Video Article

One-step Negative Chromatographic Purification of *Helicobacter pylori* Neutrophil-activating Protein Overexpressed in *Escherichia coli* in Batch Mode

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Abstract

Helicobacter pylori neutrophil-activating protein (HP-NAP) is a major virulence factor of *Helicobacter pylori* (*H. pylori*). It plays a critical role in *H. pylori*-induced gastric inflammation by activating several innate leukocytes including neutrophils, monocytes, and mast cells. The immunogenic and immunomodulatory properties of HP-NAP make it a potential diagnostic and vaccine candidate for *H. pylori* and a new drug candidate for cancer therapy. In order to obtain substantial quantities of purified HP-NAP used for its clinical applications, an efficient method to purify this protein with high yield and purity needs to be established.

In this protocol, we have described a method for one-step negative chromatographic purification of recombinant HP-NAP overexpressed in *Escherichia coli* (*E. coli*) by using diethylaminoethyl (DEAE) ion-exchange resins (*e.g.*, Sephadex) in batch mode. Recombinant HP-NAP constitutes nearly 70% of the total protein in *E. coli* and is almost fully recovered in the soluble fraction upon cell lysis at pH 9.0. Under the optimal condition at pH 8.0, the majority of HP-NAP is recovered in the unbound fraction while the endogenous proteins from *E. coli* are efficiently removed by the resin.

This purification method using negative mode batch chromatography with DEAE ion-exchange resins yields functional HP-NAP from *E. coli* in its native form with high yield and purity. The purified HP-NAP could be further utilized for the prevention, treatment, and prognosis of *H. pylori*-associated diseases as well as cancer therapy.

Video Link

The video component of this article can be found at https://www.jove.com/video/54043/

Introduction

Helicobacter pylori (*H. pylori*) is a major cause of gastritis and peptic ulcer. This bacterium has also been classified as a carcinogen in humans by the International Agency for Research on Cancer, part of the World Health Organization, in 1994. It has been estimated that the prevalence of *H. pylori* infection is 70% in the developing countries and 30-40% in the industrialized countries[1]. Even though the infection rate of *H. pylori* is decreasing in the industrialized countries, the infection rate of *H. pylori* in the developing countries is still high[2]. The standard treatment to eradicate *H. pylori* infection consists of the administration of a proton pump inhibitor, PPI, and two antibiotics, clarithromycin plus amoxicillin or metronidazole[3]. However, the rise of antibiotic resistance in *H. pylori*-related ulcer therapy urges the development of new strategies to prevent or cure the infection. Development of preventive and/or therapeutic vaccination against *H. pylori* could provide an alternative approach to control *H. pylori* infection.

Helicobacter pylori neutrophil-activating protein (HP-NAP), a major virulence factor of *H pylori*, was first identified in water extracts of *H. pylori* with the ability to activate neutrophils to adhere to endothelial cells and produce reactive oxygen species (ROS)[4]. Neutrophil infiltration of gastric mucosa found in *H. pylori*-infected patients with active gastritis may result in inflammation and tissue damage of the stomach. Thus, HP-NAP may play a pathological role by activating neutrophils to induce gastric inflammation, which further causes ulcer or *H. pylori*-associated gastric diseases. Nevertheless, HP-NAP is a potential candidate for clinical applications[5,6]. Due to the immunogenic and immunomodulatory properties of HP-NAP, this protein could be used to develop vaccines, therapeutic agents, and diagnostic tools. A clinical trial has been conducted for using recombinant HP-NAP as one of the components of a protein vaccine against *H. pylori*. This vaccine consists of recombinant HP-NAP, cytotoxin-associated gene A (CagA), and vacuolating cytotoxin A (VacA) proteins formulated with aluminum hydroxide and has further been demonstrated to be safe and immunogenic in humans[7]. Also, HP-NAP acts as a potent immunomodulator to trigger T helper type 1 (Th1)-polarized immune responses for cancer therapy[8] and to down regulate Th2-mediated immune responses elicited by allergic reactions and parasitic infections[9,10]. As for diagnostics, recombinant HP-NAP-based ELISA has been applied to detect serum antibodies against HP-NAP in

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H. pylori-infected patients[11]. One study showed that the level of HP-NAP-specific antibodies in sera from *H. pylori*-infected patients with gastric cancer was significantly higher than that from patients with chronic gastritis[12]. Another study also showed that serum antibodies against HP-NAP are associated with the presence of non-cardia gastric adenocarcinoma[13]. Thus, recombinant HP-NAP-based ELISA may be applied to detect serum antibodies against HP-NAP for prognosis of gastric cancer in *H. pylori*-infected patients. Taken together, the purified HP-NAP could be further utilized for the prevention, treatment, and prognosis of *H. pylori*-associated diseases as well as cancer therapy.

Among the several methods used for purification of recombinant HP-NAP expressed in *Escherichia coli* (*E. coli*) in its native form reported so far, a second purification step involving gel-filtration chromatography is needed to obtain highly pure HP-NAP[14-16]. Here, a method using negative mode batch chromatography with diethylaminoethyl (DEAE) ion-exchange resins is described for purification of HP-NAP overexpressed in *E. coli* with high yield and high purity. This purification technique was based on the binding of host cell proteins and/or impurities other than HP-NAP to the resin. At pH 8.0, almost no other proteins except HP-NAP are recovered from the unbound fraction. This purification approach using DEAE ion-exchange chromatography in negative mode is simple and time saving by allowing purification of recombinant HP-NAP via one-step chromatography through the collection of the unbound fraction. In addition to HP-NAP, several other biomolecules, such as viruses[17], Immunoglobulin G (IgG)[18], hemoglobin[19], protein phosphatase[20], and virulence factor flagellin[21], have also been reported to be purified by ion-exchange chromatography in negative mode. The negative mode is preferred for ion-exchange chromatography if impurities are the minor components present in the sample subjected to be purified[22]. The application of negative chromatography in purification of natural or recombinant biomolecules has been recently reviewed[23].

The present report provides a step by step protocol for expression of recombinant HP-NAP in *E. coli*, lysis of the cells, and purification of HP-NAP using negative mode batch chromatography with DEAE ion-exchange resins. If a protein desired for purification is suitable for ion-exchange chromatography in negative mode, the described protocol could also be adapted as a starting point for development of a purification process.

Protocol

Human blood was collected from healthy volunteers with prior written informed consent and approval from the Institutional Review Board of the National Tsing Hua University, Hsinchu, Taiwan.

1. Expression of Recombinant HP-NAP in *E. coli*

1. Prepare the plasmid pET42a-NAP containing the DNA sequence of HP-NAP from *H. pylori* 26695 strain as previously described[16]. Prepare the plasmids containing the DNA sequence of HP-NAP with the desired point mutations as described (see Protocol, step 8).
2. Transform 10 ng of the above DNA plasmids into *E. coli* BL21 (DE3) competent cells by heat shock for 45 seconds at 42 °C, streak the cells on lysogeny broth (LB) agar plates containing 50 µg/ml kanamycin, and incubate the plates at 37 °C for 16 hr.
3. Inoculate single colonies in individual tubes containing 5 ml of LB broth with 50 µg/ml kanamycin and grow them as precultures at 37 °C for 16 hr with shaking at 170 rpm.
4. Inoculate 2 ml of the above pre-culture cells into 200 ml of LB broth containing 50 µg/ml kanamycin in a 1 L flask. Incubate the inoculated culture flask at 37 °C for 2 hr with shaking at 170 rpm until the absorbance at 600 nm reaches approximately 0.4-0.5 detected by an UV/VIS spectrophotometer.
5. Add 80 µl of 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) into the above culture to a final concentration of 0.4 mM to induce the expression of HP-NAP. Incubate the culture for 3 hr until the absorbance at 600 nm reaches approximately 1.6-1.7.
6. Centrifuge the cells at 6,000 x g at 4 °C for 15 min to remove the supernatant. Store the cell pellet at −70 °C until purification.

2. Preparation of the Soluble Protein Fraction Containing HP-NAP

Note: All of the following steps are carried out at 4 °C.

1. Resuspend 50 ml of the cell pellet prepared in Protocol step 1.6 in 20 ml of 20 mM Tris-HCl, pH 9.0, 50 mM NaCl containing 0.13 mM phenylmethylsulfonyl fluoride (PMSF), 0.03 mM N-alpha-tosyl-L-lysinyl-chloromethylketone (TLCK), and 0.03 mM N-tosyl-L-phenylalaninyl-chloromethylketone (TPCK) at 4 °C as previously described[16].
2. Disrupt the bacteria cells by passing the bacterial suspension through a high pressure homogenizer operated at a range of 15,000 to 20,000 psi for 7 times at 4 °C.
3. Centrifuge the cell lysate at 30,000 × g at 4 °C for 1 hr to separate the soluble and insoluble protein fractions by using an ultracentrifuge. Transfer the supernatant as the soluble protein fraction to a beaker.
4. Measure the protein concentration of the soluble protein fraction by the Bradford method using a commercial kit with bovine serum albumin (BSA) as the standard according to the manufacturer's instructions.
5. Add 177.6 µl of 1 N HCl into 20 ml of the soluble protein fraction obtained from Protocol step 2.3 to adjust its pH value from pH 9.0 to pH 8.0.
6. Add 20 mM Tris-HCl, pH 8.0, 50 mM NaCl to the above protein solution to make the final protein concentration to be 0.5 mg/ml.

3. Purification of Recombinant HP-NAP From *E. coli* by Negative Mode Batch Chromatography with DEAE Ion-exchange Resins

1. Prepare 15 ml of DEAE resins.
    1. Weigh out 0.6 g dry powder of DEAE resins and suspend it in 30 ml of 20 mM Tris-HCl, pH 8.0, 50 mM NaCl at room temperature for at least 1 day.
    2. Centrifuge the resin at 10,000 x g at 4 °C for 1 min.
    3. Remove and discard the supernatant.
    4. Add 15 ml of 20 mM Tris-HCl, pH 8.0, 50 mM NaCl.

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   5. Repeat Protocol steps 3.1.2 to 3.1.4 four more times.
   6. Store the 15 ml settled resin (50% slurry in 30 ml Tris buffer) at 4 °C for subsequent use.
      Note: All of the following steps are carried out at 4 °C.

2. Add 45 ml of the soluble proteins prepared in Protocol step 2.6 to 15 ml of the resin and stir the protein/resin slurry with a magnetic stirrer at 4 °C for 1 hr.
3. Pour the protein/resin slurry into a plastic or glass column fitted with a stopcock. Allow the resin to settle under gravity.
4. Open the stopcock to allow the protein solution to run through the column by gravity flow until the liquid level in the column is just above the resin. Collect the flow-through as the unbound fraction, which contains the purified HP-NAP.
5. Add 15 ml of ice-cold 20 mM Tris-HCl, pH 8.0, 50 mM NaCl into the column.
6. Open the stopcock to allow the wash buffer to run through the column by gravity flow until the liquid level in the column is just above the resin. Collect the flow-through as the wash fraction.
7. Repeat Protocol steps 3.5 to 3.6 four more times to collect the additional wash fractions.
8. Add 15 ml of ice-cold 20 mM Tris-HCl, pH 8.0, 1 M NaCl into a column.
9. Open the stopcock to allow the elution buffer to run through the column by gravity flow until the liquid level in the column is just above the resin. Collect the flow-through as the elution fraction.
10. Repeat Protocol steps 3.8 to 3.9 four more times to collect the additional elution fractions.

4. Buffer Exchange and Endotoxin Removal of HP-NAP Purified by Negative Mode Batch Chromatography with DEAE Resins

Note: The purified HP-NAP expressed in *E. coli* needs to be subjected to buffer exchange and endotoxin removal prior to stimulate neutrophils.

1. Perform dialysis to change the buffer of the purified HP-NAP to Dulbecco's phosphate-buffered saline (D-PBS), pH 7.2, at 4 °C by using dialysis tubing with molecular weight cutoff of 14 kDa as previously described[24].
2. Filter the dialyzed HP-NAP through a syringe filter with a positively-charged, hydrophilic membrane attached to a 20 ml disposable syringe at flow rates ranging from 2.5 to 4 ml/minute at 4 °C to remove endotoxin.

5. Characterization of the Molecular Properties of Purified Recombinant HP-NAP by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), Western Blotting, Native-PAGE, Gel Filtration Chromatography, and Circular Dichroism Spectroscopy

1. Analyze the purified recombinant HP-NAP by SDS-PAGE and western blotting with hybridoma culture supernatants containing mouse monoclonal antibody MAb 16F4[25] as previously described[24].
2. Analyze the purified HP-NAP by native-PAGE and gel filtration chromatography to examine its oligomeric status as previously described[26].
3. Analyze the purified recombinant HP-NAP by circular dichroism spectroscopy to examine its secondary structure as previously described[26].

6. Evaluation of the Purity of HP-NAP by Silver Staining of a SDS-PAGE Gel

1. Prepare the fixing solution, sensitizing solution, silver solution, developing solution, stop solution, and washing solution according to the manufacturer's instructions of a silver staining kit.
2. Perform silver staining of a SDS-PAGE gel according to the manufacturer's instructions of a silver staining kit.
3. Acquire the image of the gel with an imaging system.

7. Measurement of ROS Production From Neutrophils Induced by HP-NAP

1. Isolate human neutrophils from human heparinized blood by dextran sedimentation followed by density gradient centrifugation as previously described[24].
2. Measurement of ROS production from neutrophils stimulated with HP-NAP by using a luminol-dependent chemiluminescence assay.
   1. Turn on a plate reader and set the temperature at 37 °C. Place an empty flat bottom 96-well white plate inside the plate reader chamber, allowing it to warm to 37 °C.
   2. Prepare the stimulus mixtures in the D-PBS, pH 7.2, containing 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 13.3 µM 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) with the presence and absence of 0.67 µM endotoxin-removed HP-NAP prepared from Protocol step 4.2.
   3. Adjust the concentration of human neutrophils prepared from Protocol step 7.1 to 2 x 10^6 cells/ml in D-PBS, pH 7.2, containing 5 mM glucose.
   4. Add 50 µl of neutrophil suspension into each well of the 96-well while plate.
   5. Add 150 µl of the stimulus mixtures prepared from Protocol step 7.2.2 into the well containing neutrophils at a time interval of 5 seconds per well.
   6. Measure the emission of chemiluminescence for 5 sec per well over a period of three hours by using a plate reader.

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8. Construction of DNA Plasmids Harboring HP-NAP Mutants by Polymerase Chain Reaction (PCR)-based Site-direct Mutagenesis

Note: PCR-based site-direct mutagenesis was generated basically as described previously[27] except that the "silent" restriction sites were introduced to the mutagenesis primers by site-directed mutagenesis (SDM)-assist software[28].

1. Perform PCR Reaction.
    1. Add 10 ng of plasmid pET42a-NAP, 1 µM of mutagenesis primer pairs listed in **Table 1**, 200 µM of deoxynucleoside triphosphates (dNTPs), and 3 units of high-fidelity PCR enzyme mix into a PCR tube containing deionized water, giving a final volume of 25 µl.
    2. Initiate the PCR cycles at 95 °C for 10 min to denature the template DNA, and follow with 12 amplification cycles at 95 °C for 1 minute, $T_{m\ no}$-5 °C for 1 minute, and 72 °C for 6 min.
    3. Finish the PCR cycles with an annealing step at $T_{m\ pp}$-5 °C for 1 minute and an extension step at 72 °C for 30 min.
2. Treat 15 µl of the PCR product with 0.4 µl of Dpn I restriction enzyme at 37 °C for 2 hr and then analyze 2 µl of the Dpn I-treated PCR product by agarose gel electrophoresis.
3. Screen mutants.
    1. Transform 2 µl of the PCR product into *E. coli* DH5α competent cells by heat shock for 45 sec at 42 °C.
    2. Spread the transformed cells on a LB plate containing 50 µg/ml kanamycin and incubate the plate at 37 °C for 16 hr.
    3. Isolate the plasmid DNA from the bacterial colonies using a commercial alkaline lysis kit according to the manufacturer's protocol.
    4. Treat the plasmid DNA isolated in Protocol step 8.3.3 with XhoI restriction enzyme to verify the presence of the desired silent mutations according to manufacturer's protocol.
    5. Sequence the plasmid DNA verified by Protocol step 8.3.4 with T7 promoter primer to confirm the correction of the coding sequences of HP-NAP mutants according to manufacturer's protocol.

Representative Results

The schematic diagram of the experimental procedure of negative purification of recombinant HP-NAP expressed in *E. coli* by using DEAE ion-exchange resins in batch mode is shown in **Figure 1**. This purification technique is based on the binding of host cell proteins and/or impurities other than HP-NAP to the resin. At pH 8.0, almost no other proteins except HP-NAP in its native form are recovered from the unbound fraction (**Figure 2A** and **B**). The purified HP-NAP in the unbound fraction was immunodetected by the antibody against HP-NAP (**Figure 2C**), and its purity was higher than 97% as confirmed by silver staining (**Figure 2D**). In addition, the purified recombinant HP-NAP kept its oligomeric form as analyzed by native-PAGE (**Figure 2B**) and gel filtration chromatography (**Figure 3A**). Circular dichroism spectroscopic analysis showed that the purified protein was mainly composed of α-helices (**Figure 3B**). Also, the purified HP-NAP was capable of stimulating human neutrophils to produce ROS (**Figure 3C**). Thus, the recombinant HP-NAP purified by this approach is in its native form with biological activity. Furthermore, this negative mode batch chromatography can be used to purify recombinant HP-NAP with point mutations in one step. As shown in **Figure 4**, the two recombinant HP-NAP_{Y101H} and $\text{HP-NAP}_{E97GY101H}$ mutants, which mimic HP-NAP of *H. pylori* NCTC 11639 and NCTC 11,637 strains, respectively, were purified by this negative mode batch chromatography with purity higher than 95%.

For performing negative mode batch chromatography using DEAE resins to purify HP-NAP, the pH value of the buffer used for purification should be adjusted to 8.0 to ensure that the majority of HP-NAP is present in the unbound fraction. Lesser amount of HP-NAP was present in the unbound fractions when the pH value of the buffer was higher or lower than 8.0 (**Figure 5**). Even though the purity of HP-NAP present in the unbound fractions was increased just a little bit as pH increased from 7.0 to 9.0, the amount of HP-NAP present in the unbound fractions was the highest when the pH value of the buffer was pH 8.0 (**Figure 5**). The amount of the soluble proteins from cell lysates loaded onto the resin is another important factor for this purification. Here, the ratio is 1.5 mg of proteins per milliliter resin for achieving maximum capacity of the resin to absorb the impurities from *E. coli* (**Figure 6**). In addition, the purity and yield of HP-NAP obtained by this negative mode batch chromatography can be substantially increased by increasing the amount of HP-NAP present in the soluble fraction of *E. coli* lysates. Recombinant HP-NAP was almost fully recovered in the soluble fraction upon cell lysis at pH 9.0 (**Figure 7**). Even though the solubility of recombinant HP-NAP in *E. coli* lysates was markedly increased when cells were lysed in the buffer with pH higher than 7.5 (**Figure 7**), less than 90% of HP-NAP was present as the soluble protein when cells were lysed in the buffer with pH lower than 9.0.

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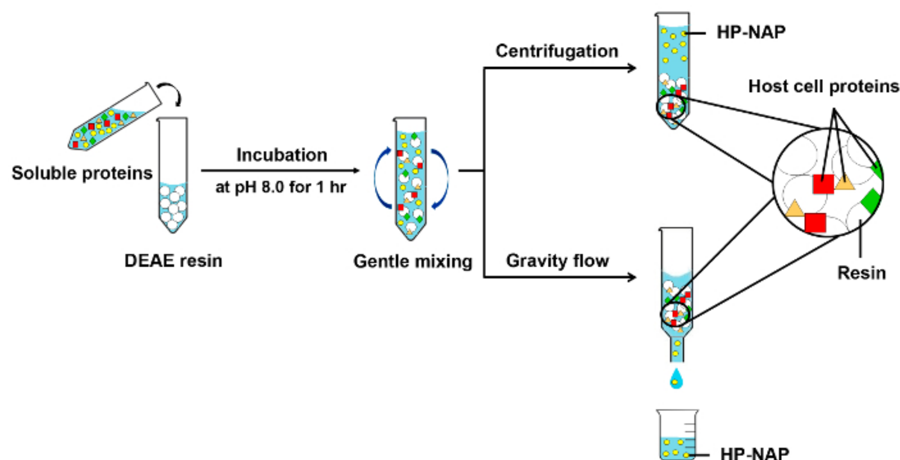

Figure 1: Schematic Outline of the Negative Purification of HP-NAP by DEAE Resins in Batch Mode. The purification starts with a batch-binding procedure. The soluble protein fraction containing HP-NAP obtained from bacterial lysates is added to the DEAE resin pre-equilibrated with Tris-buffer at pH 8.0. The proteins/resin slurries are then incubated at 4 °C for 1 hr with gentle mixing. During the incubation, the host cell proteins bind to the resin. HP-NAP present in the unbound fraction is obtained by either collecting the supernatant after centrifugation or the flow-through fraction from a column run by gravity flow. Please click here to view a larger version of this figure.

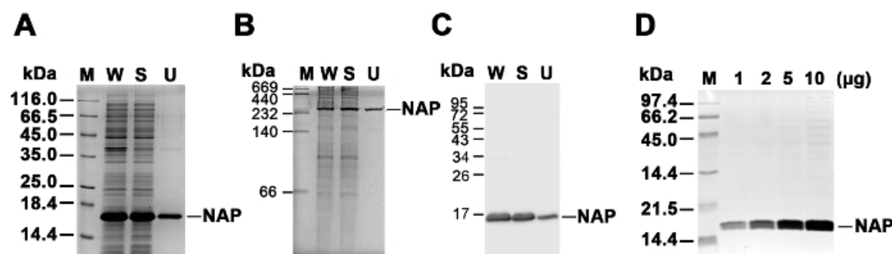

Figure 2: Exemplary Result of Purification of HP-NAP by Negative Mode Batch Chromatography with DEAE Ion-exchange Resins. The whole cell lysate (W) and soluble protein fraction (S) of *E. coli* BL21(DE3) expressing HP-NAP and the unbound fraction (U) containing HP-NAP from DEAE ion-exchange chromatography were analyzed by SDS-PAGE (**A**), native-PAGE (**B**), and western blotting (**C**). The unbound fraction with the indicated amount of proteins ranging from 1 µg to 10 µg was analyzed on a silver-stained SDS-PAGE gel (**D**). Molecular masses (M) in kDa are indicated on the left of the stained gels and the blot. (Adapted from reference[24]) Please click here to view a larger version of this figure.

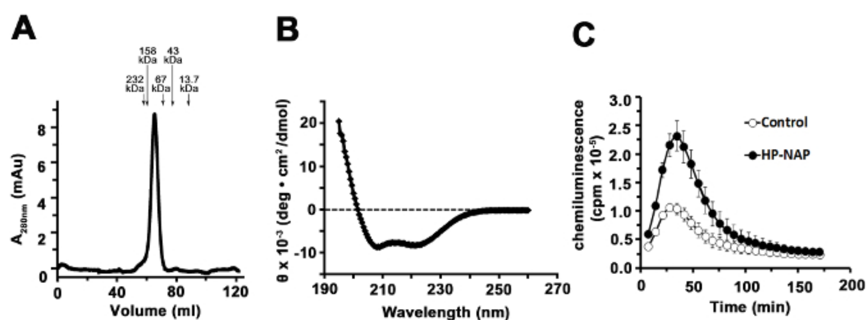

Figure 3: Structural and Functional Characterization of Recombinant HP-NAP Purified From *E. coli* by Negative Mode Batch Chromatography. (**A**) The UV absorbance profile was recorded for HP-NAP eluted from a gel filtration column. The molecular masses of the protein markers were indicated on the chromatogram. (**B**) The far UV circular dichroism spectrum of HP-NAP was recorded at the wavelength range of 195 to 260 nm. (**C**) Human neutrophils (1 x 10^5 cells) were treated with 0.5 µM HP-NAP and D-PBS, pH 7.2, as a negative control at 37 °C. The content of ROS generated from neutrophils was measured continuously by using a luminol-dependent chemiluminescence assay. Data were represented as the mean ± standard deviation of an experiment in triplicate. (Adapted from reference[24]) Please click here to view a larger version of this figure.

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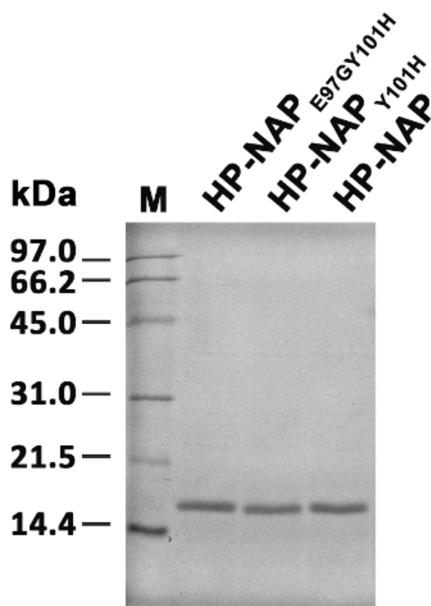

Figure 4: Purification of Recombinant HP-NAP Mutants Expressed in *E. coli* by Negative Mode Batch Chromatography. The soluble protein fractions of *E. coli* BL21(DE3) expressing two recombinant HP-NAP$_{Y101H}$ and HP-NAP$_{E97GY101H}$ mutants and wild-type HP-NAP were purified by the negative mode chromatography with DEAE resins. The unbound fractions were analyzed by SDS-PAGE**.** Molecular masses (M) in kDa are indicated on the left of the stained gels. Please click here to view a larger version of this figure.

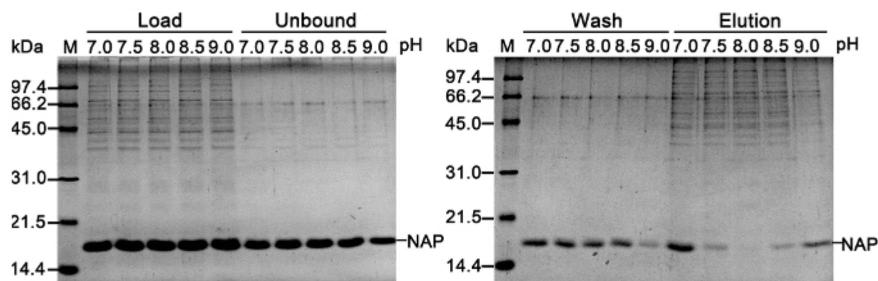

Figure 5: Effect of the Buffer pH on Purification of Recombinant HP-NAP Expressed in *E. coli* by Negative Mode Batch Chromatography. The soluble fraction of *E. coli* BL21(DE3) expressing HP-NAP lysed at pH 9.0 were adjusted to the indicated pH ranging from 7.0 to 9.0 and a protein concentration of 0.3 mg/ml. These adjusted fractions, indicated as load, were then loaded onto DEAE resins to purify recombinant HP-NAP by negative mode batch chromatography at 4 °C. The unbound, wash, and elution fractions were analyzed by SDS-PAGE. Molecular masses (M) in kDa are indicated on the left of the stained gels. (Adapted from reference[24]) Please click here to view a larger version of this figure.

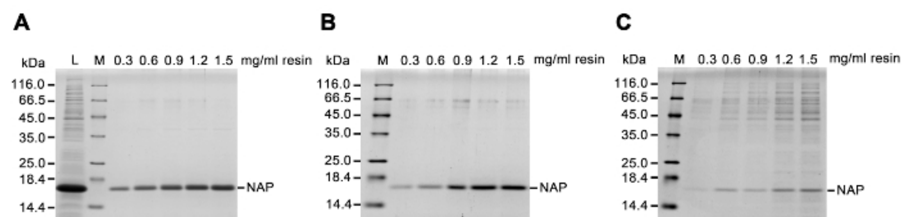

Figure 6: Effect of the Amount of Proteins Loaded onto DEAE Resins for Purifying Recombinant HP-NAP Expressed in *E. coli*. The soluble protein fractions of *E. coli* BL21(DE3) expressing HP-NAP were loaded onto DEAE resins according to the indicated ratio of mg proteins per milliliter of resins to purify recombinant HP-NAP by the negative mode batch chromatography at pH 8.0. The soluble proteins, indicated as load (L), the unbound fraction (**A**), wash fraction (**B**), and elution fraction (**C**) were analyzed by SDS-PAGE. Molecular masses (M) in kDa are indicated on the left of the stained gels. (From reference[24]) Please click here to view a larger version of this figure.

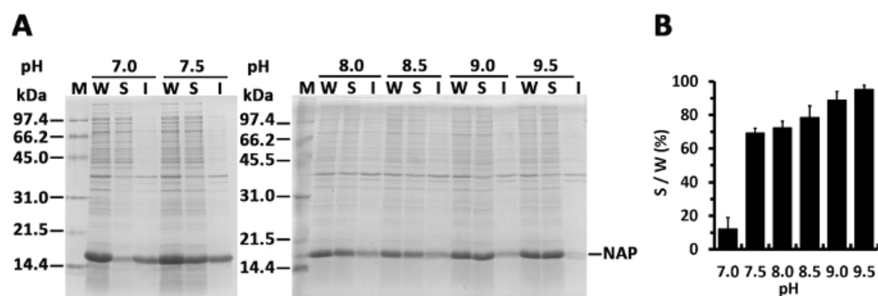

Figure 7: Effect of pH on the Solubility of HP-NAP in *E. coli* Lysates. (**A**) *E. coli* BL21(DE3) expressing HP-NAP was suspended in ice-cold Tris-HCl buffer at the indicated pH ranging from 7.0 to 9.5. Cells were lysed and then whole cell lysates (W) were centrifuged to separate soluble fractions (S) and insoluble pellets (I). The proteins were analyzed by SDS-PAGE. Molecular masses (M) in kDa are indicated on the left of the stained gels. (**B**) The percentage of solubility of recombinant HP-NAP in the whole cell lysate at each pH was calculated from the intensity of HP-NAP band on SDS gels for the soluble fraction (S) divided by that for the whole cell lysate (W). Data were represented as the mean ± standard deviation of at least two experiments. (From reference[24]) Please click here to view a larger version of this figure.

HP-NAP mutants	Primers	Sequences (5'-3') [a, b]	$T_{m\,pp}$ (°C) [c]	$T_{m\,no}$ (°C) [d]	Inserted silent restriction sites
HP-NAP$_{E97GY101H}$	Hp26695-NAP14415+(E97GY101H)+XhoI+	ACAAACATCTCGAGAAAGAATTTAAAGAGCTCTCTAACACCG	54	62	Xho I
	Hp26695-NAP14415+(E97GY101H)+XhoI-	TTCTTTCTCGAGATGTTTGTAGTCCCCTAGAATTTCTTTAAAGAT	54	62	
HP-NAP$_{Y101H}$	Hp26695-NAP14427+(Y101H)+XhoI+	ACAAACATCTCGAGAAAGAATTTAAAGAGCTCTCTAACACC	54	62	Xho I
	Hp26695-NAP14427+(Y101H)+XhoI-	TTCTTTCTCGAGATGTTTGTAGTCCTCTAGAATTTCTTTAAAGA	54	62	

[a] Primer-primer overlapping sequences are in italic.
[b] Inserted silent restriction sites are underlined.
[c] $T_{m\,pp}$ (°C) was calculated from the primer-primer overlap sequence.
[d] $T_{m\,no}$ (°C) was calculated from the primer sequence matched to the template.

Table 1: Primers Used for Mutagenesis. Please click here to view a larger version of this table.

Discussion

The negative mode batch chromatography with DEAE anion-exchange resins presented here is suitable for purification of recombinant HP-NAP overexpressed in *E coli*. The pH values of the buffers used in the steps of cell lysis and purification are very critical to ensure the solubility of HP-NAP in *E. coli* lysates and efficient separation of recombinant HP-NAP from host cell impurities, respectively. Bacterial cells should be lysed at pH 9.0, and the negative purification should be performed at pH 8.0 to obtain HP-NAP in high yield and high purity. Typical recovery of HP-NAP is 90%, and typical purity is 95%[24]. Since HP-NAP is present in the unbound fraction, a minimal but sufficient amount of the resin should be used to achieve its maximal capacity to absorb the impurities. In our case, the maximum loading capacity is 1.5 mg of the soluble proteins from *E. coli* lysates per milliliter resin.

The provided protocol is designed for purification of recombinant HP-NAP from a 50 ml *E. coli* culture. The yield of HP-NAP is around 15 mg. The purification process can be either scaled-down or scaled-up accordingly. For example, the two recombinant HP-NAP$_{Y101H}$ and HP-NAP$_{E97GY101H}$ mutants were purified from a 1 ml *E. coli* culture by using this negative mode batch chromatography. Both HP-NAP mutants with purity higher than 95% were obtained by collecting the unbound fraction (**Figure 4**). This negative mode batch chromatography have also been applied to purify recombinant HP-NAP from a 860 ml *E. coli* culture. The recovery of the step using DEAE negative mode batch chromatography has reached 97% with purity higher than 90%.

HP-NAP expressed in *Bacillus subtilis* (*B. subtilis*) has also been successfully purified by this DEAE negative mode batch chromatography in one step[26]. In our *B. subtilis* expression system, the expression level of HP-NAP is low. HP-NAP only accounts for around 5% of the total soluble proteins from the bacterial lysates. Even though HP-NAP targeted for purification is present in a small amount, HP-NAP with purity higher than 90% can be obtained by reducing the ratio of the amount of proteins from cell lysates loaded onto the resin to efficiently remove almost all the endogenous proteins from *B. subtilis*[26]. Thus, this method may also be applied to purify recombinant HP-NAP expressed in other bacterial hosts.

Several methods have been reported for the purification of recombinant HP-NAP in its native form[14-16]. However, an additional gel filtration chromatographic step is required to obtain HP-NAP in high purity. This negative chromatographic purification can efficiently yield functional recombinant HP-NAP with high purity in one step. In addition, the desalination step is not needed due to the low salt concentration in the unbound fraction. The batch-mode purification could also obviate the need for a column. Thus, this negative mode batch chromatography using DEAE resin offers a simple and efficient method to purify HP-HAP in its native form with high yield and purity. HP-NAP purified by this method

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could be further utilized for the development of vaccines, new drugs, and diagnostics for *H. pylori*-related diseases or for other new therapeutic applications.

Disclosures

HWF and YCY are inventors of patents TW I 432579 and US 8,673,312 for the method of one-step purification of *Helicobacter pylori* neutrophil-activating protein. All materials described in the manuscript will be available for research purposes. The authors confirm that this does not alter their adherence to all of the JoVE's policies on sharing data and materials.

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