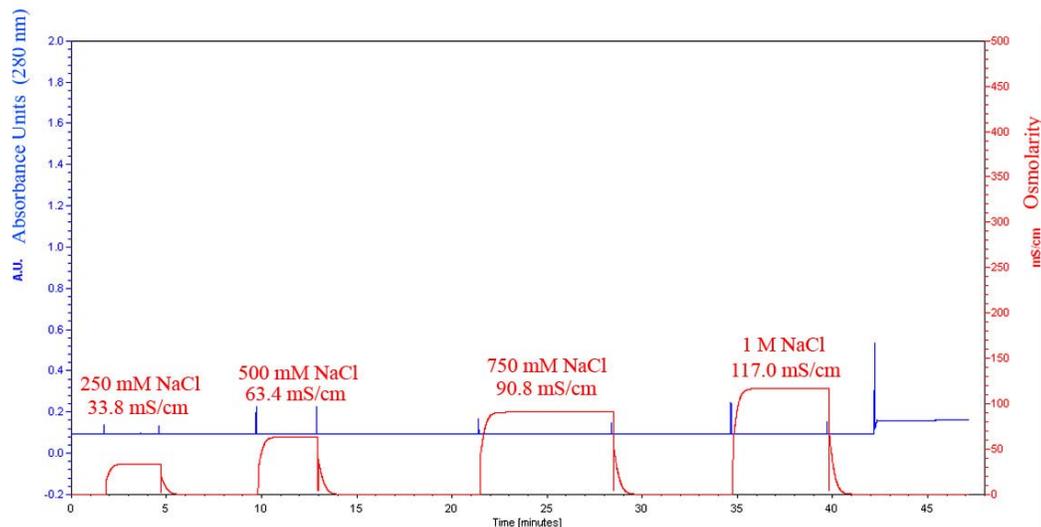


Fig 2: Four standard salt concentrations. The blue curve corresponds to the absorbance measurements with units on the left y-axis measured in Absorbance Units (AU) at 280 nm. The red curve corresponds to conductivity with units of mS/cm on the right y-axis. Time is measured in minutes across the x-axis. Results indicated that 250 mM NaCl, 500 mM NaCl, 750 mM NaCl, and 1M NaCl, correspond to values of 33.8 mS/cm, 63.4 mS/cm, 90.8 mS/cm, and 117.0 mS/cm, respectively.

The first attempts to purify Mpl made use of a 1 mL ion-exchange column (Q-Sepharose Fast Flow). A total of 3.85 mg of proteins were added to the column. Protein

elution and conductivity were recorded (Figure 3). The blue line indicates the OD₂₈₀ of eluted fractions as an indication of protein concentration, while the red line represents the conductivity in mS/cm. The flow thru peak represents proteins that were unable to form ionic bonds with the resin. The second taller peak represents proteins that were eluted at low salt concentration indicative that they formed weak ionic interactions with the resin. Smaller humps tailing the tallest blue peak represent separation of proteins based on different affinities to the column under different salt concentrations.

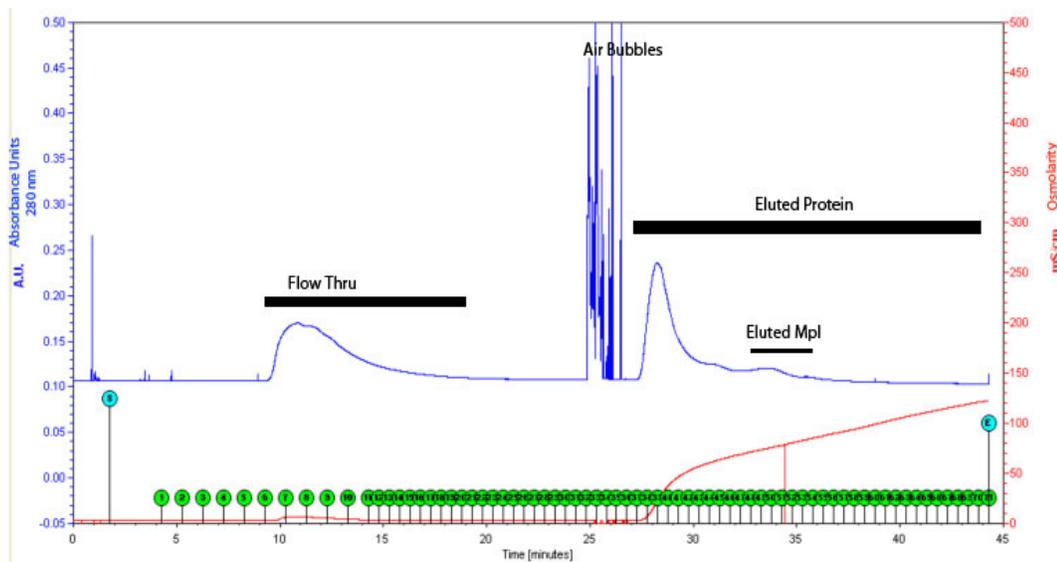


Fig 3: Determining optimal salt concentration to elute Mpl from the anionic-exchange column. The legend is formatted identically to that of Fig 1. After initiating the program, we added the protein to the column with blue peaks indicating rises in protein levels. At 10 minutes, a large peak (OD 280 nm) appeared. This was indicative of protein that did not bind to the column. Then, 28 minutes into the program, a linear salt gradient began, which caused for proteins, including Mpl, to sequentially be eluted off the ion-exchange column. Elutions 1-11 contained 5 mL each (flow rate was 5 ml/min), whereas elutions 12 and above contained 1 ml each (flow rate was 2 ml/min). Determination of where Mpl eluted required that samples be individually tested by Western immunoblot.

Fractions containing Mpl were identified by Western immunoblot (Figure 4). All of the Mpl in the sample bound to the resin, as none of it appeared in the flow thru. Mpl eluted from the column in its greatest concentration at ~64 mS/cm, which corresponds to 500 mM salt, though degradation products less than 20 kDa in size eluted in earlier fractions (data not shown) and some portions of the full length Mpl eluted both before and after reaching ~500 mM salt concentration. The purity of Mpl was assessed on a Coomassie stained protein gel loaded with concentrated Mpl-containing fractions. The results indicated that additional polypeptides eluted with Mpl (Figure 5)

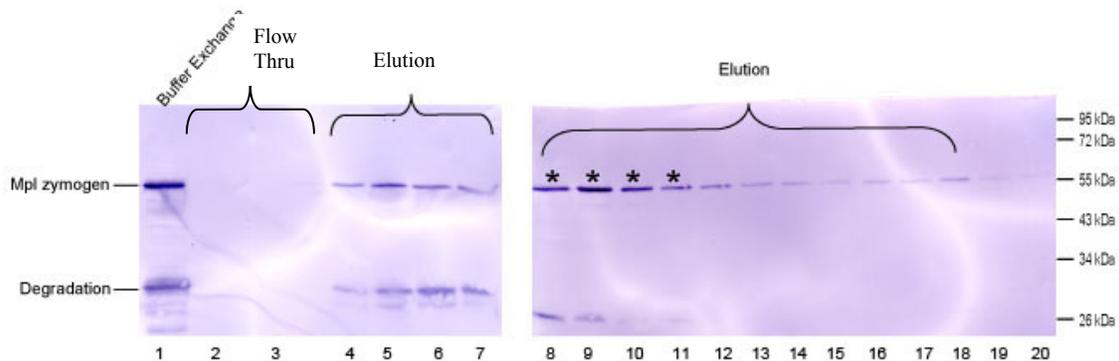


Fig 4: Samples were resolved on 12% SDS-PAGE gels and Mpl was detected by Western immunoblot. Lane 1 represents the sample that was loaded on the ion-exchange column after doing a buffer exchange. Lanes 2-3 represent proteins that did not bind to the ion-exchange column; no Mpl is detected. Lanes 7-20 represent Mpl-containing eluted protein fractions from the ion-exchange column. Lane number does not correspond to elution fractions. * = most concentrated amount of Mpl eluted.

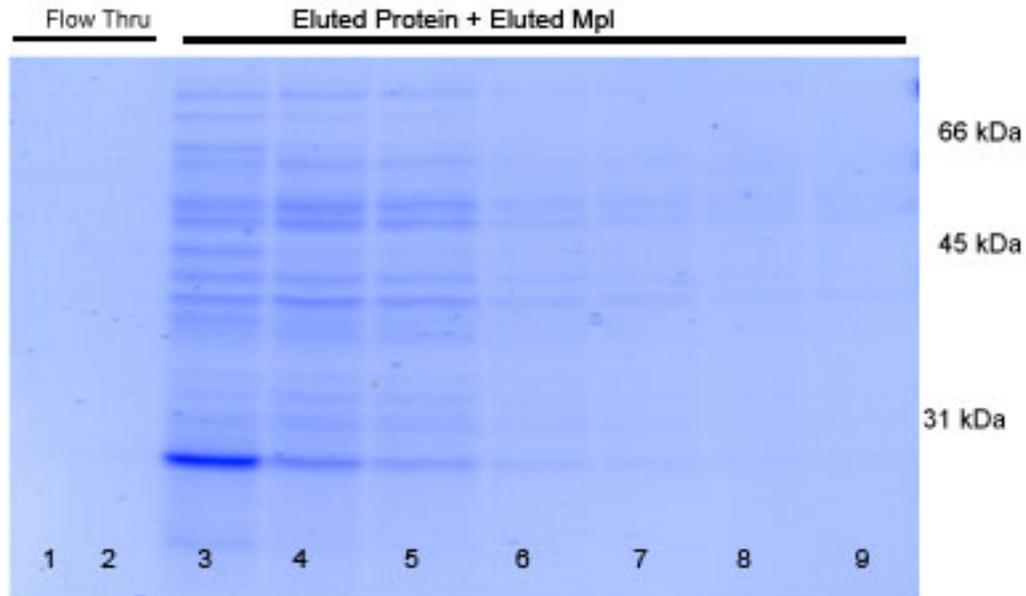


Fig 5: Samples were resolved on a 12% SDS-PAGE gel and proteins were stained with Coomassie Blue. Lanes 1-2 represent unbound protein from the ion-exchange column. Lanes 3-9 represent Mpl-containing eluted protein fractions from the ion-exchange column. Lane number does not correspond to elution fractions. These correspond to lanes 8-14 identified on the western immunoblot of Fig. 4. Mpl is not distinguishable from the other contaminants.

Considering that Mpl-containing fractions contained many other polypeptides and that Mpl was not a major protein species within these fractions, we considered eluting the proteins using a pH gradient instead of a salt gradient to improve the purification process. The experiment was repeated as described above except that a pH gradient from pH 7.8 to pH 5.0 was run to determine if pH affects the affinity of Mpl for the positively charged resin and if non-specific proteins could be further eliminated.

According to the gradient graph, two prominent absorbance curves appeared (Figure 6). The first curve represents protein unable to bind to the column (denoted as the flow thru). The second curve represents protein that eluted off the column in a pH-dependent fashion around pH 7.0. Proteins not eluted by the pH gradient were removed from the column by addition of 2 M NaCl. We observed that Mpl remained bound to the

column throughout the experiment between pH 5-7.0 and was only eluted off the column by the addition of 2M NaCl (Figure 7). Thus it was determined to keep the pH of the buffers constant at pH 7.0 and elute Mpl from the Ion-Exchange Column using a salt gradient.

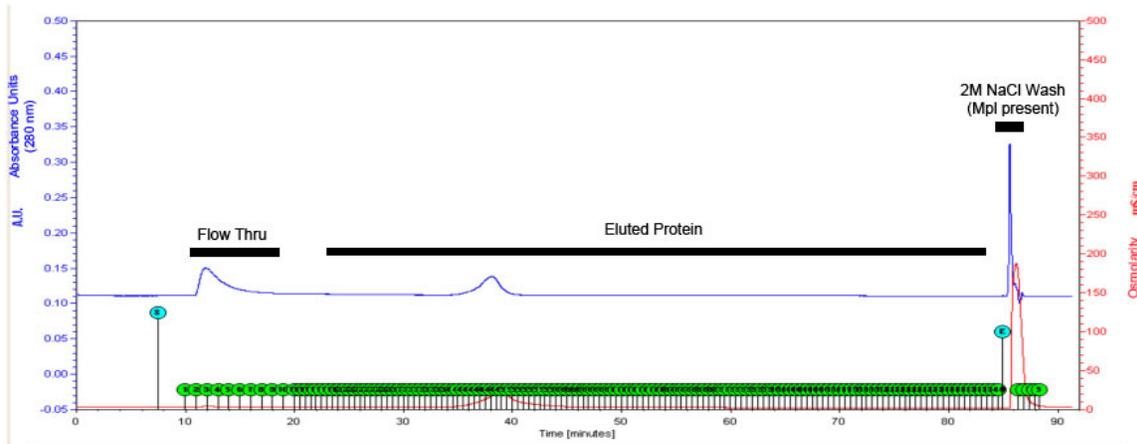


Fig 6: Determining the optimal pH to elute Mpl from the anionic-exchange column. Salt concentration was kept constant while pH varied over a linear gradient from 7.0 to 5.0. The protocol for Ion-Exchange on a 1 mL column followed the standard protocol for its volume size except the binding buffer was 20 mM Tris-HCl pH 7.0, 5 mM CaCl₂ while the eluting buffer was 20 mM Tris-HCl pH 5.0, 5 mM CaCl₂.

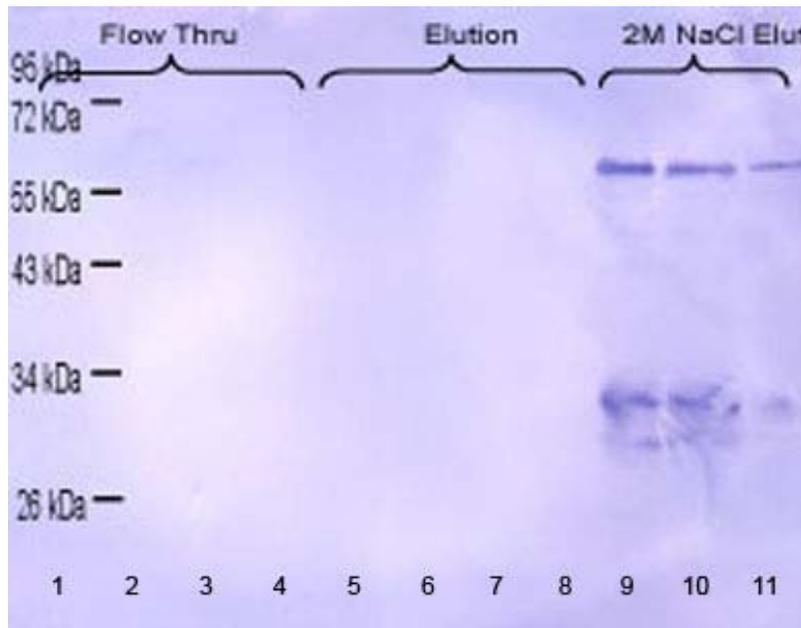


Fig 7: Samples were resolved on a 12% SDS-PAGE gels, and Mpl was detected by Western immunoblot. Lanes 1-4 represent unbound protein from the ion-exchange column. Lanes 5-8 represent non-Mpl eluted protein fractions from the ion-exchange column. Lanes 9-11 represent 2M NaCl wash fractions that do contain Mpl. Lane number does not correspond to elution fractions.

Thus to better purify Mpl, we lowered the pH of the binding buffer from 7.8 to 7.0 to remove more nonspecific protein from fractions eluting Mpl. This was achieved by performing another ion-exchange purification where the buffer's pH was kept constant at pH 7.0, and protein was eluted exclusively on the basis of a molar gradient from 0-1M NaCl. The gradient graph shows that accessory proteins modified their binding affinity as they eluted out at different times in one large flow thru peak and four small individual peaks (compare Figure 3 to Figure 8). Mpl-containing fractions were identified by Western immunoblot (data not shown). Mpl was found to elute entirely at ~500 mM NaCl as previously observed. Concentrated fractions were resolved by SDS-PAGE and stained with coomassie blue. Unfortunately, Mpl was barely visible on the stained gel (data not shown). Therefore, we increase culture size and column size in order to increased the yield of Mpl and to continue binding Mpl with equal efficiency.

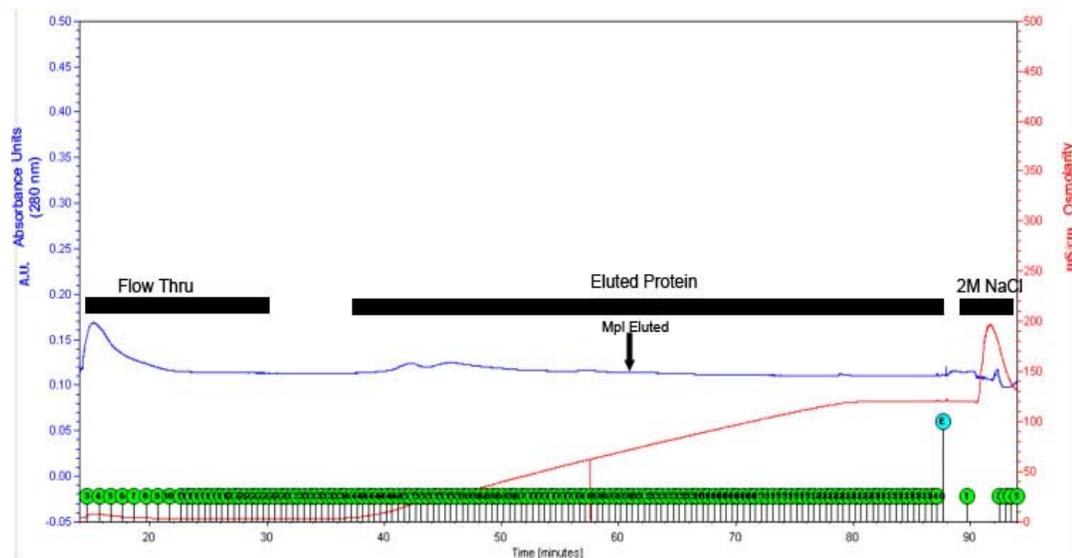


Fig 8: Confirming optimal conditions (pH=7.0 + linear salt gradient) for Mpl elution using ion-exchange chromatography. The legend remains the same as that of Fig 2. Two minutes after the addition of protein to the column, unbound protein came out of the system for 10 minutes. Non-specific protein eluted once the gradient commenced. As determined from Fig 3, Mpl eluted entirely at 500 mM (~62 minutes), thus showing that the lower pH does not affect the effectiveness of the salt to elute Mpl from the column.

Further purification made use of affinity chromatography by binding the Mpl to a resin that binds FLAG peptides. The strain of Mpl utilized contains a FLAG-tag fused to its C-terminus, and thus upon filtration through the FLAG-affinity column, Mpl will exclusively bind to the column. Mpl then is eluted from the affinity-column by adding a large concentration of FLAG peptide that outcompetes Mpl's interaction with the anti-Flag antibody. This FLAG peptide displaces Mpl. Protein without the FLAG-tag fused to it will not bind to the column and thus allows for greater separation of proteins that could not be otherwise separated through pH and conductivity.

Previous attempts to purify Mpl exclusively via Flag-tag column purification yielded concentrated amounts of Mpl, but there were several degradation products and at least 16 other protein contaminants (data supported by Alan Bitar and Gabriela Wagner in separate experiments). Therefore, decided to Mpl was purified sequentially using ion-exchange chromatography followed by affinity purification.

To continue harvesting more Mpl, a larger, 500 mL culture of *L. monocytogenes* was grown, with 20 mL ion-exchange column continuing to be employed. After growing the culture, harvesting the precipitated protein, and buffer exchanging it, the protein sample then was directly added to the ion exchange column (Figure 9). Mpl-containing fractions were identified by Western immunoblot. No Mpl zymogen was lost in the flow thru. Mpl eluted over 32 fractions (from fractions 23-55). No Mpl remained bound to the column after fraction 55, as nothing was further eluted during the 2M NaCl purge (Figure10).

These samples then were pooled and further used for Flag-tag column purification. Samples collected over the entire experiment were resolved via Western immunoblot (Figure 11). The full-length Mpl zymogen and various sizes of degradation products were loaded on the Flag-tag affinity column. However, the large majority of the degradation products were eliminated during purification as the fractions eluted from the Flag-tag affinity column consisted of the full-length zymogen (~57 kDa) and a single degradation product (~30 kDa). Concentrated fractions were then resolved on a separate gel and stained with Coomassie Blue (Figure 12). Mpl was detected with some minor contaminants. Although tests such as mass spectroscopy would be required to determine the identity of the accessory bands, there may be up to 5 proteins that co-purified. The sample is thus mostly pure.

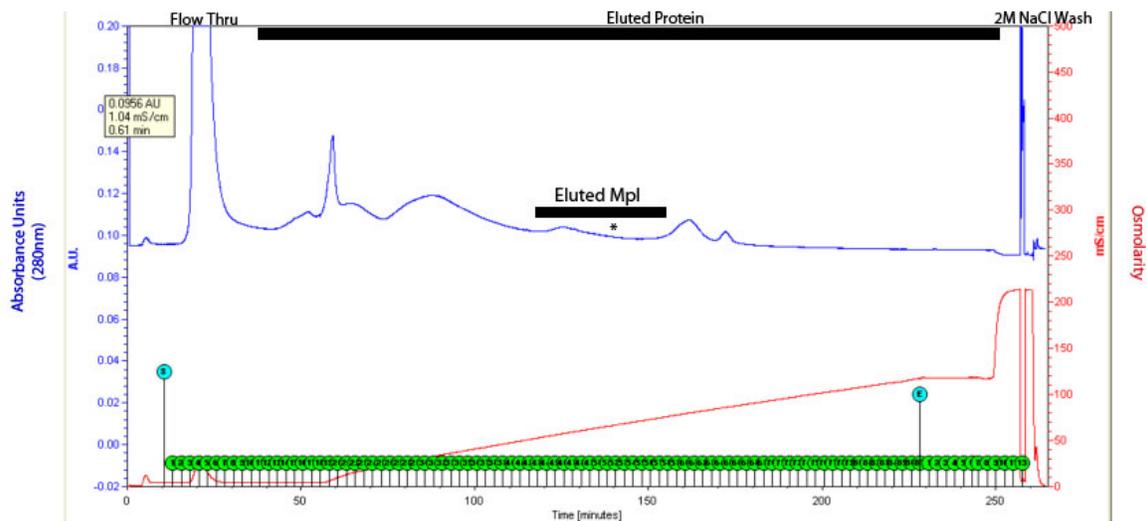


Fig 9. Ion-exchange column purification of a 500 mL culture on a 20 mL resin column. Legend is identical to that in Fig 3. High amounts of flow thru appeared 2 minutes after direct addition of protein to the column. Nonspecific protein eluted throughout the experiment and increased as the gradient began. Western immunoblotting determined the location of Mpl (Fig 11), and it is denoted on this figure above a black bar labeled “Eluted Mpl”. The asterisk indicates the location of the most heavily concentrated elution of Mpl.

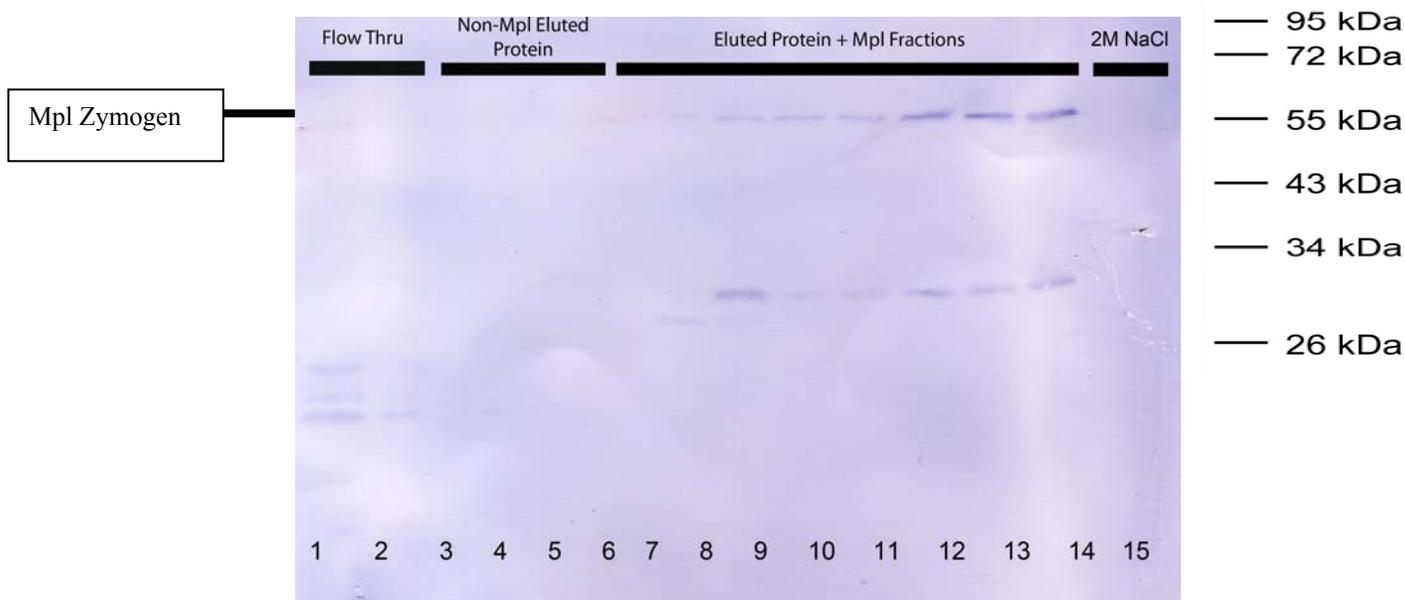


Fig 10: Samples were resolved on a 12% SDS-PAGE gel and Mpl was detected by Western immunoblot. Lanes 1-3 are protein unable to bind to the Ion-exchange column (denoted as flow thru); lanes 4-6 are non-Mpl eluted protein; lanes 7-14 are Mpl-containing fractions. Lane 15 is a fraction of 2M NaCl wash.

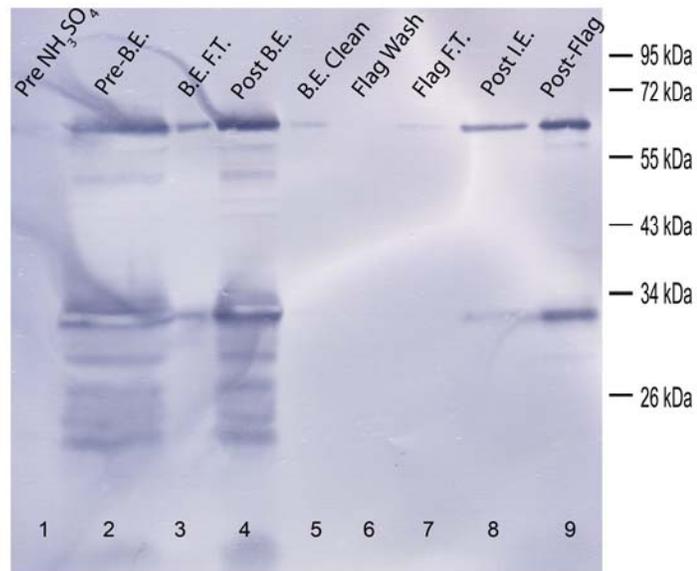


Fig 11: Results from sequential steps of purification are shown in this figure. As it is on the gel from lanes 1 to 9 sequentially: Pre-NH₃SO₄ represents the crude supernatant (0.005%). Pre-B.E. represents the concentrated NH₃SO₄ solution (0.42%). B.E. F.T. represents buffer exchange flow thru (0.83%). B.E. Clean represents protein lost from the buffer exchange column (0.125%). Flag Wash (0.1%) and Flag F.T. (0.83%) (Flag Flow Thru) represent unbound protein during FLAG-Affinity purification. Post- I.E. (0.06%) is Mpl recovered after ion-exchanger purification. Post-FLAG (0.53%) is Mpl recovered after affinity purification.

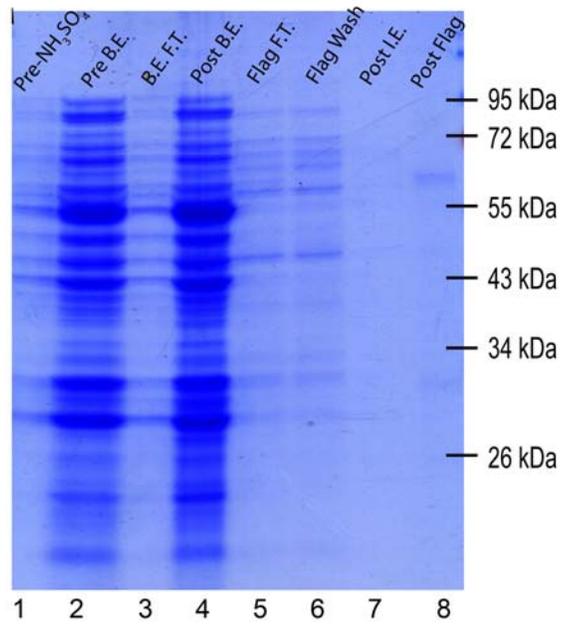


Fig 12: Coomassie stained gel from various fractions collected during Mpl purification. The samples were loaded in the same order as described in Figure 11 except that each sample is ten times more concentrated.

Discussion:

The purpose of this study was to isolate Mpl in the greatest quantity and, most importantly, with the greatest purity. This highest purity was achieved by purifying Mpl first on an ion-exchange column at pH 7.0 eluting at 500 mM, followed by Flag-tag affinity purification.

Initially we tried to express the catalytic mutant of Mpl in *E. coli*, but consistently the yield of Mpl was too low and the protein was mostly degraded. Thus purification in the host bacterium, *L. monocytogenes*, became the obvious alternative and proved successful.

Mpl secreted in the supernatant of a bacterial culture was purified in two steps. First, the protein was purified by ion-exchange chromatography, using an anion-exchange resin. Using this approach, Mpl eluted from the column at a salt concentration of 500 mM with a few contaminating polypeptides. Second, the Mpl-containing fractions from the ion-exchange column were further purified by Flag-tag affinity chromatography. After this second step, Mpl was the predominant protein found in the eluted fractions, but there were still some contaminants. The identity of the contaminating bands remain unknown. In previous experiments where exclusively ion-exchange was conducted followed by Coomassie Blue Staining of Mpl-rich samples, a secondary band co-eluted slightly below where Mpl is expected to resolve upon gel fractionation. This protein (~54.5 kDa), which remains difficult to view in figure 12 due to being present in a low concentration, was identified by mass spectroscopy to be the fumarate reductase flavoprotein subunit. It is thus presumed that this less concentrated band present below the predominant band of Mpl in the Coomassie Blue Stain after Flag-tag affinity

purification is also fumarate reductase flavoprotein subunit. To determine the other four proteins, mass spectroscopy could also be utilized.

The fact that one must scrutinize the final purification product to view the contaminating proteins that co-eluted with Mpl implies that very little of these proteins contaminated the Mpl sample. A BCA assay was conducted to determine the percent total protein lost between each step yielding purification to thus determine the percentage of Mpl in the total amount of cultured *L. monocytogenes* supernatant. In an effort not to waste protein, too little Mpl was utilized in the BCA conducted, and thus I was unable to quantify the proper amount.

Accessory proteins that co-eluted with Mpl may be appearing due to an interaction these proteins have with our protein of interest, such as retaining it in its conformation and/or serving as chaperones. These proteins may be associating with Mpl simply due to nonspecific weak intermolecular forces of attraction, such as van der waal forces, ionic bonds, covalent bonds, and hydrogen bonds.

Other methods to further purify Mpl could include knocking out the genes to the respective co-eluting proteins identified. Alternatively, fractions after ion-exchange that possess Mpl could then be excised from the gel and eluted from the gel by dialysis. This method often leads to a significant loss of protein. Size exclusion chromatography remains a possible candidate for further purification as it may separate some proteins of contrasting molecular size, but proteins such as the nearly co-migrating presumed fumarate reductase flavoprotein subunit would not be separated.

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