

Video Article

Expression, Purification, and Antimicrobial Activity of S100A12

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Abstract

Calgranulin proteins are important mediators of innate immunity and are members of the S100 class of the EF-hand family of calcium binding proteins. Some S100 proteins have the capacity to bind transition metals with high affinity and effectively sequester them away from invading microbial pathogens in a process that is termed "nutritional immunity". S100A12 (EN-RAGE) binds both zinc and copper and is highly abundant in innate immune cells such as macrophages and neutrophils. We report a refined method for the expression, enrichment and purification of S100A12 in its active, metal-binding configuration. Utilization of this protein in bacterial growth and viability analyses reveals that S100A12 has antimicrobial activity against the bacterial pathogen, *Helicobacter pylori*. The antimicrobial activity is predicated on the zinc-binding activity of S100A12, which chelates nutrient zinc, thereby starving *H. pylori* which requires zinc for growth and proliferation.

Video Link

The video component of this article can be found at <https://www.jove.com/video/55557/>

Introduction

S100 proteins are a class of the EF-hand family of calcium binding proteins with a diverse array of functions¹. They are expressed in a tissue and cell specific manner, and regulate a broad spectrum of cellular functions^{2,3}. Unique to calcium binding proteins, S100 proteins exhibit both intracellular and extracellular functions^{4,5}. Within the cell, Ca²⁺ binding induces a conformational change that exposes a hydrophobic surface that specifically targets protein binding partners⁶. This intracellular mechanism regulates important processes such as cell proliferation, differentiation and energy metabolism. In the extracellular milieu, S100 proteins exhibit two functions⁷. In one, they act as damage associated molecular pattern (DAMP) proteins and initiate a pro-inflammatory immune response through interaction with pattern recognition receptors^{8,9}. Additionally, several members of the S100 protein class sequester transition metals, a function that serves to starve microbial pathogens in a process termed nutritional immunity^{10,11}.

S100A12 (also known as calgranulin C and EN-RAGE) is highly expressed in macrophages and neutrophils and has been identified as a potential biomarker for inflammatory diseases^{12,13}. In addition to binding calcium at its EF-Hand sites, S100A12 has two high affinity transition metal binding sites located at opposite ends of the dimer interface^{14,15}. Each binding site is comprised of three histidine residues and one aspartic acid residue and can chelate zinc or copper^{16,17}. Recently, we reported that S100A12-dependent zinc starvation is important in regulating *Helicobacter pylori* growth and the activity of pro-inflammatory virulence factors¹⁸.

H. pylori infects the stomach of about half of the world's human population; making it arguably one of the most successful bacterial pathogens¹⁹. Infection with *H. pylori* can lead to significant gastric disease outcomes including gastritis, peptic and duodenal ulcer, mucosa associated lymphoid tissue (MALT) lymphoma, and invasive gastric adenocarcinoma (stomach cancer). Stomach cancer is the leading cause of non-cardia cancer-associated death in the world, and the single biggest associated risk factor for stomach cancer is infection with *H. pylori*.

H. pylori persists in the gastric niche despite a robust immune response to the pathogen, underscoring the need for a better understanding of immune mechanisms of controlling this bacterial infection^{20,21,22,23}. *H. pylori*-associated inflammation is characterized by a profound infiltration of polymorphonuclear cells, or neutrophils, which deposit a repertoire of antimicrobial proteins, including S100A12, at the site of infection^{18,24,25}. In an effort to understand the complex dialogue between host and pathogen, we sought to refine the technique to purify S100A12 and use it to study the antimicrobial affect it exerts upon this medically relevant pathogen. The protocol below outlines an improved technique for S100A12 purification in its biologically active state; capable of binding nutrient metals with high affinity and chelating them away from invading microorganisms. Furthermore, the methods below highlight the utility of this protein as a critical reagent to study the mechanism by which innate antimicrobial molecules restrict the growth of bacterial pathogens.

S100A-family proteins have gained appreciation as an important group of innate immune system molecules which participate in immune signaling as well as host defense²⁷. The most well-studied of these is calprotectin (MRP-8/14, calgranulin A/B, S100A8/A9)^{28,29,30}. Calprotectin

is a neutrophil-associated protein which forms a heterodimer of the S100A8 and S100A9 subunits which binds transition metals at the dimer interface³¹. Calprotectin has been shown to possess two metal binding sites: Site 1 can bind Zn²⁺, Mn²⁺, or Fe²⁺, and Site 2 can bind Zn²⁺^{31,32}. Numerous reports have demonstrated that calprotectin has antimicrobial activities against diverse pathogens including *Staphylococcus aureus*, *Candida albicans*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Escherichia coli*, and *H. pylori*, and that the inhibitory effects are due to the metal chelation activity of calprotectin^{25,28,29}.

Previous work demonstrated calprotectin exerts numerous activities on *H. pylori* including altering the lipid A structure in the outer membrane, repressing the *cag*-Type IV secretion system (which is a major proinflammatory virulence factor within *H. pylori*), inducing biofilm formation, and repressing *H. pylori* growth and viability in a dose-dependent manner^{25,33}. Furthermore, genetic and biochemical assays revealed the antibacterial activity of calprotectin against *H. pylori* was largely derived from its ability to bind nutrient zinc²⁵. *H. pylori* requires zinc, as was determined by previous research which utilized a chemically defined medium to ascertain the micronutrient requirements for this pathogen to grow and proliferate³⁴. Additionally, calprotectin was highly abundant within *H. pylori*-infected tissues, and associated with neutrophilic infiltrates, indicating the host may be employing calprotectin as an antimicrobial strategy during infection and subsequent inflammation^{25,35}.

Recent evidence from unbiased proteomics screening techniques suggests that under conditions where reactive oxygen species are plentiful, calprotectin undergoes post-translational modifications which alter the hexa-histidine binding site, thereby inhibiting the metal-binding activity of the protein³⁶. As such, we hypothesized that other S100A-family proteins among the wide repertoire of these molecules could potentially act as auxiliary metal-chelators. We selected S100A12 for further study because it was not identified in the aforementioned screen for post-translational modification, it has the capacity to bind zinc, and it is highly abundant in human tissues derived from *H. pylori*-infected individuals.

Our work indicates that S100A12 can inhibit *H. pylori* growth and viability in a dose-dependent manner in the G27 strain of *H. pylori*, and that the antimicrobial activity of this protein can be reversed by the addition of excess nutrient zinc. This work complements our previous work indicating S100A12 exerted antimicrobial activity against PMSS1 and 7.13 strains of *H. pylori*, demonstrating its broad antibacterial activity against numerous clinical isolates and laboratory-adapted strains of *H. pylori*¹⁸. Together, these results confirm the importance of S100A12 as a mechanism to control bacterial growth and proliferation via nutritional immunity. Future studies of this important host-bacterial interaction could include exploiting the activity of S100A12 to reduce bacterial burden within host tissues, or determining the contribution of this protein to immune signaling in the context of *H. pylori* infection.

Protocol

1. Expression of S100A12

1. Transform competent BL21 DE3 cells with a pGEMEX-S100a12 plasmid using a standard heat shock protocol¹⁸. Add 1 to 5 μ L of plasmid to 50 μ L of bacteria in a microcentrifuge tube on ice. Incubate for 20 min.
2. Heat shock the cells at 42 °C for 30 s.
3. Incubate the cells on ice for 2 min.
4. Add 500 μ L of SOC media to the cells. Incubate at 37 °C with shaking at 250 rpm on an orbital shaker for 1 h.
5. Plate 150 μ L of the transformation reaction on LB-agar medium (supplemented with 100 μ g/mL ampicillin). Incubate for 12-16 h at 37 °C.
6. Pick one colony. Inoculate 2 mL of LB (supplemented with 100 μ g/mL ampicillin).
7. Incubate for 4-6 h at 37 °C on an orbital shaker (300 rpm). The OD₆₀₀ should read between 1-3 absorbance units.
8. Add 500 μ L of starter culture to 50 mL of ZYM-5052 autoinduction media²⁶ supplemented with 100 μ g/mL of ampicillin. For best aeration and maximum expression, use a 250 mL baffled Erlenmeyer flask. Shake (300 rpm) for 24 h, at 37 °C.
9. Transfer bacterial suspension to a centrifuge tube. Pellet the cells by centrifugation (4,000 x g, 10 min) at 4 °C.
10. Decant the media, log sample, and store cell paste at -80 °C. Sample is stable for years.

2. Purification of S100A12 Using Low Pressure Chromatography

1. Resuspend cells in 30 mL of 20 mM Tris, pH 8.0.
2. Sonicate the suspension on ice to lyse cells. Use ~20 W output, 5 s on and 5 s off cycle for 5 min.
3. Transfer the solution to high-speed centrifuge tubes. Clarify the cell lysate by centrifugation, 20,000 x g for 30 min at 4 °C.
4. Decant the supernatant and transfer to a clean 100 mL polypropylene beaker. Cool the solution by placing the beaker on ice. Add a stir bar and slowly add 11.20 g of ammonium sulfate. Allow the solution to stir on ice for an additional 1 h. This will create a 60% solution of ammonium sulfate and precipitate most of the *E. coli* endogenous proteins. S100A12 will remain soluble.
5. Centrifuge the solution at 4 °C, 20,000 x g for 20 min to pellet the precipitated protein.
6. Decant the supernatant and transfer to dialysis tubing (MWCO 3,500 kDa). Dialyze against 1 L of 20 mM Tris, pH 8.0 at 4 °C. Change the dialysis buffer twice. Allow 4 h in between changes.
7. **Anion exchange chromatography**
 1. Perform chromatography on a low pressure system. Typical flow rate is 1 mL/min.
 2. Equilibrate a 5 mL Sepharose column with 10 mL of 20 mM Tris, pH 8.0.
 3. Load the ~40 mL of S100A12 solution using the sample pump (collect the flow through).
 4. Wash the column with 10 mL of 20 mM Tris, pH 8.
 5. Develop the column with a 0-30% gradient (Buffer B is 20 mM Tris, pH 8.0, 1 M NaCl) over 19 column volumes (CV, 95 mL). Collect 5 mL fractions.
 6. Take a 10 μ L aliquot of each fraction and analyze using MES SDS PAGE with Coomassie staining. Run gel using constant voltage (20 V/cm) for 30 min.
 7. Pool fractions containing S100A12. S100A12 runs at ~10 kDa protein on a denaturing gel.
 8. Concentrate fractions to 5 mL using an ultrafiltration device (MWCO 10 kDa). Centrifuge at 3,000 x g for ~8 min. Take the top fraction.

8. Size-exclusion chromatography

1. Equilibrate S75 column with 1 CV (120 mL) of 20 mM Tris pH 8, 100 mM NaCl.
2. Inject < 5 mL of concentrated S100A12 fractions (from the ion exchange chromatography).
3. Develop the column at a flow rate of 1 mL/m over 120 mL. Collect 5 mL fractions.
4. Take a 10 μ L aliquot of each fraction and analyze using SDS PAGE with Coomassie staining. Validate protein identity using western blot or mass spectrometry (calculated molecular mass of monomeric subunit 10,575.0 Da, measured 10575.4 Da).

9. Pool fractions

1. Measure the absorbance of the protein A_{280} using a spectrophotometer. Use the SEC buffer as a blank. The calculated extinction coefficient for S100A12 homodimer is $5960 \text{ M}^{-1} \text{ cm}^{-1}$.
NOTE: Typical yields are 35-45 mg of S100A12 per 50 mL of culture.
2. Aliquot S100A12 into 1.5 mL microcentrifuge tubes (1 mg/tube), flash freeze in liquid nitrogen and store at $-80 \text{ }^\circ\text{C}$.

3. Antimicrobial Activity Assays

1. Streak *H. pylori* strain G27 onto tryptic soy agar plates supplemented with 5% sheep blood (blood agar plates). Grow 2-3 days at $37 \text{ }^\circ\text{C}$ in room air supplemented with 5% carbon dioxide.
2. Inoculate *H. pylori* into Brucella broth supplemented with 1x cholesterol. Culture overnight shaking (250 rpm) at $37 \text{ }^\circ\text{C}$ in room air supplemented with 5% carbon dioxide.
3. Dilute *H. pylori* 1:10 into 50% Brucella broth, 50% calprotectin buffer plus 1x cholesterol and culture in medium alone or supplemented with 100 μ M zinc chloride plus 0, 100 or 1,000 μ g/mL of purified S100A12. Culture overnight shaking (250 rpm) at $37 \text{ }^\circ\text{C}$ in room air supplemented with 5% carbon dioxide.
4. The following day, perform serial dilutions and plate onto blood agar plates. Allow bacterial colonies to grow for 2-3 days at $37 \text{ }^\circ\text{C}$ in room air supplemented with 5% carbon dioxide. Enumerate the colony forming units to calculate bacterial growth in the presence or absence of S100A12 and/or exogenous zinc.

Representative Results

S100A12 expression and purification

A three-step purification produced ~40 mg of recombinant S100A12 from 50 mL of bacterial culture. The first step was an ammonium sulfate precipitation of endogenous *E. coli* proteins. This step was followed by anion-exchange chromatography (**Figure 1A**). The protein is tracked by a SDS-PAGE stained with Coomassie Brilliant Blue (**Figure 1B**). The last step of the purification procedure involved pooling fractions containing S100A12 for size-exclusion chromatography which separates proteins by molecular weight and shape (**Figure 2A**). S100A12 is a homodimer (92 amino acids per subunit) and has a total molecular weight of about 21 kDa. Fractions collected from size-exclusion chromatography were analyzed by SDS-PAGE and visualized by Coomassie staining (**Figure 2B**).

S100A12 represses bacterial growth via zinc chelation activity

To investigate the antimicrobial activity of S100A12, bacterial viability analyses were performed via quantitative microbiological culture techniques (**Figure 3**). Enumeration of bacterial cells reveals that exposure to 100 μ g/mL of S100A12 in the presence or absence of an exogenous source of nutrient zinc does not significantly inhibit bacterial viability compared to controls without S100A12 added ($P > 0.05$, One way ANOVA). However, exposure to 1000 μ g/mL of S100A12 in medium alone results in a 69-fold decrease in bacterial viability compared to medium alone ($P = 0.0193$, Student's *t* Test, $P > 0.05$ One Way ANOVA); a result that was reversed by the addition of an exogenous source of nutrient zinc ($P = 0.023$, Student's *t* Test). These results demonstrate the antimicrobial activity of S100A12 is dependent upon its zinc sequestration activity.

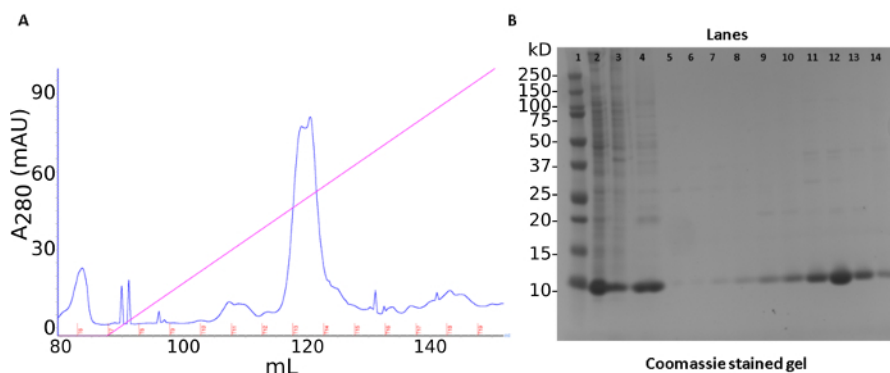


Figure 1: Cell lysis and S100A12 purification by anion exchange chromatography. (a) Chromatogram of ion exchange purification. Trace of UV absorbance at 280 nm shown in blue, salt gradient depicted in pink, collected fractions marked in red. (b) SDS PAGE gel of purification steps. Lanes: 1) molecular weight standard 2) soluble lysate fraction 3) ammonium sulfate pellet 4) ammonium sulfate supernatant 5-14) Q chromatography fractions 6-15. [Please click here to view a larger version of this figure.](#)

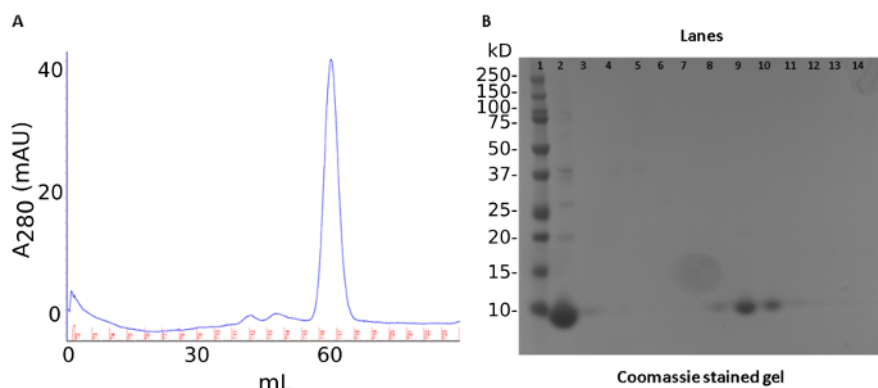


Figure 2: Size exclusion chromatography results of S100A12 purification. (a) Chromatogram of size-exclusion results. Trace of UV absorbance at 280 nm shown in blue, collected fractions marked in red. (b) SDS PAGE gel of size-exclusion chromatography. Lanes: 1) molecule weight marker 2) sample load 3-15) fractions 10-21. [Please click here to view a larger version of this figure.](#)

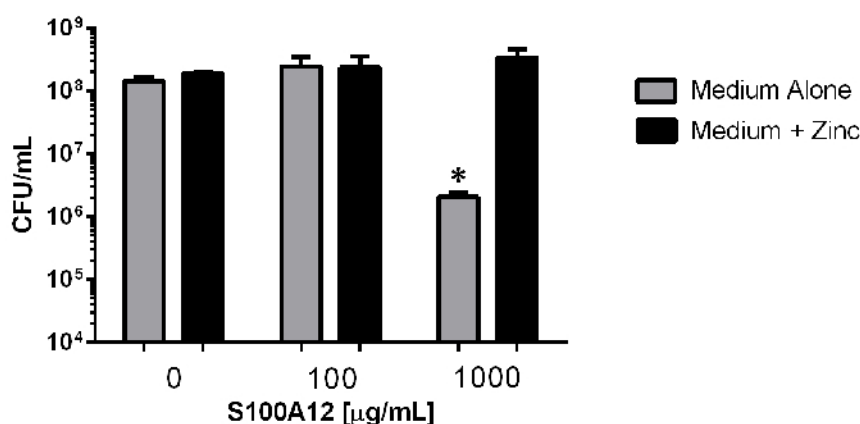


Figure 3: Quantitative culture analyses of bacterial viability in response to exposure to S100A12-dependent zinc chelation. Bacteria were exposed to 0, 100, or 100 µg/mL of S100A12 in medium alone (grey bars), or medium supplemented with 100 µM zinc chloride (black bars). Exposure to 1,000 µg/mL in the absence of an exogenous source of nutrient zinc results in significant inhibition of bacterial viability (*P < 0.05, Student's t test compared to medium + zinc condition). [Please click here to view a larger version of this figure.](#)

Discussion

An efficient protocol for both expression and purification of human S100A12 is presented. The *E. coli* expression system is the most common tool used for the production of recombinant proteins, particularly when mg quantities are required for biochemical and biophysical studies. A key enhancement of the procedure described here is the use of auto-induction media²⁶ which increases the yield of purified protein by a factor of almost thirty as compared to expression with standard Luria-broth media¹⁸. Additionally, auto-induction media greatly simplifies the protein-expression workflow. Using conventional growth media, cultures must be continuously monitored so that an inducing agent can be added during the exponential growth phase. There is no need to monitor the doubling times when using auto-induction media. The cultures are allowed to grow to saturation. During the initial stages of growth with auto-induction media, *E. coli* uses glucose as a carbon source. Once the glucose is depleted, the bacteria switch to lactose as a carbon source which induces protein expression²⁶. The composition of the auto-induction media is exquisitely tuned to allow for the growth of high density cultures and therefore increased expression of recombinant protein. In our experience with S100A12, auto-induction media greatly increases the amount of expressed protein, however we caution that this may be protein dependent and should be experimentally verified.

S100A12 is expressed in the cytoplasmic fraction of *E. coli* which suggests that it is soluble and well folded. The first step of the purification involves adding a high percentage of ammonium sulfate which precipitates many of the endogenous *E. coli* proteins. This step leverages the high stability and solubility of the S100 family of proteins. S100A12 is moderately acidic (pI 5.81). Thus S100A12 is purified further with a strong anion exchange resin. The last step of the purification process is a size-exclusion chromatography column which ensures that S100A12 is monodisperse and dimeric. This step of the protocol is crucial as S100A12 has been shown to form soluble oligomers¹⁵ and it is unknown what affect oligomerization may have on its antimicrobial activity. Since members of the S100 class share high sequence and structural homology, their physical properties are similar²⁷. Hence, this method of purification of S100A12 may be broadly applicable to all S100 proteins that are expressed in the soluble fraction of *E. coli*.

Previous work has demonstrated that S100 proteins, such as calprotectin, have antimicrobial activity against bacterial pathogens such as *H. pylori*, and that this activity is dependent upon zinc-binding activity²⁵. The antimicrobial activity of S100A12 is also zinc dependent, but the growth inhibition demonstrated in bacterial growth assays reveals that significantly more S100A12 is required to inhibit growth compared to other S100-family proteins such as calprotectin. Thus, it is critical to use higher concentrations of S100A12 than calprotectin to achieve similar phenotypes

(growth inhibition or alterations in virulence). Future applications of this protocol could be applied to determine global changes in bacterial gene expression, metabolomic, or proteomic alterations in response to the metal sequestration imposed by S100A12.

Disclosures

The authors declare that they have no competing financial interests.

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