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A Systematic Approach Toward Stabilization of CagL, a Protein Antigen from *Helicobacter pylori* That Is a Candidate Subunit Vaccine

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Abstract

An important consideration in the development of subunit vaccines is loss of activity caused by physical instability of the protein. Such instability often results from suboptimal solution conditions related to pH and temperature. Excipients can help to stabilize vaccines, but it is important to screen and identify excipients that adequately contribute to stabilization of a given formulation. CagL is a protein present in strains of *Helicobacter pylori* that possess type IV secretion systems. It contributes to bacterial adherence via $\alpha 5\beta 1$ integrin, thereby making it an attractive subunit vaccine candidate. We characterized the stability of CagL in different pH and temperature conditions using a variety of spectroscopic techniques. Stability was assessed in terms of transition temperature (T_m) with the accumulated data then incorporated into an empirical phase diagram (EPD) that provided an overview of CagL physical stability. These analyses indicated maximum CagL stability at pH 4–6 up to 40 °C in the absence of excipient. Using this EPD analysis, aggregation assays were developed to screen a panel of excipients with some found to inhibit CagL aggregation. Candidate stabilizers were selected to confirm their enhanced stabilizing effect. These analyses will help in the formulation of a stable vaccine against *H. pylori*.

Introduction

The gram-negative bacterium *Helicobacter pylori* establishes lifelong infection of the human gastric mucosa.^{1,2} The bacterium is considered an important human pathogen because over half of the world's population is infected, with rates as high as 90% in many developing nations. It is recognized as the etiologic agent of most peptic ulcer disease³ and is a significant risk factor for the development of gastric adenocarcinoma, classified by the WHO International Agency for Research on Cancer as a Class I carcinogen.⁴ Although less than 20% of infected individuals develop disease, the large number of infected individuals and the nature of *H. pylori*-related gastric disorders create a heavy burden on health care systems world-wide. The U.S. Department of Health and Human Services estimate that in 2004 alone, direct costs associated with peptic ulcer disease reached \$2.6 billion in the U.S. with additional indirect costs associated with loss of work productivity estimated to be over \$518 million. Gastric cancer costs in 2004 were estimated to be \$487 million with indirect costs reaching \$1.4 billion. Although efficacious antimicrobial therapies to treat *H. pylori* are used routinely, it remains an important pathogen because most infected individuals

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remain asymptomatic for years or decades before presenting with disease, especially in the case of gastric cancer. Therefore, in parts of the world where gastric cancer remains one of the primary causes of death due to cancer, a vaccine for *H. pylori* is highly desirable.⁵

Development of a vaccine against *H. pylori* is complicated by its nature as a noninvasive bacterium. The bacteria remain strictly luminal and do not cross the epithelium. An effective vaccine therefore must induce the appropriate Th memory cells that can be recruited to mucosal surfaces. Multiple strategies and adjuvants have been tested for vaccination against *H. pylori* in mice, nonhuman primates, and in clinical trials.⁶ These studies have employed mucosal delivery systems *via* orogastric or intranasal applications, however, the use of systemic immunization has also been investigated. Most vaccines that have been tested in human trials have relied on the delivery or expression of a single *H. pylori* antigen, typically the urease protein.^{6,7} There is evidence, however, that improved efficacy can be achieved with multivalent subunit vaccines.⁸ Thus, the identification and characterization of additional candidate antigens would be advantageous for the continued advancement of a *H. pylori* vaccine. One such candidate for use in a subunit vaccine is the protein CagL.

The pathogenesis of *H. pylori* is promoted by both inflammatory and carcinogenic events. Strains possessing the type IV secretion system (T4SS) encoded by the Cag pathogenicity island (PAI) are generally considered to be more virulent and can induce more severe disease. Contact of the T4SS with the host epithelium induces IL-8 production⁹ and also provides a means for the bacteria to “inject” the CagA oncoprotein into the host cell where it influences a number of cellular processes.^{10–14} As part of the *Helicobacter* T4SS, CagL participates in attachment to the host cell through contact with $\alpha 5\beta 1$ integrin and this interaction is important the induction of IL-8 secretion.^{15,16} CagL is therefore a highly conserved protein present in what are considered the more virulent and carcinogenic strains of *H. pylori* where it plays a role in inducing both host inflammatory responses and carcinogenic events. A detailed characterization of the physical properties of this 26 kDa protein would provide important information for incorporating CagL into an experimental subunit vaccine. Toward this end, we have purified CagL and subjected it to extensive biophysical analysis to provide insight into its solution properties and to explore conditions suitable to stable vaccine formulation. Spectroscopic methods were used to monitor CagL secondary, tertiary and quaternary structures. The accumulated data were then summarized in an empirical phase diagram (EPD) to allow for the screening of excipients that may be useful in the future formulation of a stable CagL-containing vaccine against *H. pylori* infection.¹⁷

Materials and Methods

Protein Preparation

The *cagL* coding sequence was enzymatically amplified by standard PCR from *H. pylori* strain 26695 (ATCC #700392) and the gene lacking the region encoding an N-terminal signal peptide was cloned into pET15b, which was then used to transform *Escherichia coli* Tuner (DE3).¹⁵ The CagL sequence was analyzed by blast analysis against several dozen clinical isolates whose genomes have been deposited with NCBI, and it was found to be highly conserved between isolates (95–100% identify at the nucleotide level). The CagL protein was then expressed cytoplasmically and purified using Immobilized Metal Affinity Chromatography (IMAC) by virtue of an N-terminal histidine tag as reported.¹⁵ For preparing large amounts of CagL, the protein was produced in a 10 L fermenter. After overnight growth, the culture was induced using a final concentration of 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). The bacteria were harvested by centrifugation at $4500 \times g$ for 20 min and the bacterial pellet was resuspended in CW buffer (25 mM potassium phosphate monobasic, 25 mM potassium phosphate dibasic, 200 mM NaCl pH 7.5) with

addition of a protease inhibitor cocktail (Roche Corp.). After cell disruption by microfluidizing, the insoluble material was collected by centrifugation at $22,000 \times g$ for 15 min. The overexpressed protein formed inclusion bodies which were solubilized into LW buffer (25 mM potassium phosphate monobasic, 25 mM potassium phosphate dibasic, 200 mM NaCl pH 7.5 containing 6 M guanidine hydrochloride) and homogenized. The homogenized suspension was centrifuged at $22,000 \times g$ for 15 min and the supernatant was collected. The supernatant was diluted with 2.4 L of ice cold refolding buffer (67.5 mM Tris-HCl, 20 mM NaCl, 0.8 mM potassium chloride, 1.1 mM EDTA 2 mM reduced glutathione and 0.2 mM oxidized glutathione) with stirring at 4 °C for 4 h. Meanwhile, a column of TALON IMAC resin (BD Biosciences) was charged with CoCl_2 and then equilibrated with refolding buffer. The protein sample was then loaded onto the column with subsequent washing. The bound protein was released using elution buffer (150 mM imidazole, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, 137 mM sodium chloride and 2.7 mM potassium chloride, pH 7.4). Fractions were collected and analyzed by SDS-PAGE with Coomassie blue staining where CagL protein migrated as a single species with a molecular mass of approximately 25 kDa and the final purity estimated to be > 90% by densitometry (Suppl. Fig. S1). The fractions containing CagL were pooled, dialyzed into PBS, filtered and stored at -80°C .

For the majority of the spectroscopic analyses used in this study, the protein was dialyzed using 10-kDa molecular weight cut-off Slide-A-Lyzer units (Fisher Scientific) into 20 mM citrate/20 mM phosphate (CP) buffer adjusted to an ionic strength of 150 mM with sodium chloride, at a pH ranging from 3 to 8. The protein was filtered after dialysis using 0.45 μm filter, and the concentration was determined using UV visible spectroscopy with a molar extinction coefficient of $10,555 \text{ M}^{-1}$. The final protein concentration was adjusted to 0.2 mg/ml for use in most of the biophysical studies described, however, the concentration was made to 3 mg/ml for the near-UV circular dichroism measurements.

Far-UV Circular Dichroism (CD) Spectroscopy

Circular dichroism (CD) spectroscopy was used to monitor the CagL secondary structure as a function of pH and temperature.¹⁸ CD spectra and CD-based thermal unfolding profiles were acquired in 0.1 cm path length cuvettes, using a Jasco J-815 CD spectrometer equipped with a Peltier temperature controller. The spectra were obtained in the far-UV region 260–190 nm and the thermal unfolding profiles were obtained using the minimum at 222 nm to monitor the loss of α -helical structure as a function of temperature from 10 to 90 °C. The signal was recorded every 2.5 °C using a temperature ramp rate of 15 °C/h and the samples were equilibrated to within 0.1 °C of the target temperature for 5 sec prior to each data point measurement. Full spectra were obtained at 10 °C with a resolution of 0.5 nm at 50 nm/min and a data integration time of 2 sec. Readings were collected in triplicate ($n=3$) at each pH and buffer readings were subtracted. The CD signals reported here are presented as molar ellipticity after conversion using Jasco Spectral manager and Origin 8.6 software.

Near-UV CD Spectroscopy

Near-UV CD was performed using the same instrument used for far-UV CD analyses (Jasco J-815). Experimental variables were kept the same as those used for far-UV CD measurements except for the use of 1-cm pathlength cuvettes. The protein concentration was increased to 3 mg/mL to ensure acquisition of sufficient signal for these analyses. The CD signal was converted to molar ellipticity and buffer readings were subtracted prior to data analysis. The transition temperature for each thermal unfolding curve was calculated using second order derivative function in Origin 8.6.

Static Light Scattering

Static light scattering (SLS) data were acquired using a Photon Technology International (PTI) spectrofluorometer equipped with a turreted four-position cuvette holder and temperature controller. The sample was excited at 275 nm and scattering intensity at the same wavelength was recorded using a detector located at an angle of 90° to the sample and as a function of temperature at every 2.5 °C from 10 to 85 °C. The samples were equilibrated prior to each measurement at each temperature for 3 min. Each pH sample was measured in triplicate (n=3) and the buffer blank reading was subtracted prior to data analysis. SLS provided a measure of quaternary structure changes (*e.g.* protein aggregation) that may occur as the solution conditions change.^{19,20}

Extrinsic 8-Anilino-1-Naphthalene Sulfonate (ANS) Fluorescence

8-Anilino-1-naphthalene sulfonate (Invitrogen) was used as an extrinsic fluorescent probe to monitor changes in the solvent exposure of apolar regions of CagL.²¹ These regions become increasingly exposed to the solvent as the protein is subjected to different stress conditions that cause regional unfolding of the structure. The ANS was used at a 10-fold molar excess relative to the protein concentration. The sample was excited at 385 nm and the spectra were collected from 10 to 85 °C with measurements taken every 2.5 °C. The samples were equilibrated at each temperature for 3 min prior to taking a measurement. Excitation and emission slit widths were kept 3 nm and 4 nm, respectively, for collecting the ANS fluorescence data. Buffer containing ANS was used as a blank and the sample readings were corrected for this blank prior to data analysis. Emission at 475 nm was monitored as a function of temperature and readings were acquired in triplicate (n=3) in all cases.

Empirical Phase Diagram (EPD)

Accumulated thermal stability data from far-UV and near-UV CD, and static light scattering spectroscopy were incorporated into a color map called an empirical phase diagram (EPD). The EPD was generated using Matlab software to transform the data from a multidimensional vector map on a pH-temperature plane into a color map. The data from n sets of experiments were first represented as an n -dimensional vector at every pH and temperature point on the pH-temperature plane. The vectors were then assembled to produce an $n \times n$ density matrix from which the orthogonal eigenvectors and eigenvalues were determined. The transformed dataset was then truncated to three dimensions based on the three eigenvectors with the highest eigenvalues. The transformed and truncated dataset, now expressed in terms of a new three-dimensional orthogonal coordinate system, was converted to a color map by expressing each of the three coordinates as red, green or blue and basing the intensity of the color on the value within the transformed dataset. This resulted in formation of a color map depicting the different structural states of the protein. A detailed description of the method used to generate an EPD is described elsewhere.¹⁷ This EPD enabled visualization of the different structural states of the protein at various conditions of pH and temperature.

Aggregation Assays

Compounds (excipients) from a generally regarded as safe (GRAS) library were screened for their ability to maintain optimal stability of the protein in the absence of cold storage.²² The conditions used for these assays were selected based upon the EPD phase boundaries determined for CagL. From the CagL EPD, aggregation assays were performed at a pH of 5.0 and at a temperature of 50 °C. The light scattering data indicated that CagL aggregates as a function of time at this pH and temperature. The compounds tested included carbohydrates, polyols, amino acids, detergents and polymers. Excipients were dissolved in CP buffer at 150 mM ionic strength and the pH was adjusted to 5. The protein was dissolved

in the same buffer at pH 5 with the optimum protein concentration for these experiments determined to be 0.3 mg/mL. The optical density at 360 nm (OD_{360}) was monitored every 60 sec for 90 min. The effect of each excipient formulation on aggregation was determined in triplicate. The OD_{360} for the protein in the absence of any excipient (C in equation 1) was found to reach a maximum value at 40 min so this time was chosen for analysis of excipient effects on aggregation. The OD_{360} at 0 min was subtracted from the OD_{360} at 40 min (ΔOD_{360}) and the percentage of aggregation of the protein in the presence of excipients (E in Equation 1) was determined. This value was subtracted from 100 to give the percentage inhibition of aggregation as follows:

$$\% \text{ Inhibition of Aggregation} = 100 - \left(\frac{\Delta OD_{360}(E)}{\Delta OD_{360}(C)} \times 100 \right) \quad \text{Equation 1}$$

where $\Delta OD_{360}(E)$ represents difference in the OD at 360 nm of the protein in the presence of excipients and $\Delta OD_{360}(C)$ represents change in OD at 360 nm of the protein with no excipient added, which served as the negative control.

The Stabilizing Effect of Inhibitors of Aggregation on the Protein Conformation

After analyzing data from the aggregation assays, four excipients (arginine, diethanolamine, sucrose and Brij 35) were selected to confirm their stabilizing effect on the secondary and tertiary structure of CagL using near- and far-UV CD, and light scattering spectroscopies. The CD unfolding profiles were obtained by monitoring the CD signal at 222 nm for far-UV and 281 nm for near-UV CD, as a function of temperature. Change in the conformational stability for CagL in the presence of these excipients was determined in terms of midpoint of transition temperature (T_m) values. Respective buffer blanks were subtracted from the sample readings prior to data analysis. The CagL protein without any excipient was subjected to the same spectroscopic analyses to serve as another control. Transition temperature from the curves obtained was calculated using Origin lab 8.6 software by applying a second order derivative function. An increase in the transition temperature provided evidence of increased structural stability of the protein in the presence of a given excipient.

Results and Discussion

Circular Dichroism

The Far-UV circular dichroism (260–190 nm) provides valuable information on the secondary structure content of a protein.^{23,24} The CD spectrum of purified recombinant CagL from *H. pylori* possesses double minima at 208 nm and 222 nm suggesting a dominant alpha helical structure (Fig. 1A). The signal at these two wavelengths is more intense at pH 4 and 5 than at other pH conditions, indicating that the protein is probably folded correctly at these pHs while the alpha helical structure is partially lost at pH 3, 6, 7 and 8. Thermal unfolding of the secondary structure of CagL was then monitored at 222 nm as a function of temperature from 10 to 90 °C (Fig. 1B). The CD thermal unfolding plots reveal susceptibility of the CagL secondary structure to the thermal stress applied at different pH conditions as indicated by differences in the observed transition temperatures. The unfolding at pH 4, 5 and 6 starts at around 45–50 °C, while at pH 3, 7 and 8 the unfolding begins at around 30–35 °C. The midpoint of CagL thermal unfolding (T_m) is observed to occur at higher temperatures within the intermediate pH range (pH 4, 5 and 6). Meanwhile, CagL stability appears to decrease at pH 3, 7 and 8, where the CD spectra reveal comparatively less alpha helical structure. Compromised stability is also reflected in terms of lower T_m values (Suppl. Table S1).

The stability of CagL tertiary structure was investigated using near-UV CD spectroscopy (250–320 nm) as a function of temperature. The near-UV CD spectral signals arise from the environmental sensitivity of aromatic amino acid residues and disulfide bonds. The near-UV CD spectra for CagL (Fig. 2A) reveal two major peaks at 281 nm and 287 nm arising from tyrosyl residues and two minor peaks at 266 nm and 259 nm arising from phenylalanyl residues, respectively.²⁵ The magnitude of the intensity for each peak differs at the different pH conditions tested at 10 °C, which can be attributed to many factors including the nature of the environment of the residues, their mobility and their spatial disposition.²⁶ Thermal stress applied at every pH disturbs this spatial disposition and causes exposure of the residues to the polar environment which results in a decreased signal. Therefore, the near-UV CD signal at 281 nm was monitored to track changes in CagL tertiary structure at different pHs and the loss of CagL folding as a function of temperature (Fig. 2B). The T_m value for each curve was calculated and is presented in Supplemental Table S1. In agreement with the far-UV CD data, the near-UV CD data indicate maximum stability for CagL at pH 4 and 5. In fact, the near-UV CD spectrum at pH 4 showed the greatest absorbance at 281 nm and the thermal melt at this pH indicated maximum physical stability for CagL at pH 4 (Fig. 2B). Lower absorbance at neutral pH conditions could be due to either a higher mobility for the tyrosyl residue or increased exposure to the polar environment. Thermal unfolding was also monitored at 287 nm which yields similar trends across the different pH-temperature conditions examined (data not shown).

Static Light Scattering

The aggregation induced as CagL was subjected to thermal stress was monitored at 275 nm. Scattering intensity increases as a protein aggregates and the transitions are often seen as a rapid increase in scattering intensity. CagL shows a broad transition at pH 3 without a large increase in the intensity which can also be a sign of protein instability (Fig. 3). In addition to the broad transition seen at pH 3, a minor transition is observed consistently at ~24.5 °C. At pH 4, 7 and 8, however, the aggregation does not become readily apparent at these pHs until about 40 °C. At pH 5 and 6 a sharp increase in the light scattering intensity was observed suggesting that at these pHs protein aggregation occurs at elevated temperatures (45–55 °C). The overall light scattering results are presented in Supplemental Table S1 with CagL appearing to be most stable at pH 4, 5 and 6.

Extrinsic 8-Anilino-1-Naphthylene Sulfonate (ANS) Fluorescence

ANS is an environmentally sensitive dye that is essentially nonfluorescent in polar environments but shows a dramatic increase in fluorescence emission after binding to hydrophobic regions of a protein.²⁷ Thus, ANS can be used to probe thermally-induced tertiary structure perturbations in a protein as well as protein unfolding. The initial fluorescence intensity increase seen for ANS upon CagL addition indicated that at 10 °C CagL has extensive exposed hydrophobic regions at pH 3 and to a lesser extent at pH 4 (Fig. 4). At pH 5, 6, 7 and 8 the ANS intensity starts at about the same level, suggesting a compactly folded protein structure in these higher pH environments. These differences in ANS intensity indicate that hydrophobic patches exposed on CagL are more accessible or prevalent at pH 3 and 4 than in the higher pH environments. This could indicate reduced stability at low pH and this is supported by the lower thermal unfolding transitions seen as a sharp increase in the ANS intensity at these low pHs. The ANS fluorescence-based thermal transitions at each pH are summarized in Supplemental Table S1. These data show that the temperature-dependent sharp increase in ANS intensity seen at the lower pHs occurs at higher temperatures as the pH is increased. A second broader transition at a much lower temperature is observed, but only as the pH increased to 6 or higher. These data indicate that CagL is most stable under slightly acidic to near-neutral pH conditions (pH 4–6) (Fig. 4 and Suppl. Table S1). The emission spectra (400–505 nm) for ANS fluorescence intensity at the

pHs used here were seen as a well-defined peak at ~475 nm (Suppl. Fig. S2), suggesting that it is unlikely that the increasingly positive charge on the protein at acidic pH conditions is responsible for interactions with the negatively charged ANS dye.

Empirical Phase Diagram (EPD)

In this study, pharmaceutically relevant stress conditions (pH and temperature) were applied to CagL from *H. pylori* and changes in the secondary, tertiary and quaternary structure were tracked using near- and far-UV CD, and static light scattering. To compare these multiple datasets as part of a single analysis, they were incorporated into a comprehensive color map called an empirical phase diagram or EPD (Fig. 5) that defines different pH-temperature regions where the protein maintains structural similarity. Identical color regions represent structurally similar states of the protein while changes in the color pattern are recognized as transitions in the protein structure or empirical phase of the protein. Note that these diagrams are empirical and do not represent thermodynamic phases because no equilibrium is suggested across the observed phases.

The EPD shown in Figure 5 can be divided into two major phases based upon predominant color changes that are used to allow an improved understanding of the findings. The phase of maximum stability is indicated by the brown color at lower temperatures (from 10 °C to ~37–45 °C) for each pH tested. This is supported by the higher T_m values seen at pH 4, 5 and 6 conditions using the above spectroscopic techniques. In particular, the CD spectra show stable secondary structure content under these pH conditions. The near-UV CD spectra data also indicate that the tertiary structure of CagL is in its most highly folded state under these lower pH and temperature conditions. This is reinforced by the ANS fluorescence intensity data which start lower under at least two of these pH conditions (pH 5 and 6). Instability of CagL at pH 7 and 8 is indicated by the low absorbance minima examined in far-UV CD spectra and low absorbance at 287 nm and 281 nm in near-UV CD spectra, which suggest loss of properly folded conformation at these conditions. This is reflected in a greenish-brown color in the EPD. At the same time hydrophobic patches may not be exposed at these pHs as suggested by ANS fluorescence data. Though CagL possessed average physical stability at pH 3, the structural conformation at this pH was most accessible to the ANS. Static light scattering data indicated higher T_m values at pH 4, 5 and 6. The SLS data also suggested that pH 5 and 6 favor aggregation of the protein at elevated temperatures. The blue-purple phase at pH 5 and 6 between ~55–65 °C reflects severe aggregation of CagL as observed by the static light scattering data (Fig. 3). The onset of aggregation at these pHs started at lower temperatures than for the other pH values. Another major phase in the EPD is indicated by the green color at elevated temperatures, which appears to represent severely altered structural changes and denatured protein. This aggregated phase encompassed the transition region for each pH tested along with completely unfolded or denatured protein with increasing temperatures starting at ~40–45 °C. Continuation of the brown colored phase shifting toward green represents a transition caused by increasing temperature. Considering all of the presented data and the EPD, pH 4 was found to best preserve conformational stability for CagL.

Screening for Stabilizers

Based on the empirical phase diagram shown in Figure 5, CagL appears to be most thermostable at pH 4, 5 and 6, but the aggregation observed under these pH conditions, particularly at pH 5 and 6 indicates a need to screen for stabilizers that are able to inhibit aggregation and stabilize CagL. The conditions for our high throughput aggregation screen were thus determined using the color-based phase boundaries observed in the EPD. CagL at pH 5 was incubated at 50 °C (conditions shown above to stress the protein's thermal stability with regard to aggregation) in the absence or presence of excipients, and the optical

density at 360 nm was monitored for 1.5 h to monitor protein aggregation. Protein lacking excipient served as a negative control for stabilization. Each stabilizer was tested in triplicate and the percentage values for inhibition of aggregation relative to the protein without excipient were calculated. Using this approach, different compounds from a GRAS library were tested to identify inhibitors of aggregation. The compounds tested were categorized as detergents, polyols, sugars and amino acids. Of these compounds, sucrose (20% w/v), diethanolamine (0.3 M), arginine (0.3 M), Brij 35 (0.1% w/v), guanidine (0.3 M), lysine (0.3M), trehalose (20%), dextrose (20%), glycerol (20%) inhibited the aggregation most significantly (Table 1). The observed stabilization based on this aggregation assay could be due to either inhibition of aggregate formation or stabilization of the protein structure. The values for the percentage inhibition of aggregation for all the compounds tested are presented in Table 1. Notably, positively charged excipients (arginine, lysine, guanidine, diethanolamine) exhibited positive effects on stability, perhaps due to electrostatic interactions, particularly cation- π interactions between positively charged excipient molecules and molecular quadrupoles above and below aromatic side chains of Tyr and Phe.²⁸ Arginine is commonly involved in cation- π interactions at protein complex interfaces.²⁹ Thus, deleterious protein-protein interactions could be overcome by addition of arginine as an excipient which could compete for participation in cation- π interactions. In contrast, the carbohydrate excipients (*e.g.* sucrose) could stabilize the proteins in solution by preferential hydration³⁰ while the mechanism by which detergents (*e.g.* Brij 35) stabilize CagL could be by inhibiting the protein interfacial (apolar) adsorption properties.³¹

To reduce the number of compounds further examined, one representative compound from each class was selected to confirm their stabilizing effects on the stability and/or conformation of CagL using CD and light scattering spectroscopic techniques as a function of temperature. The compounds selected were arginine (0.3 M), diethanolamine (0.3 M), Brij 35 (0.1% w/v) and sucrose (20% w/v). Increased values for T_m were interpreted to be a direct effect on the structural stability of CagL or inhibition of its aggregation. Conversely, decreased values for the T_m indicated that the added compound had a destabilizing effect on CagL in solution. Table 2 summarizes the T_m values calculated for each data set ($n=3$). Of the four tested compounds, only sucrose was found to have a substantial stabilizing effect on CagL by all of these spectroscopic methods (Table 2 and Suppl. Fig. S3). Meanwhile, Brij 35 had a substantial stabilizing effect, but only with regard to preventing aggregation as indicated by static light scattering data. Furthermore, though aggregation assays indicated arginine and diethanolamine were more stabilizing than sucrose and Brij 35 (see Table 1), the thermal melts suggested that sucrose and Brij 35 delay the onset of aggregation. Meanwhile, arginine and diethanolamine simply slow the rate of aggregation. Consequently, sucrose and Brij 35 appear to be the better stabilizers.

The broad transition in the far-UV CD melt observed for Brij 35 with an onset at about 35 °C and the lower T_m seen for the near-UV CD melt suggest that Brij 35 does not stabilize the CagL tertiary structure. Overall, the T_m values reported in Table 2 confirm that Brij 35 fails to stabilize the secondary and tertiary structure of CagL. Static light scattering data did, however, reveal a stabilizing role for Brij 35 and sucrose in preventing CagL aggregation at higher temperatures relative to arginine and diethanolamine. Furthermore, the onset of aggregation with Brij 35 was at ~55 °C while for sucrose it was observed at ~40–45 °C, which suggests that its detergent properties are best suited for only the blocking of aggregative interfaces. The transition temperature values confirm that both sucrose and Brij 35 inhibit temperature-induced aggregation of CagL (Table 2). Significant aggregation was observed in the presence of arginine and diethanolamine with the onset of transition using static light scattering starting at ~28 °C for arginine and ~40 °C for diethanolamine. In addition, CagL in the presence of diethanolamine showed higher light scattering intensities at 10 °C (Suppl. Fig. S3C). This was further reflected in lower transition temperature values

(Table 2). The far-UV CD data in the presence of arginine as an excipient are not included here because arginine interfered with the far-UV CD signal.

The average T_m ($n=3$) calculated using far-UV CD for CagL without excipient is about 52 °C, while in the presence of sucrose the T_m increases to nearly 56 °C. Similarly, static light scattering data revealed that Brij 35 and sucrose each helps to stabilize CagL quaternary structure by inhibiting protein aggregation as was indicated by the low intensity signal in the presence of these excipients for CagL (Suppl. Fig. S3C). The stabilization of protein structures by sucrose is anticipated to occur through a well-known mechanism of preferential hydration.^{32,33} In contrast to sucrose, however, Brij 35, arginine and diethanolamine do not appear to stabilize the CagL structure when multiple spectroscopic methods are applied. The destabilizing effect of arginine could be due to the guanidinium group present in this amino acid which may alter the overall structural properties of the protein.

To determine whether combinations of sucrose and Brij 35 could alter the stabilizing effect of each for CagL, the ability to inhibit temperature induced aggregation by different combinations of these two excipients was tested using near- and far-UV CD and static light scattering. T_m values were calculated for each combination and are presented in Table 3. Lower T_m values seen in the presence of Brij 35 in the near- and far-UV CD data suggest that Brij 35 has a destabilizing effect despite the presence of sucrose. Broad transitions were observed for 0.1% Brij 35 and different combinations of Brij 35 with sucrose according to the far-UV CD data (data not shown), whereas sucrose (20%) indicated a sharp transition (Suppl. Fig. S3A). This indicated that Brij 35 is dominant for destabilization of CagL at different combinations of sucrose and Brij 35 based on CD spectroscopy. On the other hand, as the concentration of sucrose was increased in these combinations, a progressive increase in the T_m values was noted. This reveals a stabilizing role for sucrose with regard to CagL secondary and tertiary structure and suggests that this effect is concentration dependent. The static light scattering data did not indicate concentration dependent inhibition of aggregation of CagL by sucrose, but rather it showed that Brij 35 is able to prevent aggregation of CagL at all the tested concentrations of Brij 35 (Table 3). The onset of aggregation for all combinations of sucrose and Brij 35 tested was seen to occur at ~50 °C (data not shown). The light scattering data indicated Brij 35 alone is an effective inhibitor of aggregation, however, while sucrose also inhibited aggregation, the combination of the two appeared to attenuate the effect of Brij 35 alone (Table 3).

Conclusions

Using the systematic approach described here, we identified the optimal pH and temperature conditions that stabilize the type IV secretion system protein CagL from *H. pylori* when it is prepared as a purified recombinant protein. CagL possesses maximum stability at pH 4, 5 and 6 up to 35–40 °C in the absence of any excipient. At these conditions, particularly at pH 5 and 6, CagL demonstrated significant aggregation prior to unfolding as the temperature was increased. The EPD approach helped in the screening of stabilizers that inhibit aggregation for CagL. Ultimately, sucrose was confirmed to be a suitable excipient for a potential CagL vaccine, because it raised the thermal transition temperature of CagL based on multiple spectroscopic methods. This stabilizing effect is not surprising since sucrose is often found to be a preferred and commonly used excipient in approved vaccine formulations.^{22,34–37}

There is a large body of literature on experimental vaccines for *H. pylori* in different animal models. The role of antibody has been specifically addressed by a number of laboratories and while antibody may be participating in the protective immune response, it is not

required for protection.^{38–40} Unpublished data show that mucosal immunization with recombinant CagL using cholera toxin (CT) as an adjuvant is protective (T. Blanchard, unpublished results). This is consistent with the findings of others demonstrating that many *Helicobacter* antigens, such as urease, GroES or even bacterial lysates,^{8,39} can serve as protective antigens when administered with different adjuvants including CT, *E. coli* heat-labile enterotoxin (LT) and aluminum salts.^{39–42} It may thus be possible to confer protection using CagL with systemic immunization since aluminum salt-based vaccines are as effective as mucosal vaccines at protecting animals against challenge with *Helicobacter*.^{42–45} Hence, this study should eventually be extended to gain additional information with respect to CagL stability with multiple compatible adjuvants for the formulation of a vaccine that can be administered via multiple routes. These potential studies would involve investigating the conditions for optimum adsorption of the antigen to potential aluminum salts. In light of previous studies,^{34,46} it is likely that the excipients identified here would be compatible with aluminum salt adjuvants. It is possible that decreased thermostability upon adsorption of CagL to this adjuvant's surface might be observed as has been reported for other proteins,⁴⁷ however, such destabilization can vary and is highly protein dependent. Nevertheless, the compounds identified as stabilizers in this study would be expected to provide some level of stabilization for CagL in the presence of aluminum salt adjuvants.⁴⁸

Adjuvants like the double mutant heat-labile toxin (dmLT) from *Escherichia coli* or monophosphoryl lipid A (MPL) can also serve as adjuvants for CagL vaccine formulations. In particular, dmLT has been identified as an effective adjuvant in *H. pylori* vaccine studies.⁴⁹ Being a soluble, detoxified protein adjuvant that is co-administered with an antigen for mucosal immunization,⁵⁰ the use of dmLT could provide an alternative to adsorption to aluminum salt adjuvants. This may lessen potential destabilization that could occur during the adsorption process. Similarly, MPL can be formulated as an emulsion-based vaccine for parenteral immunization⁵¹ as yet another alternative to the use of aluminum based adjuvants.

This study has generated information regarding the stability and physical behavior of the *H. pylori* T4SS protein CagL over a range of pH and temperature conditions. Such solution extremes are often those encountered as being detrimental during the transport and storage of protein antigens. The stabilizers identified in the study can potentially be used for a successful formulation of CagL as a vaccine component with enhanced storage stability. In fact, an initial experiment shows that sucrose enhances the stability of CagL as a function of time at room temperature (see Suppl. Fig. S4). The examination of other stabilizing excipients (see Table 1) can also be considered further as CagL is developed as a potential *H. pylori* vaccine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Analysis of the secondary structure content of the CagL using circular dichroism spectroscopy. **A)** Far-UV CD spectra for CagL at different pHs at 10 °C. **B)** The thermal unfolding of CagL was monitored as a change in the minimum at 222 nm. Error bars show standard deviation from three readings. Symbols: pH 3 (■), pH 4 (□), pH 5 (●), pH 6 (○), pH 7 (▲) pH 8 (△).

Figure 2.

Thermal stability of CagL analyzed using near-UV CD. **A)** The near-UV CD spectra for CagL are shown at different pHs at 10°C. **B)** The thermal unfolding of CagL monitored as a function of temperature at 281 nm. Error bars show standard deviation from duplicate experiments. Symbols: pH 3 (■), pH 4 (□), pH 5 (●), pH 6 (○), pH 7 (▲) pH 8 (△).

Figure 3.

Static light scattering intensity was monitored as a function of temperature to observe aggregation patterns under different pH conditions for CagL. Error bars show standard deviation from three experiments. Symbols: pH 3 (■), pH 4 (□), pH 5 (●), pH 6 (○), pH 7 (▲) pH 8 (△).

Figure 4. ANS fluorescence intensity was monitored as a function of temperature to probe structural perturbations in CagL at different pHs. The values shown are an average of three experiments (with error bars showing standard deviation). Symbols: pH 3 (■), pH 4 (□), pH 5 (●), pH 6 (○), pH 7 (▲) pH 8 (△).

Figure 5.

Empirical phase diagram for CagL. The accumulated data, comprising the thermal stability data from near- and far-UV CD and static light scattering, were treated as described in methods for conversion into a color scheme. The different colors change to indicate different phases of protein physical stability of the protein observed at different pH and temperature conditions.

Table 1
Summary of excipients screening by aggregation assays

The percentage inhibition of aggregation of CagL in the presence of GRAS compounds is calculated as described in methods. Negative values indicate that the compound promoted aggregation. Concentrations of the excipients are indicated either in % w/v, or molarity.

Excipient	Concentration	% Inhibition of Aggregation
Arginine	0.3M	78.4 ± 6.4
Brij 35	0.10%	73.3 ± 2.4
Diethanolamine	0.3M	64.3 ± 2.3
Brij 35	0.05%	63.5 ± 1.6
Guanidine	0.3M	60.9 ± 1.3
Sucrose	20%	49.7 ± 1.7
Lysine	0.3M	47.1 ± 28.1
Trehalose	20%	43.2 ± 1.9
Dextrose	20%	43.0 ± 0.4
Glycerol	20%	40.0 ± 1.6
2-OH β Cyclodextrin	10%	34.9 ± 2.1
Maleic acid	0.15M	32.1 ± 16.8
Sodium Citrate	0.2M	31.9 ± 3.7
Sucrose	10%	27.9 ± 1.3
Dextrose	10%	27.7 ± 1.1
Glycerol	10%	27.7 ± 1.6
Calcium Chloride	0.2M	26.5 ± 4.3
Trehalose	10%	25.3 ± 3.1
2-OH β Cyclodextrin	5%	24.9 ± 8.2
Proline	0.3M	22.0 ± 2.8
Sorbitol	10%	21.6 ± 2.7
Mannitol	10%	20.6 ± 4.3
2-OH λ Cyclodextrin	10%	18.9 ± 0.3
Lactose	10%	17.7 ± 0.5
Lactic acid	0.15M	15.7 ± 2.4
Tween 80	0.05%	15.7 ± 2.9
Tween80	0.10%	13.0 ± 1.6
Glycine	0.3M	9.4 ± 1.8
Tween 20	0.10%	8.4 ± 4.0
2-OH λ Cyclodextrin	5%	8.3 ± 4.4
Tween 20	0.05%	7.9 ± 3.8
Pluronic F68	0.10%	-0.6 ± 3.3
Pluronic F68	0.05%	-5.0 ± 4.9
Dextran T70 3	3.5mM	-21.5 ± 7.8
Ascorbic acid	0.15M	-26.3 ± 2.0

Table 2
Exploring the stabilizing effects of four potential excipients

Changes in the thermal transition temperatures (°C) for CagL observed using CD and light scattering data, in the presence and absence of the selected GRAS compounds. These data were collected at pH 4 where CagL displayed maximum thermal stability. Because arginine interferes with the far-UV CD signal, it was not included in this analysis.

Spectroscopic Technique	Excipient (concentration)	T _m (°C)
Far-UV Circular Dichroism	CagL + Arginine (0.3M)	Not done
	CagL + Diethanolamine (0.3M)	50.6 ± 0.1
	CagL + Sucrose (20% w/v)	55.3 ± 0.2
	CagL + Brij 35 (0.1% w/v)	44.9 ± 0.4
	CagL (no excipient)	51.3 ± 0.3
Near-UV Circular Dichroism	CagL + Arginine (0.3M)	48.7 ± 0.5
	CagL + Diethanolamine (0.3M)	45.0 ± 0.3
	CagL + Sucrose (20% w/v)	53.5 ± 0.1
	CagL + Brij 35 (0.1% w/v)	45.0 ± 0.3
	CagL (no excipient)	51.1 ± 0.1
Static Light Scattering	CagL + Arginine (0.3M)	50.2 ± 0.4
	CagL + Diethanolamine (0.3M)	49.7 ± 0.5
	CagL + Sucrose (20% w/v)	57.2 ± 0.4
	CagL + Brij 35 (0.1% w/v)	63.5 ± 0.3
	CagL (no excipient)	54.4 ± 0.4

Table 3
Exploring the stabilizing effects of combinations of sucrose and Brij 35 concentration on CagL

The thermal transition temperatures (T_m) expressed in °C for CagL as observed using far- and near-UV CD and static light scattering data in the presence of different combinations and concentrations of sucrose and Brij 35.

Spectroscopic Technique	Excipient (concentration % w/v)	T_m (°C)
Far-UV Circular Dichroism	Brij 35 (0.1%)	52.5 ± 1.0
	Sucrose (5%) + Brij 35 (0.025%)	44.5 ± 1.2
	Sucrose (5%) + Brij 35 (0.075%)	45.1 ± 2.8
	Sucrose (10%) + Brij 35 (0.05%)	50.1 ± 0.4
	Sucrose (15%) + Brij 35 (0.025%)	49.5 ± 0.4
	Sucrose (20%) + Brij 35 (0.025%)	51.5 ± 0.4
	Sucrose (20%)	55.9 ± 0.2
	CagL alone	51.3 ± 0.3
Near-UV Circular Dichroism	Brij 35 (0.1%)	45.0 ± 0.3
	Sucrose (5%) + Brij 35 (0.025%)	46.8 ± 0.6
	Sucrose (5%) + Brij 35 (0.075%)	47.8 ± 0.1
	Sucrose (10%) + Brij 35 (0.05%)	48.0 ± 1.5
	Sucrose (15%) + Brij 35 (0.025%)	49.7 ± 0.7
	Sucrose (20%) + Brij 35 (0.025%)	49.8 ± 0.9
	Sucrose (20%)	53.5 ± 0.1
	CagL alone	51.1 ± 0.1
Static Light Scattering	Brij 35 (0.1%)	63.5 ± 0.3
	Sucrose (5%) + Brij 35 (0.025%)	64.9 ± 1.4
	Sucrose (5%) + Brij 35 (0.075%)	57.5 ± 2.6
	Sucrose (10%) + Brij 35 (0.05%)	55.1 ± 0.9
	Sucrose (15%) + Brij 35 (0.025%)	55.3 ± 0.2
	Sucrose (20%) + Brij 35 (0.025%)	55.5 ± 0.1
	Sucrose (20%)	57.2 ± 0.4
	CagL alone	54.4 ± 0.4