

Research Article

INTER-SUBUNIT INTERACTION AND ARRANGEMENT OF THE CENTRAL STALK SUBUNITS OF *ENTEROCOCCUS HIRAE* V-ATPASEK. M. Mozaffor Hossain^{1,2} and Ichiro Yamato²¹Department of Veterinary and Animal Sciences, Faculty of Agriculture, University of Rajshahi, Rajshahi 6205, Bangladesh²Department of Biological Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda-shi, Chiba 278-8510, Japan

Abstract: V-ATPases function as ATP-dependent ion pumps in various membrane systems of living organisms. ATP hydrolysis causes rotation of the central rotor complex, which is composed of NtpC, NtpD, and NtpG subunits. We successfully cloned, expressed, and purified the central stalk subunits of Na⁺-translocating V-type ATPase from *E. hirae*. Full length DNAs for genes of NtpC, NtpD, and NtpG subunits were amplified by conventional PCR method and cloned into the plasmid vector pET23d. Recombinant DNA transformed *E. coli* (JM109) cells were grown in LB agar plate containing ampicillin. NtpC and NtpG subunit proteins were expressed as separate His-tagged soluble proteins in *E. coli* BL21(DE3) cells at 30 °C and NtpD subunit protein was stably expressed in *E. coli* BL21(DE3) cells at 16 °C. Expressed central stalk subunit proteins were purified by affinity chromatography followed by gel-filtration method. The amount of purified NtpC, NtpD, and NtpG subunit proteins were 13.8 mg/1 liter culture, 16.6 mg/1 liter culture, and 15.2 mg/1 liter culture, respectively. Tobacco etch virus (TEV)-protease was also expressed in *E. coli* BL21(DE3) cells and purified by affinity chromatography. The amount of purified TEV-protease was 8.50 mg/1 liter culture. Here, we report the stable interaction and the complex formation mechanisms of the central stalk subunits of *E. hirae* V-ATPase. The purified NtpD could interact directly with NtpG and NtpC could bind directly to NtpD-G complex and these three subunits could form NtpD-G-C complex. NtpD could bind to NtpG and formed NtpD-G complex. NtpD or NtpD-G complex could bind to NtpA₃-B₃ and formed NtpA₃-B₃-D or NtpA₃-B₃-D-G complex, respectively. NtpC could bind to NtpA₃-B₃-D-G and formed NtpA₃-B₃-D-G-C complex but could not bind to NtpA₃-B₃, NtpA₃-B₃-D, NtpD, or NtpG.

Keywords: *Enterococcus hirae*; V-ATPase; central stalk subunit proteins; interaction.

Note : Coloured Figures available on Journal Website in "Archives" Section

Introduction

Ion-transporting ATPases are divided into three types based on their function and taxonomic origin (F-, V-, and A-type ATPases). F-ATPases function as ATP synthases in mitochondria, chloroplasts, and oxidative bacteria (Walker, 1998). Vacuolar ATPases (V-ATPases) function as ATP-dependent

proton pumps in acidic organelles and in plasma membranes of eukaryotic cells (Forgac, 2007). A-ATPases in the archaea function as ATP synthases similar to the F-ATPases (the 'A' designation in A-type refers to archaea), although the structure and subunit composition of A-ATPases are more similar to those of V-ATPases (Schafer *et al.*, 1999). These ATPases possess an overall similar structure that is composed of catalytic portion (F₁-, V₁-, or A₁-ATPase) and a membrane-embedded ion-transporting portion (F_o-, V_o-, or A_o-ATPase), and they have a similar reaction mechanism that occurs through rotation (Forgac, 2007). V-ATPases have

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Received: December 24, 2016

Accepted: February 4, 2017

Published: February 6, 2017

globular catalytic domain, V_1 , where ATP is hydrolyzed, attached by a central and peripheral stalks to intrinsic membrane domain, V_o , where ions are pumped across the membrane (Forgac, 2007). V-ATPase is an ion-translocating rotary motor (Forgac, 2007) in which hydrolysis of ATP generates rotation of the central stalk and an attached membrane ring of the hydrophobic subunits. Ions are pumped through a pathway at the interface between the rotating ring and a static membrane component, which is linked to the outside of the V_1 domain by the peripheral stalks (Forgac, 2007).

A family of V-ATPases are also found in the membranes of some bacteria (Lolkema *et al.*, 2003; Yokoyama and Imamura, 2005; Murata *et al.*, 2005a). In the simplest bacterial V_1V_o , the catalytic core of V_1 moiety consists of A, B, D, and F subunits, in which three alternately arranged A and B subunits form a hexameric A_3B_3 ring, and the central shaft, composed of D and F subunits, penetrates the central cavity of the A_3B_3 ring (Arai *et al.*, 2013). One example is V-ATPase from the thermophilic bacterium *Thermus thermophilus* (Tsutsumi *et al.*, 1991; Yokoyama *et al.*, 1994). *T. thermophilus* V-ATPase functions as an ATP synthase *in vivo* like as F-type ATP synthases (Yokoyama *et al.*, 2003). The *T. thermophilus* V-ATPase is composed of nine subunits; A, B, D, F, C, E, G, I, and L (Yokoyama *et al.*, 2000) in which D, F, and C subunits form the central stalk (Yokoyama *et al.*, 2003; Iwata *et al.*, 2004). Central stalk subunits D and F of *T. thermophilus* V-ATPase have been shown to play an important role in the regulation of enzyme activity (Imamura *et al.*, 2004) and subunit C has an important role in reversible association/dissociation of V-type ATPase (Iwata *et al.*, 2004).

A fermentative bacterium *Enterococcus hirae* (*E. hirae*) has a variant of V-ATPase which physiologically transports Na^+ rather than H^+ (Heefner and Harold, 1982). This enzyme is composed of nine subunits fewer than eukaryotic V-ATPases (Murata *et al.*, 2005a), which are encoded by nine *ntp* subunit genes (*ntpFIKECGABD*) organized in the *ntp* operon (Takase *et al.*, 1994; Murata *et al.*, 1999). Therefore, *E. hirae*, Na^+ -translocating V-ATPase, is a homolog of eukaryotic V-type ATPase. The catalytic domain (V_1) of this ATPase is consisted of $\text{NtpA}_3\text{B}_3\text{-D-G}$, where NtpG subunit corresponds to F subunit of other V-ATPases (Hosaka *et al.*, 2006). The membrane domain (V_o) in which the rotation energy is converted to Na^+ translocation is composed of oligomers of 16-kDa NtpK (corresponds to

eukaryotic subunit c) forming a membrane rotor ring and a single copy of the NtpI subunit (corresponds to eukaryotic subunit a) (Murata *et al.*, 2008). The peripheral stalks of this enzyme is composed of NtpF (corresponds to eukaryotic subunit G) and NtpE subunits together with the N-terminal hydrophilic domain of NtpI subunit (Murata *et al.*, 2005b). The central stalk of Na^+ -translocating V-ATPase in *E. hirae* is composed of NtpC, NtpD, and NtpG subunits (Figure 1). NtpA₃-B₃-D-G complex and V_o moiety are connected by a central stalk subunit NtpC of V_1 (Murata *et al.*, 2005b). The central stalk subunit NtpC of V-ATPase of *E. hirae* acts as a clutch to attach V_1 central stalk subunits (NtpD-G) onto the NtpK ring of V_o part. Subunit NtpC is also the part of the central stalk of *E. hirae* V-ATPase and serves as a socket to attach V_1 central stalk subunits (NtpD and NtpG) onto the NtpK ring of V_o part like d subunit of *T. thermophilus* V-ATPase. This region is possibly responsible for the reversible association/dissociation of the complex (Makyio *et al.*, 2005). The asymmetrical interaction between the central stalk and A_3B_3 hexamer is thought to be the primary importance in the rotational mechanism as in the case of F₁-ATPase (Yoshida *et al.*, 2001; Kabaleeswaran *et al.*, 2009). Electron microscopic studies have provided only limited information about the subunit interactions (Bernal and Stock, 2004; Zhang *et al.*, 2008).

The molecular weights of NtpC, NtpD, and NtpG subunits are 38 kDa, 27 kDa, and 11 kDa, respectively, though their structural arrangement are not clear (Murata *et al.*, 1997). It is expected that central stalk subunits of Na^+ -translocating V-type ATPase in *E. hirae* play an important role in its enzymatic activities but not yet confirmed. We expect that the biochemical and molecular biological studies of this bacterial ATPase should give us a fundamental understanding of the properties of V-type ATPases. For understanding the structure and mechanism of V-ATPase, it is prerequisite and essential to confirm the inter-subunit interaction and arrangement of the central stalk subunits of *E. hirae* V-ATPase. Here, we report the inter-subunit interaction and arrangement of the central stalk subunits of *E. hirae* V-ATPase.

Materials and methods

PCR amplification of the central stalk subunit genes
Escherichia coli (*E. coli*) harboring pET23d, pCR2.1 TOPO HisNtpC, pCR2.1 TOPO HisNtpD, and

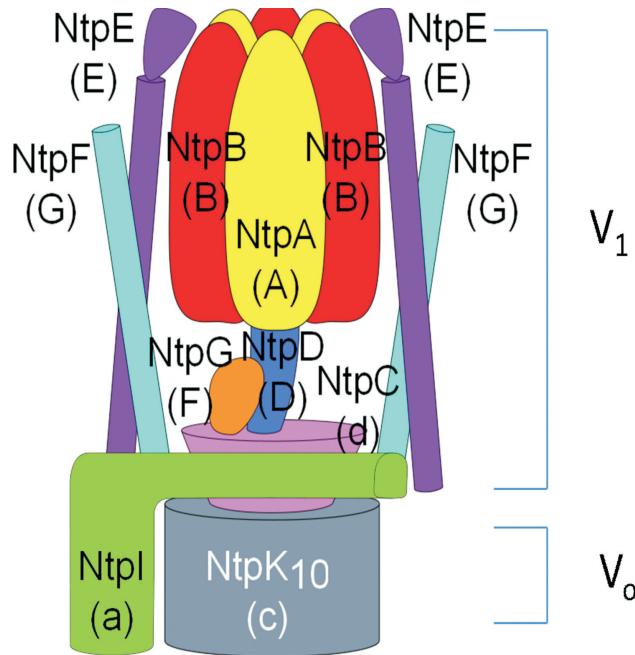


Figure 1: A model of V-ATPase from *Enterococcus hirae*.

V_1 indicates catalytic domain (consisting of NtpA₃-B₃-D-G), V_0 indicates membrane domain (consisting of NtpI-K₁₀). Peripheral stalk consists of NtpE and NtpF subunits and central stalk consists of NtpC, NtpD, and NtpG subunits (Parentheses show the names of corresponding subunits of eukaryotic V-ATPase).

pCR2.1 TOPO HisNtpG were grown. Plasmids of pET23d, pCR2.1 TOPO HisNtpC, pCR2.1 TOPO HisNtpD, and pCR2.1 TOPO HisNtpG were purified by using plasmid purification kit (Qiagen) according to the manufacturer's instructions. Concentrations of purified plasmid DNAs were measured for pET23d, pCR2.1 TOPO HisNtpC, pCR2.1 TOPO HisNtpD, and pCR2.1 TOPO HisNtpG by nanodrop spectrophotometer. The *ntpC* gene was amplified by PCR using oligonucleotide primers 5'-GACGTCGGATCC ATGGAATATCACGAAC CGCT- 3' and 5'-AAAAAAACTTGATTA GGGTGTGGTCAC GTAGTGCCT ACG- 3'. The *ntpD* gene was amplified by PCR using oligonucleotide primers 5'-GCAGCGAGATCTATGCG TCTGAATGT- 3' and 5'- AAAAAGTTGATTA GGGTGTGGTCAC GTAGTGCCT ACG- 3'. The *ntpG* gene was amplified by PCR using oligonucleotide primers 5'-CCAGGTGGATCC ATGACCTATAAAAT TGGCGTGG-3' and 5'-AAAAAAACTTGATTA GGGTGTGGTCAC GTAGTGCCT ACG- 3'. PCR was performed in a total of 50 μ l final volume; consisting of 10 μ l 5 x Taq buffer, 4 μ l 1.25 mM nucleotides (dNTPs), 0.1 μ l of each primer (10

pmol), 1 μ l template, 0.5 μ l DNA polymerase, and 34.3 μ l distilled water. A thermal cycler (PTI-100, MJ Research Inc.) was used and the PCR program was: denaturation at 95 °C for 20 seconds, primer annealing at 65 °C for 20 seconds, and extension at 72 °C for 20 seconds (30 cycles).

Cloning and sequencing of the central stalk subunit genes

Amplified *ntpC*, *ntpD*, and *ntpG* genes were digested by *Sty*I and *Dra*III, *Bg*III and *Dra*III, *Bam*HII and *Dra*III, restriction enzymes, respectively. At the same time the vector DNA(pET23d) was digested separately by corresponding restriction enzymes to clone *ntpC*, *ntpD*, and *ntpG* genes. Digested DNAs were run in agarose gel and resulting corresponding bands were purified by using QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions. The concentrations of restriction enzyme digested vector (pET23d) DNA and insert (*ntpC*, *ntpD*, and *ntpG*) DNAs were measured by nanodrop spectrophotometer. All recombinant technologies (DNA digestion by restriction endonucleases, T4-DNA ligase mediated ligation, plasmid preparation, and growth of bacterial cultures) were performed according to Sambrook and Russell (2001) to generate pET23d-HisNtpC, pET23d-HisNtpD, and pET23d-HisNtpG. Recombinant DNAs were transformed into *E. coli* JM109 competent cells and grown on LB agar plates containing ampicillin (50 μ g/ml) for cloning purposes. Recombinant plasmids were purified from *E. coli* JM109 cells by using plasmid purification kit (Qiagen) according to the manufacturer's instructions and amplified by PCR using BigDye premix and sequence buffer. Amplified regions were checked by sequencing using BigDye Terminator v3.0 sequencing kit (Applied Biosystem) and ABI 3100-Avant sequencer. Sequencing results were compared with the sequences of *ntpC*, *ntpD*, and *ntpG* genes using CLUSTALW program (Thomson *et al.*, 1994) to ensure successful cloning.

Expression of the central stalk subunit proteins

Plasmids of pET23d-HisNtpC, pET23d-HisNtpD, and pET23d-HisNtpG were purified from *E. coli* JM109 cells and transformed into *E. coli* BL21(DE3) cells. *E. coli* BL21(DE3) cells containing *ntpC*, *ntpD*, and *ntpG* genes were inoculated separately in one liter of m-DM-CA culture medium [3.4 gm KH₂PO₄,

7.3 gm Tris, 0.5 gm Na-citrate, 0.01% $MgSO_4$, 0.4% Bacto casamino acid (Difco, Technical), 0.4% lactate, containing ampicillin (50 $\mu g/ml$), (pH 7.4); (Mogi and Anraku, 1984)]. Bacterial cultures were incubated at 30 °C for *ntpC* and *ntpG* genes and at 16 °C for *ntpD* gene with shaking at 100 rpm. After the OD_{600} reached 0.4-0.6, IPTG was added at the concentration of 0.2 mM and the cell cultures were further incubated at the same temperature for 6 hours for *ntpC* and *ntpG* genes and 8 hours for *ntpD* gene. *E. coli* BL21(DE3) cells were harvested by centrifugation at 6000 rpm for 10 minutes at 4 °C. Cells were suspended in 30 ml of buffer A [50 mM Tris-HCl (pH 8.5), 10 mM EDTA] and suspensions were centrifuged at 3000 rpm for 30 minutes at 4 °C. Collected cells were suspended again in 30 ml of buffer B [50 mM Tris-HCl (pH 8.5), 1 mM EDTA, 20% sucrose] and treated with lysozyme (100 $\mu g/ml$) to obtain spheroplasts. Spheroplasts were resuspended in 30 ml of buffer C [20 mM Tris-HCl (pH 8.5), 150 mM NaCl] and disrupted with sonication (Branson sonifier, output 5, duty cycle 50%, five times of 5 min at 4 °C). NtpC, NtpD, and NtpG polypeptides were recovered in the supernatant cell lysate after centrifugation at 15000 rpm for 10 minutes at 4 °C.

Expression of TEV protease

The tobacco etch virus protease (TEV-protease) gene containing *E. coli* BL21(DE3) cells were inoculated in one liter of m-DM-CA medium containing ampicillin (50 $\mu g/ml$) and chloramphenicol (30 $\mu g/ml$) and incubated at 22 °C with shaking at 100 rpm. After the OD_{600} reached 0.4-0.6, IPTG was added at the concentration of 0.5 mM and the culture was incubated at the same temperature for 6 hours. *E. coli* BL21(DE3) cells were harvested by centrifugation at 6000 rpm for 10 minutes at 4 °C. Cells were washed with 30 ml of buffer D [15 mM Tris-HCl (pH 8.5), 0.5 M KCl] and suspended in 30 ml of buffer E [50 mM Tris-HCl (pH 8.5), 0.5 M KCl, 1 mM DTT, 1% Triton X-100] and treated with 100 $\mu g/ml$ lysozyme to obtain spheroplasts. Spheroplasts were disrupted by using sonication (Branson sonifier, duty cycle 50%, output 5, five times of 5 min at 4 °C). Protein was recovered and undisrupted cells were removed by centrifugation at 18000 rpm for 10 minutes at 4 °C.

Purification of subunit proteins

Total cell lysates of different subunits were added separately to 5 ml bed volume of Ni Sepharose 6

Fast Flow equilibrated with buffer F (20 mM Tris-HCl (pH 8.5), 0.5 M KCl, 5 mM imidazole, 0.1 mM DTT) and incubated at 4 °C with gentle agitation (end-over-end rotation) for 1 hour. Supernatants were removed by aspiration after sedimentation. Resins were resuspended in 10 ml of buffer F and transferred into 20 ml chromatography columns (Bio-Rad). Flow-through fractions were discarded and bound proteins were eluted (sequentially) with 25 ml buffer H (20 mM Tris-HCl (pH 8.5), 0.5 M KCl, 400 mM imidazole, 0.1 mM DTT) at a flow rate of 0.3 ml/min. Purified protein samples were pooled and concentrated to 1 ml volume by ultrafiltration with Amicon Ultra Centrifugal Filter Devices (pore size; MW 10 kDa for NtpC and NtpD, 3 kDa for NtpG). Concentrated proteins were further purified by gel-filtration method (by using Superdex 75 5/150 GL column and gel-filtration buffer consisting of 20 mM Mes-Tris (pH 6.5), 10% glycerol, 50 mM KCl, 5 mM $MgSO_4$, 0.1 mM DTT). Protein concentrations were measured according to the Lowry method (Lowry *et al.*, 1951) with bovine serum albumin as the standard and kept at -80 °C with 10% glycerol.

Purification of TEV-protease

Total cell lysate was added to 3 ml bed volume of Ni Sepharose 6 Fast Flow equilibrated with buffer G (50 mM Tris-HCl (pH 8.5), 0.5 M KCl, 20 mM imidazole, 1 mM DTT, 10% glycerol) and incubated at 4 °C with gentle agitation (end-over-end rotation) for 1 hour. Supernatant was removed by aspiration after sedimentation. Resins were resuspended with 5 ml of buffer I (50 mM Tris-HCl (pH 8.5), 0.5 M KCl, 200 mM imidazole, 1 mM DTT, 10% glycerol) and transferred into a falcon tube. Bound proteins were eluted with 5 ml of buffer I by centrifugation (1000 rpm for 20 minutes) for three times. Protein samples that were eluted with buffer I were pooled and concentrated to 1 ml volume by ultrafiltration by using Amicon Ultra Centrifugal Filter Devices (pore size; MW 10 kDa). Concentrated sample was diluted with 14 ml of stock buffer (20 mM sodium phosphate (pH 6.0), 100 mM NaCl, 1 mM DTT, 10% glycerol). Furthermore, diluted proteins were concentrated to 1 ml volume by ultrafiltration by using Amicon Ultra Centrifugal Filter Devices (pore size; MW 10 kDa). Concentrated sample was transferred into 1.5 ml eppendorf tube and centrifuged at 15000 rpm for 10 minutes at 4 °C. Supernatant was collected as a pure protein and concentration was measured by Lowry method

(Lowry *et al.*, 1951) with bovine serum albumin as the standard.

SDS-PAGE analysis

SDS-PAGE was carried out according to Laemmli (1970). Protein markers used were phosphorylase b (97.0 kDa), BSA (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (21.1 kDa), and α -lactalbumin (14.4 kDa) (low molecular weight marker, GE Healthcare). All expressed and purified proteins were analyzed by SDS-PAGE on 15% gel and subsequently stained with CBB.

Synthesis and purification of individual NtpA and NtpB subunit

The DNA fragment corresponding to *ntpA* or *ntpB* gene having optimal codon usages for *E. coli* expression system was cloned into the plasmid vector pET23d and expressed in *E. coli* BL21(DE3) cells by IPTG induction (Arai *et al.*, 2009). *E. coli* lysate was added separately to 5 ml bed volume of Ni Sepharose 6 Fast Flow equilibrated with buffer A (20 mM Tris-HCl (pH 8.5), 0.5 M KCl, 5 mM imidazole, 0.1 mM DTT) and incubated at 4 °C with gentle agitation (end-over-end rotation) for 1 hour. Supernatants were removed by aspiration after sedimentation. Resins were resuspended in 10 ml of buffer A and transferred into 20 ml chromatography columns (Bio-Rad). Flow-through fractions were discarded and bound proteins were eluted (sequentially) with 30 ml of buffer B (20 mM Tris-HCl (pH 8.5), 0.5 M KCl, 400 mM imidazole, 0.1 mM DTT) at a flow rate of 0.3 ml/min. Purified protein samples were pooled and concentrated to 1 ml volume by ultrafiltration with Amicon Ultra Centrifugal Filter Devices (pore size; MW 30 kDa). Concentrated proteins were further purified by gel-filtration method (by using Superdex 75 5/150 GL column and gel-filtration buffer consisting of 20 mM Mes-Tris (pH 6.5), 10% glycerol, 50 mM KCl, 5 mM MgSO₄, 0.1 mM DTT). Protein concentrations were measured according to the Lowry method (Lowry *et al.*, 1951) with bovine serum albumin as the standard.

Pull-down assay using one tagged subunit and co-purification of the complex

To obtain non-tagged subunit protein, the histidine-affinity tag was removed by tobacco etch virus protease treatment at 4 °C for 12 hours (Yamamoto

et al., 2008). Non-tagged subunit proteins were purified by gel filtration method and collected as flow-through fractions. Collected non-tagged subunit proteins were concentrated by ultrafiltration using Amicon Ultra Centrifugal Filter Devices (pore size; MW 10 kDa for NtpC and NtpD, 3 kDa for NtpG). His-tagged sample and molar excess of non-tagged sample were mixed and incubated on ice for 90 minutes (Yamamoto *et al.*, 2008). The mixture was added to 1 ml bed volume of Ni Sepharose 6 Fast Flow equilibrated with buffer C (20 mM Tris-HCl (pH 8.5), 0.5 M KCl, 0.1 mM DTT) and incubated at 4 °C with gentle agitation (end-over-end rotation) for 1 hour. The mixture was loaded into a chromatography column which was washed with 5 column volumes of buffer C and flow-through fraction was collected. Bound proteins were eluted with buffer D (20 mM Tris-HCl (pH 8.5), 0.5 M KCl, 400 mM imidazole, 0.1 mM DTT) at a flow rate of 0.3 ml/min. Flow-through fraction and eluted fraction were analyzed by SDS-PAGE on 15% gel with CBB stain.

Gel-filtration analysis

Protein-protein interaction was analyzed using Superdex 200 5/150 GL column (GE Healthcare) connected with BioLogic HR system (Bio-Rad Laboratories). For gel-filtration, buffer E (20 mM MES-Tris (pH 6.5), 10% glycerol, 50 mM KCl, 5 mM MgSO₄, 0.1 mM DTT) was used as basic buffer solution. Elution was done at flow rate of 0.2 ml/min at room temperature. The NtpA₃-B₃ heterohexamer was reconstituted with NtpA and NtpB monomers and further the NtpA₃-B₃-D by mixing diluted NtpD with NtpA₃-B₃. First, 50 µg of purified NtpC and 15 µg of NtpG subunit proteins were mixed (1:1 molar ratio) in a total of 50 µl of reconstitution buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM DTT) and incubated on ice for one hour and analyzed by gel-filtration. Next, 50 µg of reconstituted NtpA₃-B₃-D-G and 15 µg of NtpC subunit proteins were mixed (1:3 molar ratio) in a total of 50 µl of same reconstitution buffer and incubated on ice for one hour and analyzed by gel-filtration. Furthermore, 60 µg of reconstituted NtpA₃-B₃-D and 30 µg of NtpC subunit proteins were mixed (1:5 molar ratio) in a total of 50 µl of same reconstitution buffer and incubated on ice for one hour and analyzed by gel-filtration. Corresponding elution fractions were collected and assessed by SDS-PAGE on 15% gel with silver stain.

Results

Amplification, cloning and sequencing of the central stalk subunit genes

Concentrations of purified plasmid DNAs of pET23d, pCR2.1 TOPO HisNtpC, pCR2.1 TOPO HisNtpD, and pCR2.1 TOPO HisNtpG were 121.7 ng/ μ l, 1005.3 ng/ μ l, 1123.9 ng/ μ l, and 952.7 ng/ μ l, respectively. Amplification of subunit genes by PCR resulted in the production of specified bands on agarose gel (Figure 2). The concentrations of restriction enzyme digested vector (pET23d) DNA and insert DNAs for *ntpC*, *ntpD*, and *ntpG* genes were 10.4 ng/ μ l, 9.5 ng/ μ l, 8.1 ng/ μ l, and 6.2 ng/ μ l, respectively. PCR products were successfully ligated separately to pET23d vector and cloning was verified by PCR and restriction digestion analysis. The transformed *E. coli* JM109 cells were grown well in LB agar plate containing ampicillin. Sequenced data of three samples were the same with those of the original *ntpC*, *ntpD*, and *ntpG* genes. Multiple alignments of nucleotides by CLUSTALW program confirmed the identity of clones showing 100%

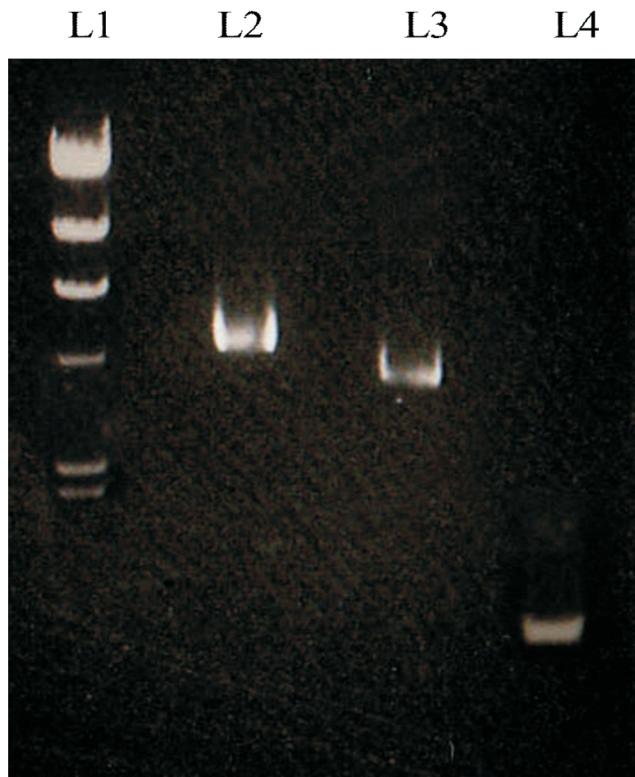


Figure 2: Agarose gel profile of cloned *ntpC*, *ntpD*, and *ntpG* genes.

L1, L2, L3, and L4 indicate marker, cloned *ntpC*, cloned *ntpD*, and cloned *ntpG* genes, respectively. Lane 1 was loaded with 3 μ l marker and each sample lane was loaded with 10 μ l of corresponding DNA.

homology with *ntpC*, *ntpD*, and *ntpG* genes of *E. hirae* V-ATPase.

Expression of the central stalk subunit proteins

In vivo expression of NtpC, NtpD, and NtpG proteins were performed successfully. First, we tried to express NtpC, NtpD, and NtpG proteins in *E. coli* BL21(DE3) cells at 30 °C. We succeeded in the expression of only NtpC and NtpG proteins at 30 °C, whereas, expressed NtpD protein precipitated as inclusion bodies at 30 °C. Subsequently, we could express NtpD protein *in vivo* by reducing the bacterial culture temperature from 30 °C to 20 °C though it was unstable. Finally, we succeeded in the expression of NtpD protein in stable form by reducing culture temperature from 20 °C to 16 °C. The NtpC, NtpD, and NtpG proteins were expressed as His-tagged in *E. coli* BL21(DE3) cells with induction of IPTG. Expressed NtpC, NtpD, and NtpG proteins were confirmed as apparent molecular masses of 38 kDa, 27 kDa, and 11 kDa, respectively as assessed by SDS-PAGE on 15% gel (Figure 3a).

Purification of subunit proteins

NtpC, NtpD, and NtpG proteins were individually purified by using affinity chromatography followed by gel-filtration method. Concentrations of purified NtpC, NtpD, and NtpG proteins were 13.8 mg/1 liter culture, 16.6 mg/1 liter culture, and 15.2 mg/1 liter culture, respectively. Purified NtpC and NtpG proteins were stable at 4 °C for long time and NtpD was unstable at high concentration. But at low concentration NtpD was stable at 4 °C for at least one week. Purified NtpC, NtpD, and NtpG proteins were confirmed as assessed by SDS-PAGE on 15% gel (Figure 3b).

Expression and purification of TEV protease

TEV-protease was successfully expressed as a soluble protein in *E. coli* BL21(DE3) cells and purified by affinity chromatography. Expressed and purified TEV-protease was confirmed as assessed by SDS-PAGE on 15% gel (Figure 3c). The amount of purified TEV-protease was 8.50 mg/1 liter culture.

Complex formation of central stalk subunits determined by pulldown assay

Protein-protein interaction of these central stalk subunit proteins was examined by pulldown assay.

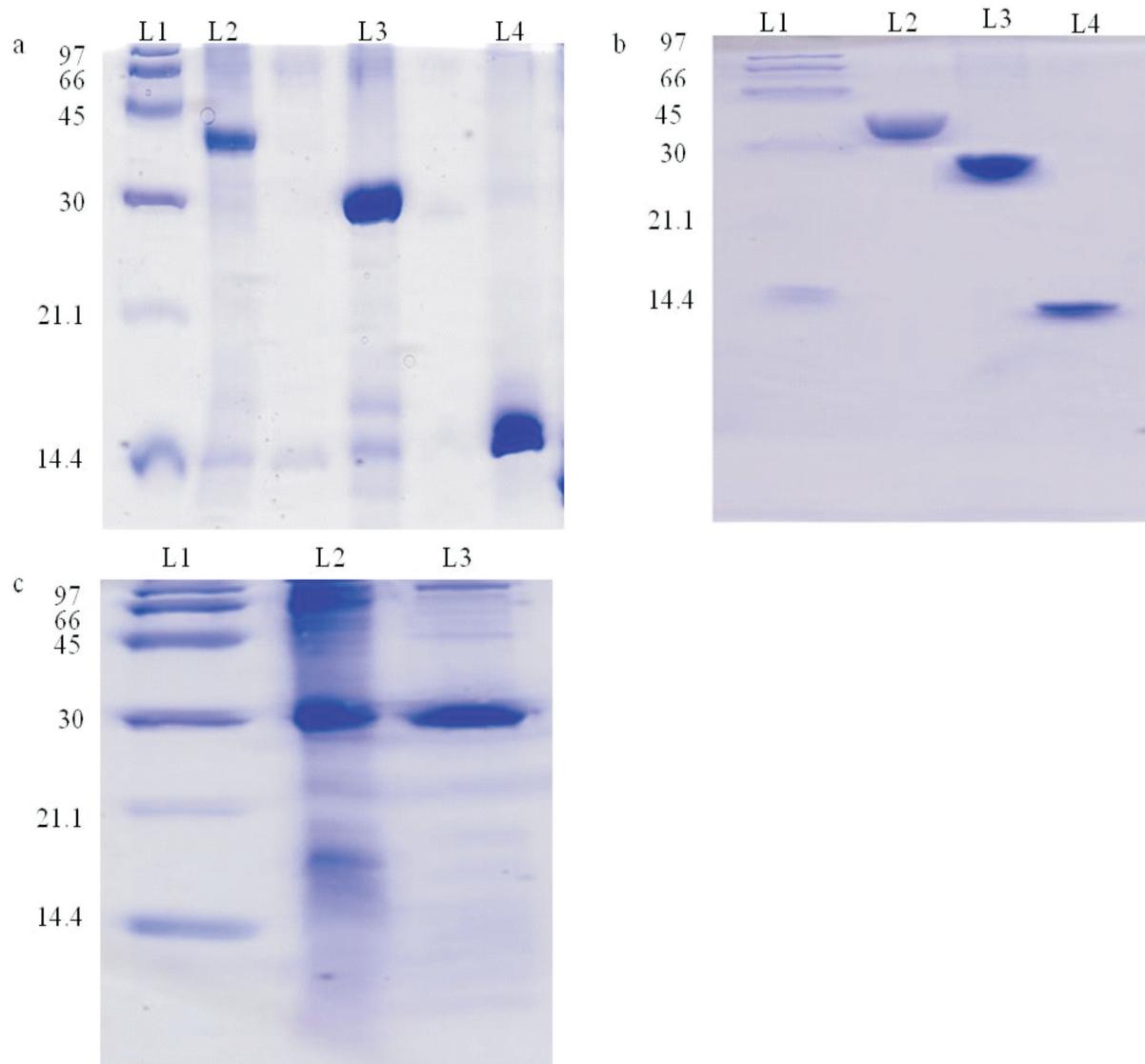


Figure 3: SDS-PAGE profile of expressed and purified NtpC, NtpD, NtpG subunits and TEV protease proteins.

(a) SDS-PAGE profile of expressed NtpC, NtpD and NtpG subunits proteins. L1, L2, L3, and L4 indicate LMW marker, expressed NtpC, expressed NtpD, and expressed NtpG, respectively. Lane 1 was loaded with 2 µl marker and each sample lane was loaded with 20 µg of cell lysate and stained with CBB. (b) SDS-PAGE profile of purified NtpC, NtpD, and NtpG subunit proteins. L1, L2, L3, and L4 indicate LMW marker, purified NtpC, purified NtpD, and purified NtpG, respectively. Lane 1 was loaded with 2 µl marker and each sample lane was loaded with 5 µg of purified protein and stained with CBB. (c) SDS-PAGE profile of expressed and purified TEV-protease. L1, L2, and L3 indicate LMW marker, expressed TEV-Protease, and purified TEV-protease, respectively. Lane 1 was loaded with 2 µl marker, L2 was loaded with 20 µg of cell lysate and L3 was loaded with 5 µg of purified TEV-protease and stained with CBB. The molecular weights (kDa) of the standard proteins in the marker (L1) are indicated in the gel.

His-tagged portion was removed successfully from His-tagged NtpC, His-tagged NtpD, and His-tagged NtpG by TEV-protease treatment at 4 °C for 12 hours at the ratio of 25:1. Concentrations of non-tagged NtpC, NtpD, and NtpG were 6.0 mg/ml, 7.2 mg/ml, and 6.8 mg/ml, respectively. In this assay only one kind of subunit was attached with Ni Sepharose 6 Fast Flow through its His-tagged portion. Non-tagged NtpD interacted with His-

tagged NtpG in pulldown assay and formed NtpD-G complex as assessed by SDS-PAGE showed two specific bands for NtpD and His-tagged NtpG in elution fraction (Figure 4a). However, non-tagged NtpC could not bind to His-tagged NtpG in this assay. Non-tagged NtpC was eluted with flow-through fraction and His-tagged NtpG was eluted with elution fraction as assessed by SDS-PAGE (Figure 4b).

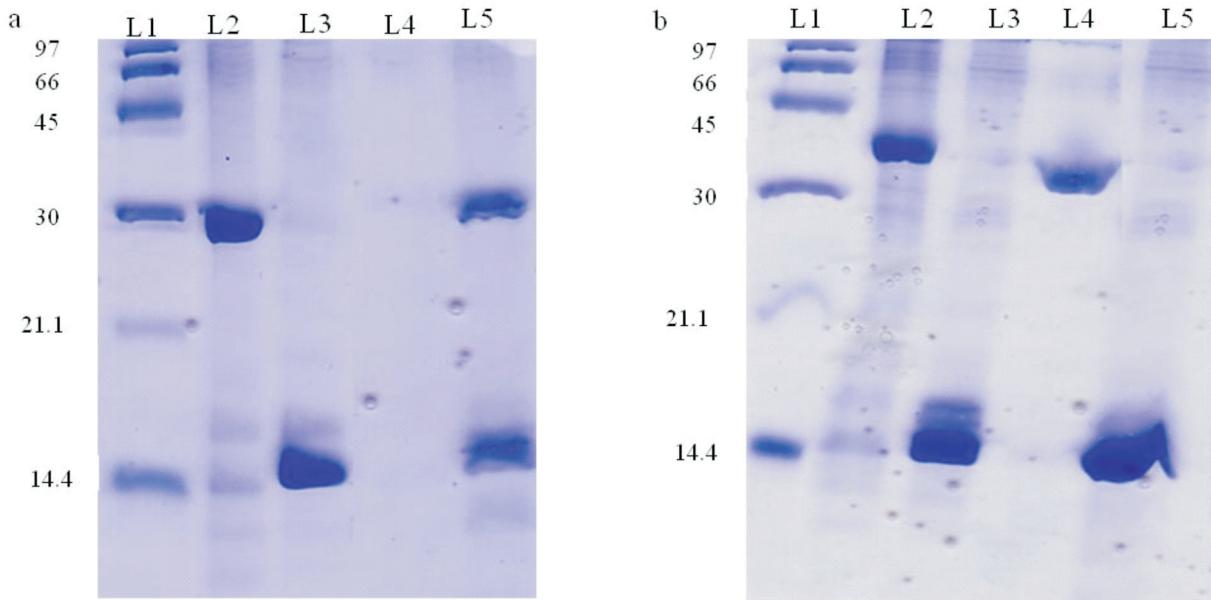


Figure 4: Subunit-subunit interaction by pulldown experiment.

(a) SDS-PAGE pattern of the flow-through fraction and elution fraction of NtpD and His-tagged NtpG mixture is shown. L1, LMW marker; L2, NtpD as a control; L3, His-tagged NtpG as a control; L4, flow-through fraction, and L5, elution fraction. L2 and L3 were loaded with 5 µg of purified NtpD and His-tagged NtpG, respectively. L4 and L5 were loaded with 20 µl of flow-through fraction and elution fraction, respectively. (b) SDS-PAGE pattern of the flow-through fraction and elution fraction of NtpC and His-tagged NtpG mixture is shown. L1, LMW marker; L2, NtpC as a control; L3, His-tagged NtpG as a control; L4, flow-through fraction, and L5, elution fraction. L2 and L3 were loaded with 5 µg of purified NtpC and His-tagged NtpG, respectively. L4 and L5 were loaded with 20 µl of flow-through fraction and elution fraction, respectively. The molecular weights (kDa) of the standard proteins in the marker (L1) are indicated in the gel.

Stable interactions of NtpC, NtpD, and NtpG subunits demonstrated by analytical gel-filtration assay

Protein-protein interaction of these above proteins was also examined with gel-filtration analysis. NtpC and NtpG did not show interaction in the gel-filtration assay as assessed by elution profile and confirmed by checking these eluted fractions by SDS-PAGE on 15% gel with silver stain (Figure 5). Whereas, NtpA₃-B₃-D-G and NtpC showed stable interaction in this assay as assessed by elution profile (a clear peak shift can be seen for NtpC) and confirmed by checking eluted fractions corresponding to NtpA₃-B₃-D-G by SDS-PAGE on 15% gel with silver stain (Figure 6). As the control experiment, NtpA₃-B₃-D and NtpC did not show interaction in the gel-filtration assay as assessed by elution profile and confirmed by checking these eluted fractions by SDS-PAGE on 15% gel with silver stain (Figure 7). These results indicated that NtpC have the stable interaction with NtpA₃-B₃-D-G probably through NtpD-G complex because it could not interact with NtpA₃-B₃-D or NtpG alone.

Discussion

It was expected that central stalk subunits of Na⁺-translocating V-type ATPase of *E. hirae* play an important role in its enzymatic activities. The rotational mechanism of the central stalk of V-ATPase still remains unknown. To study the structure and mechanism of V-ATPase, it was essential to clone, express, and purify all subunits genes of Na⁺-translocating V-type ATPase from *E. hirae*. Here, the central stalk subunit genes of Na⁺-translocating V-type ATPase from *E. hirae* were amplified by PCR using suitable primer pairs. The concentrations of purified plasmid DNAs were high enough for restriction enzyme digestion. Amplified DNAs were successfully digested with suitable restriction endonucleases and ligated with pET23d vector DNA. Recombinant DNAs were transformed in *E. coli* JM109 cells and grown well in LB agar plate containing ampicillin for cloning purposes. The number of transformed cells on LB agar plate containing ampicillin indicated that ligation and transformations were successful.

Different expression systems have been developed for expression of proteins such as *E. coli*

prokaryotic system, yeast expression system; insect and mammalian cell expression systems. Each of these systems has own advantages and disadvantages (Sambrook and Russell, 2001). It has been shown that *E. coli* system is a very rapid, inexpensive, and efficient for the production of recombinant genes (Li *et al.*, 2008). So, *E. coli* could be a suitable expression system for the expression of all different subunit proteins of V-type ATPase from *E. hirae*. It has been reported that the expression of NtpD alone was unstable and did not

give high yield (Arai *et al.*, 2009). Here, we succeeded in expression of NtpC and NtpG proteins at 30 °C and NtpD protein at 16 °C. From the present study it is concluded that central stalk subunits of Na⁺-translocating V-type ATPase from *E. hirae* can be expressed in *E. coli* BL21(DE3) cells and purified by affinity chromatography as well as gel-filtration method. Furthermore, the expression and purification of TEV-protease was also successful. The purification and concentration of TEV-protease was good enough for the removal

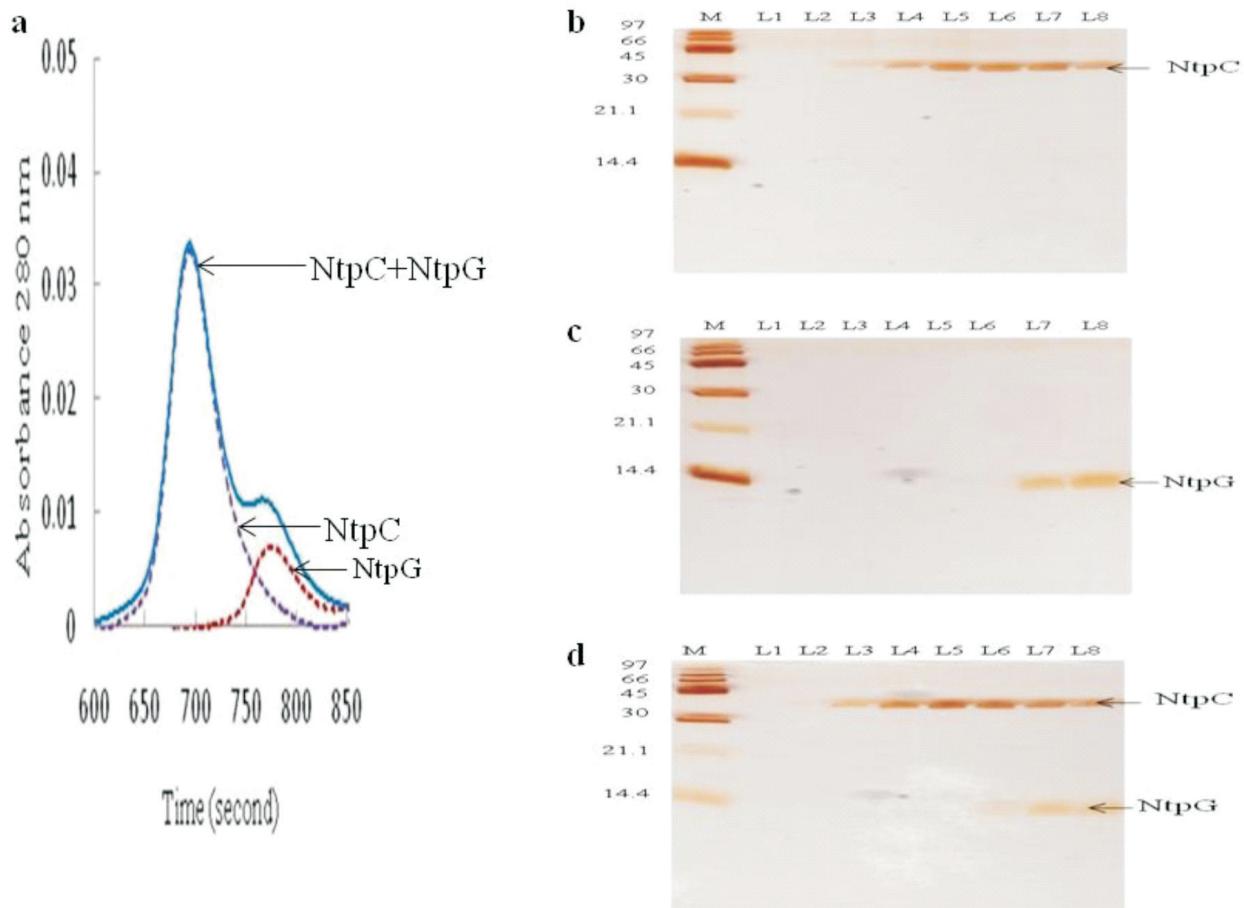


Figure 5: Complex formation of NtpC as assayed with gel-filtration.

(a) Gel-filtration elution profiles of isolated NtpC (violet colored dotted line) and NtpG (pink colored dotted line) as well as premixed NtpC and NtpG (blue colored continuous line) on a Superdex 200 5/150 GL column. Protein concentration is represented as absorbance 280 nm and abscissa is shown by time of elution. Fifty µg of NtpC was mixed with 15 µg of NtpG (1:1 molar ratio) and subjected to gel-filtration under the same condition for control experiments using individual components. Proteins were eluted as double peaks in the order of molecular mass (NtpC 38 kDa and NtpG 11 kDa). No complex formation between NtpC and NtpG was observed because the two components eluted as separate entities. Chromatography of isolated NtpC and NtpG were shown for comparison. (b) Silver stained 15% SDS-PAGE pattern of peak fractions corresponding to NtpC molecular size. (c) Silver stained 15% SDS-PAGE pattern of peak fractions corresponding to NtpG molecular size. (d) Silver stained 15% SDS-PAGE pattern of peak fractions corresponding to NtpC and NtpG molecular sizes. M indicates low molecular weight marker and L1 to L8 indicate corresponding elution time from 650 sec to 810 sec with 20 sec interval. Each lane was loaded with 20 µl of eluted fraction. The molecular weights (kDa) of the standard proteins in the marker (L1) are indicated in the gel.

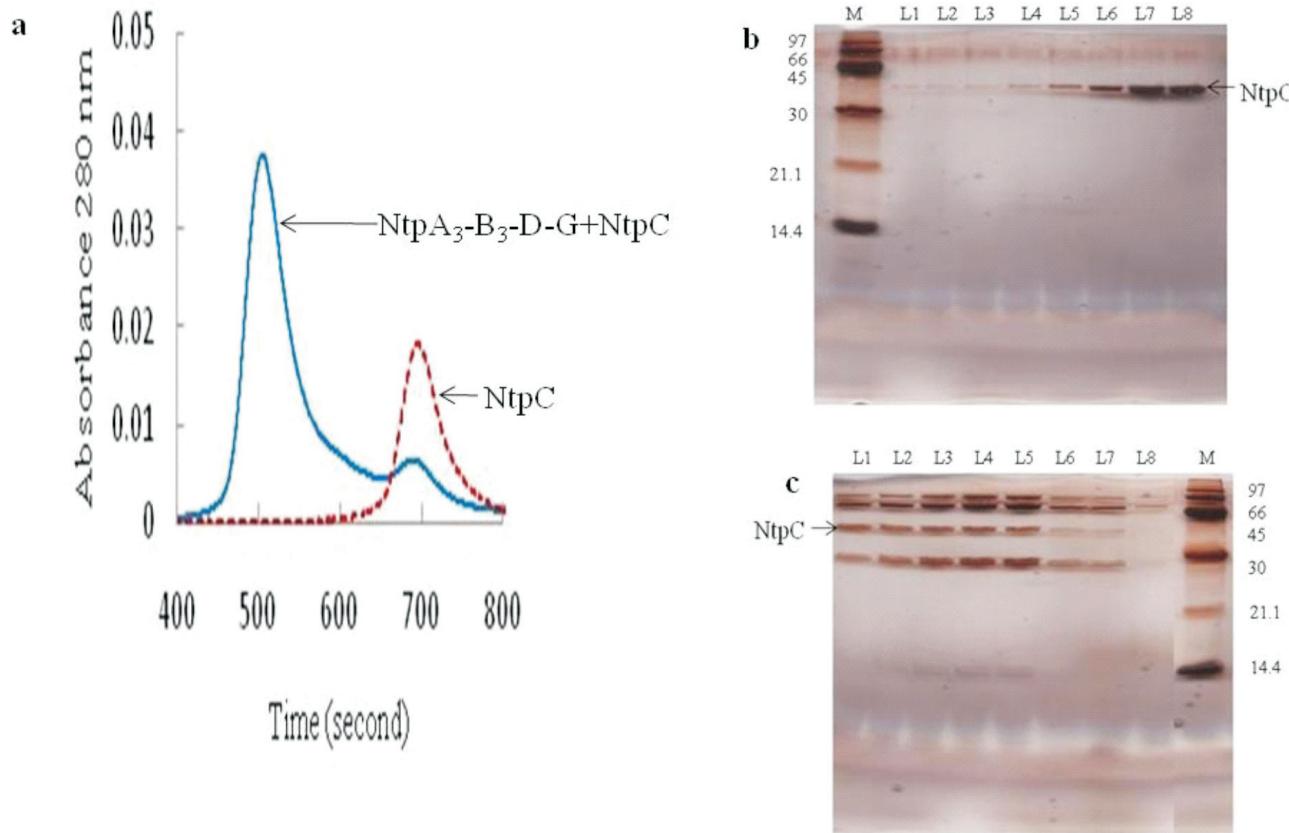


Figure 6: Complex formation of NtpC as assayed with gel-filtration.

(a) Gel-filtration elution profiles of isolated NtpC (pink colored dotted line) and premixed NtpA₃-B₃-D-G and NtpC (blue colored continuous line) on a Superdex 200 5/150 GL column. Protein concentration is represented as absorbance 280 nm and abscissa is shown by time of elution. Fifty µg of NtpA₃-B₃-D-G was mixed with 15 µg of NtpC (1:3 molar ratio) and subjected to gel-filtration under the same condition for control experiment using individual component. Chromatography of NtpC alone was shown for comparison. (b) Silver stained 15% SDS-PAGE pattern of peak fraction corresponding to NtpC molecular size. (c) Silver stained 15% SDS-PAGE pattern of peak fractions corresponding to NtpA₃-B₃-D-G and NtpC molecular sizes. M indicates low molecular weight marker and L1 to L8 indicate corresponding elution time from 450 sec to 810 sec with 45 sec interval. Each lane was loaded with 20 µl of eluted fraction. The molecular weights (kDa) of the standard proteins in the marker are indicated in the gel.

of His-tagged portion from these His-tagged subunit proteins.

The NtpD was shown to interact with NtpG directly by pulldown assay and these two subunits formed NtpD-G complex. However, NtpD or NtpG alone could not form complex with NtpC in pulldown and gel-filtration assay. From these findings, it was predicted that NtpD has binding affinity for NtpG but NtpD or NtpG alone has no binding affinity for NtpC. Gel-filtration profile of NtpD-G complex and NtpC mixture would be complicated because of the similar molecular sizes. Therefore, we did not perform the gel-filtration assay of NtpD-G complex with NtpC to check the complex formation. NtpC was shown to interact with NtpA₃-B₃-D-G but not with NtpA₃-B₃-D. Thus NtpC must

have interacted with NtpA₃-B₃-D-G through NtpD-G. Purified NtpD interacted with NtpA₃-B₃ and formed NtpA₃-B₃-D complex as reported previously by Arai *et al.* (2009) but NtpC or NtpG alone could not bind to NtpA₃-B₃. Therefore, it is probable that reconstituted NtpA₃-B₃ binds NtpD, then NtpG binds to NtpD in the complex, and finally that of NtpC binds to NtpD-G in the NtpA₃-B₃-D-G complex forming the V₁ with central stalk.

Acknowledgements

This work was supported by Targeted Protein Research Program (T. M.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), and by Chiba University Young Research-Oriented Faculty Member Development Program in Natural Science Areas (T. M.) from MEXT. The Japanese agencies are highly acknowledged.

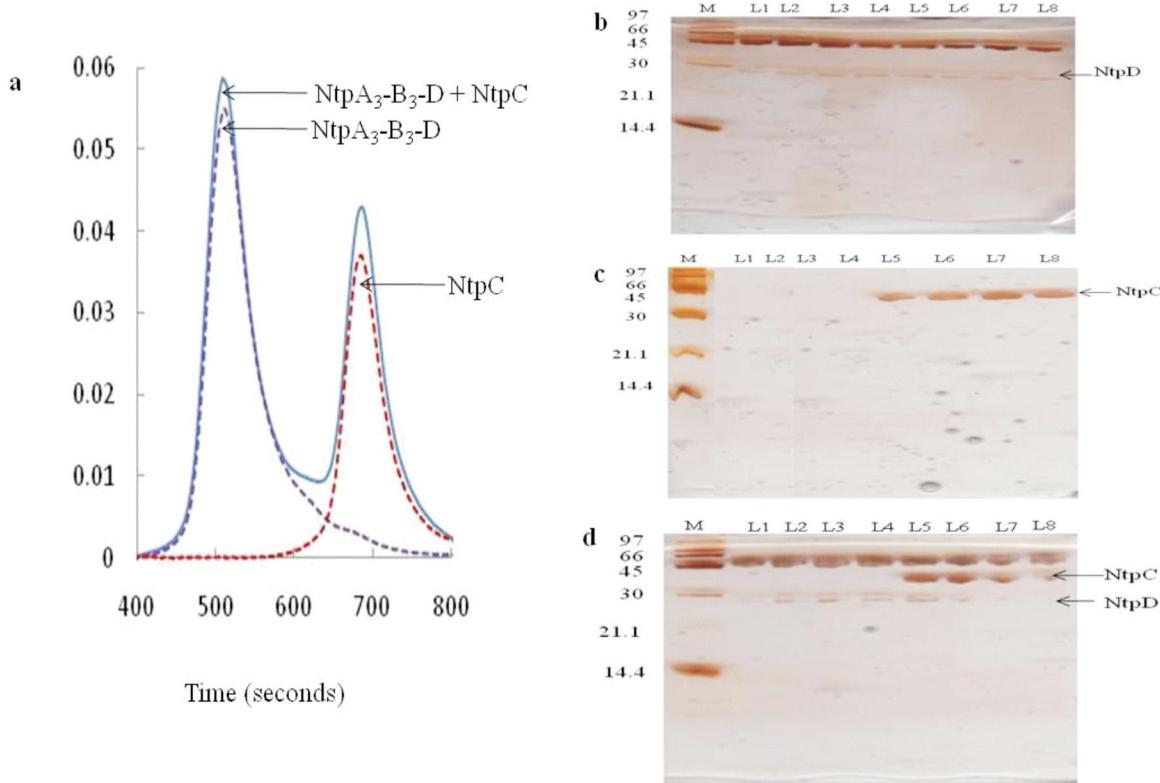


Figure 7: Complex formation of NtpC as assayed with gel-filtration.

(a) Gel-filtration elution profiles of isolated NtpA₃-B₃-D (violet colored dotted line) and NtpC (pink colored dotted line) as well as premixed NtpA₃-B₃-D and NtpC (blue colored continuous line) on a Superdex 200 5/150 GL column. Protein concentration is represented as absorbance 280 nm and absicca is shown by time of elution. Sixty µg of NtpA₃-B₃-D was mixed with 30 µg of NtpC (1:5 molar ratio) and subjected to gel-filtration under the same condition for control experiments using individual components. Proteins were eluted as double peaks in the order of molecular mass (NtpA₃-B₃-D 375 kDa and NtpC 38 kDa). No complex formation between NtpA₃-B₃-D and NtpC was observed because the two components eluted as separate entities. Chromatography of individual NtpA₃-B₃-D alone and NtpC alone were shown for comparison. (b) Silver stained 15% SDS-PAGE pattern of peak fractions corresponding to NtpA₃-B₃-D molecular size. (c) Silver stained 15% SDS-PAGE pattern of peak fractions corresponding to NtpC molecular size. (d) Silver stained 15% SDS-PAGE pattern of peak fractions corresponding to NtpA₃-B₃-D and NtpC molecular sizes. M indicates low molecular weight marker and L1 to L8 indicate corresponding elution time from 450 sec to 810 sec with 45 sec interval. Each lane was loaded with 20 µl of eluted fraction. The molecular weights (kDa) of the standard proteins in the marker are indicated in the gel.

Conflict of interest

There is no conflict of interest.

Abbreviations

PCR, polymerase chain reaction; DNA, deoxyribonucleic acid; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; ATP, adenosine 5' triphosphate; dNTP, deoxynucleotide triphosphate; IPTG, isopropyl (thio) β -D-galactoside; BSA, bovine serum albumin; DTT, dithiothreitol; CBB, Coomassie brilliant blue; EDTA, ethylenediaminetetraacetic acid; LB, Luria-Bertani; m-DMCA, modified-Davis Mingoli-casamino acid; OD600, optical density at 600 nm.

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