African Journal of Biotechnology Vol. 10(13), pp. 2406-2413, 28 March, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2126
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Expression and purification of the central stalk subunits of Na^+-translocating V-type ATPase from *Enterococcus hirae*

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Accepted 09 December, 2010

***Enterococcus hirae* (*E. hirae*) vacuolar ATPase (V-ATPase) is composed of a soluble catalytic domain (V_1; $NtpA_3$-B_3-D-G) and an integral membrane domain (V_o; NtpI-K_{10}) connected by a central and peripheral stalks. Central stalk of Na^+-translocating V-type ATPase of *E. hirae* is composed of NtpC, NtpD and NtpG subunits. The aim of the present study was cloning and expression of these central stalk subunits of *E. hirae* V-type Na^+-ATPase. Here we cloned the synthesized DNA fragments, corresponding to *ntpC*, *ntpD* and *ntpG* genes, into the plasmid vector, pET23d. NtpC, NtpD and NtpG subunit proteins were expressed, separately as His-tagged soluble proteins in *Escherichia coli* BL21(DE3) cells and then, purified by Ni Sepharose 6 fast flow column. Purification of expressed protein was confirmed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). The amount of purified NtpC, NtpD and NtpG subunit proteins were measured as 14, 17 and 15 mg/1 liter culture, respectively.**

Key words: *Enterococcus hirae*, V-ATPase, central stalk subunits, expression.

INTRODUCTION

Vacuolar ATPases (V-ATPases) function as ATP-dependent proton pumps in acidic organelles and in plasma membranes of eukaryotic cells (Forgac, 2007). This acidification is involved in concentration of neurotransmitters, processing of secretory proteins, endocytosis and other important cellular processes (Forgac, 2007). V-ATPases have 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

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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-DM-CA,** modified-Davis Mingioli-casamino acid; **OD600,** optical density at 600 nm.

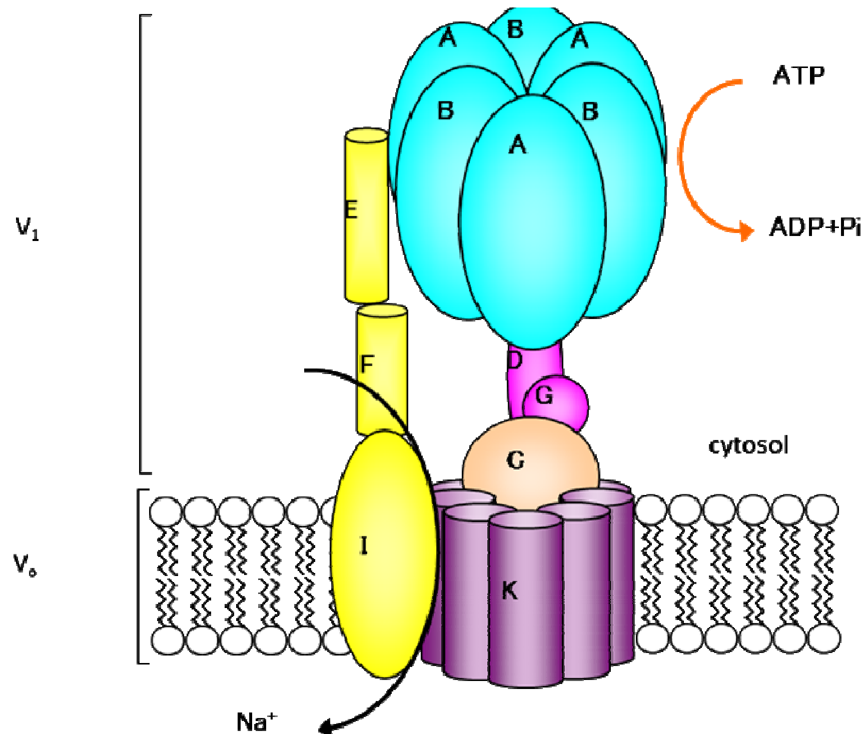

Figure 1. Structural model of V-ATPase from *E. hirae*. (V_1 indicates catalytic domain and V_o indicates membrane domain; Murata et al., 2008).

of some bacteria (Lolkema et al., 2003; Yokoyama and Imamura, 2005; Murata et al., 2005a). 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). *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., 2005b), 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 $NtpA_3$-B_3-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). $NtpA_3$-B_3-D-G complex and V_o moiety are connected by a central stalk subunit NtpC of V_1 (Murata et al., 2005b). The molecular weights of NtpC, NtpD and NtpG subunits are 38, 27 and 11 kDa, respectively, though their structural arrangement is 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 (Murata et al., 2005a). 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 pre-requisite and essential to express and purify different subunits of Na^+-translocating V-type ATPase from *E. hirae*. In this study, we expressed NtpC, NtpD and NtpG subunit proteins individually in *Escherichia coli* BL21 (DE3) cells by isopropyl (thio) β-D-galactoside (IPTG) induction and purified by Ni Sepharose 6 fast flow column.

MATERIALS AND METHODS

Cloning and sequencing of the central stalk subunit genes

Synthesized DNA fragments corresponding to *ntpC*, *ntpD* or *ntpG* gene having optimal codon usage for *E. coli* expression system was collected from the Takara Co. Ltd., Japan. The DNA fragments of *ntpC*, *ntpD* and *ntpG* genes were 984, 630 and 309 bp, respectively (Takase et al., 1994). The plasmid vector pET23d was digested by *Sty*I and *Dra*III restriction enzymes separately to clone *ntpC*, *ntpD* and *ntpG* genes, respectively. All recombinant processing (DNAs digestion by restriction endonucleases, T4-DNA ligase, plasmid preparation and growth of bacterial cultures) were performed, for each previously mentioned gene, according to Sambrook and Russell (2001) so that we can generate pET23d-HisNtpC, pET23-HisNtpD and pET23-HisNtpG. Recombinant DNAs were transformed into *E. coli* JM109 competent cells and grown on Luria-Bertani (LB) agar plates containing ampicillin (50 µg/ml) for cloning purposes. Recombinant plasmids were purified from *E. coli* JM 109 cells separately using a 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 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 (Figure 2). Positive clones of *ntpC*, *ntpD* and *ntpG* genes were preserved separately at -80°C with 10% glycerol as constructs for expression of NtpC, NtpD and NtpG subunit proteins.

Expression of the central stalk subunit proteins

Cloned *ntpC*, *ntpD* and *ntpG* genes were transformed separately into *E. coli* BL21 (DE3) cells. *E. coli* BL21(DE3) cells containing *ntpC*, *ntpD* or *ntpG* genes were inoculated separately into one liter modified-Davis Mingioli-casamino acid (m-DM-CA) culture medium (3.4 gm KH_2PO_4, 7.3 gm Tris, 0.5 gm Na-citrate, 0.01% (w/v) $MgSO_4$, 0.4%(w/v) Bacto casamino acid (Difco, Technical), 0.4% lactate, (pH 7.4) containing ampicillin (50 µg/ml)) (Mogi and Anraku, 1984). Bacterial cultures were incubated at 30°C for *ntpC* and *ntpG* genes for 12 h and at 20°C for *ntpD* gene for 18 h with shaking at 100 rpm. After the OD_{600} reached 0.4 to 0.6, IPTG was added at the concentration of 0.2 mM and the cultures were incubated at the same temperature for further 6 h for *ntpC* and *ntpG* genes and 8 h for *ntpD* gene. *E. coli* BL21(DE3) cells were harvested separately by centrifugation at 6,000 rpm for 10 min at 4°C. Cells were suspended separately in 30 ml of buffer A (50 mM Tris-HCl (pH 8.5), 10 mM EDTA) and suspensions were centrifuged separately at 3,000 rpm for 30 min at 4°C. Collected cells were suspended again separately in 30 ml of buffer B (50 mM Tris-HCl (pH 8.5), 1 mM EDTA, 20% sucrose) and treated with lysozyme (100 µg/ml) to obtain spheroplasts. Sphero-plasts were resuspended separately 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 3 min at 4°C). NtpC, NtpD and NtpG polypeptides were recovered sepa-rately in the supernatant cell lysate after centrifugation at 15,000 rpm for 10 min at 4°C.

Purification of NtpC, NtpD and NtpG subunit proteins

Total cell lysates of different subunits were added separately to 5 ml bed volume of Ni Sepharose 6 fast flow (GE Healthcare) equilibrated with buffer D (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 h. Supernatants were removed separately by aspiration after sedimentation; resins were resuspended separately in 10 ml of buffer D and transferred separately into 20 ml chromatography columns (Bio-Rad). Flow through fractions were discarded and bound proteins were eluted separately (sequentially) with 25 ml buffer E (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 separately to 1 ml volume by ultrafiltration with Amicon ultra centrifugal filter devices (pore size; MW 10 kDa for NtpC and NtpD and 3 kDa for NtpG). Protein concentrations were measured according to the 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 (20.1 kDa) and α-lactalbumin (14.4 kDa) (low molecular weight marker, GE Healthcare). All eluted and purified proteins were analyzed by SDS-PAGE on 15% gel and subsequently stained with Coomassie brilliant blue (CBB).

RESULTS AND DISCUSSION

Cloning and sequencing of the central stalk subunit genes

Synthesized DNA fragments corresponding to *ntpC*, *ntpD* and *ntpG* genes were successfully ligated separately to pET23d vectors. Cloning was verified by PCR and restriction digestion analysis. The transformed cells (JM109) were grown well in LB agar plate containing ampicillin. The number of transformed cells on LB agar plate containing ampicillin indicated that ligation and transformations were successful. Sequenced data of three samples were the same with those of the original *ntpC, ntpD* and ntp*G* genes. Multiple alignments of nucleotide and amino acid sequences by CLUSTALW program confirmed the identity of clones as showed 100% homology with *ntpC*, *ntpD* and *ntpG* genes of *E. hirae* V-ATPase. In Figure 2, the alignments of nucleotide and amino acid sequences of cloned *ntpC*, *ntpD* and *ntpG* genes and their products with the sequences of those from *E. hirae* were shown.

Expression of the central stalk subunit proteins

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 its 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 proteins (Li et al., 2008). So, *E. coli* could be used as suitable expression system of different subunit proteins of V-type ATPase from *E. hirae*. *In vivo* synthesis of NtpC, NtpD and NtpG were performed as described in

a

		. . . . . .
E.hirae	1	ATGGAGTATCATGAATTAAATCCCTTGATACGTGGTAGAGAATTAGAGTTGATTTCAAAA
	1	M E Y H E L N P L I R G R E L E L I S K
optimal codon	1	ATGGAATATCACGAACTGAACCCGCTGATTCGTGGTCGTGAACTGGAACTGATCAGCAAA
	1	M E Y H E L N P L I R G R E L E L I S K
		. . . . . .
E.hirae	61	GACACGTTTGAGCAAATGATCCAAACCGATTCGATCGATTCACTTGGAGAAATCTTACAA
	21	D T F E Q M I Q T D S I D S L G E I L Q
optimal codon	61	GATACCTTCGAACAAATGATCCAGACCGATAGCATTGATAGCCTGGGCGAAATTCTGCAA
	21	D T F E Q M I Q T D S I D S L G E I L Q
		. . . . . .
E.hirae	121	TCCACGATCTATCAGCCGTATATCTATGACGGCTTTGACAAGGATTTTGAAGCCAATCTC
	41	S T I Y Q P Y I Y D G F D K D F E A N L
optimal codon	121	AGCACCATCTATCAGCCGTATATCTATGATGGCTTCGATAAAGATTTCGAAGCGAACCTG
	41	S T I Y Q P Y I Y D G F D K D F E A N L
		. . . . . .
E.hirae	181	TCTCAGGAACGCAGCAAATTATTCCAGTGGTTGAAAGAATCTGCACCAGAACCAGAAATC
	61	S Q E R S K L F Q W L K E S A P E P E I
optimal codon	181	AGCCAGGAACGTAGCAAACTGTTCCAGTGGCTGAAAGAAAGCGCGCCGGAACCGGAAATT
	61	S Q E R S K L F Q W L K E S A P E P E I
		. . . . . .
E.hirae	241	GTTTGGATCTATACGATGCGTTACACTTTCCATAATTTGAAAGTATTGACAAAGGCTGAG
	81	V W I Y T M R Y T F H N L K V L T K A E
optimal codon	241	GTTTGGATCTATACCATGCGCTACACCTTCCATAACCTGAAAGTGCTGACCAAAGCGGAA
	81	V W I Y T M R Y T F H N L K V L T K A E
		. . . . . .
E.hirae	301	ATCACAGGGCAAAACCTTGATCACCTTTACATCCATGATGGATTTTATTCGCTGGAAGTG
	101	I T G Q N L D H L Y I H D G F Y S L E V
optimal codon	301	ATTACCGGCCAGAACCTGGATCATCTGTATATCCACGATGGCTTCTATAGCCTGGAAGTG
	101	I T G Q N L D H L Y I H D G F Y S L E V
		. . . . . .
E.hirae	361	TTGAAAGATGCGATTCACACGCAAGTGTCGGTGGAATTGCCAGACAGTCTCATGGATTAT
	121	L K D A I H T Q V S V E L P D S L M D Y
optimal codon	361	CTGAAAGATGCGATCCATACCCAGGTTAGCGTTGAACTGCCGGATAGCCTGATGGATTAC
	121	L K D A I H T Q V S V E L P D S L M D Y
		. . . . . .
E.hirae	421	ATTCGAGAAGTTCATGAATACTGCGAAGAATCGACTATTTTACAAGGGATCGATGTGATT
	141	I R E V H E Y C E E S T I L Q G I D V I
optimal codon	421	ATCCGCGAAGTTCACGAATATTGCGAAGAAAGCACCATTCTGCAAGGCATCGATGTGATT
	141	I R E V H E Y C E E S T I L Q G I D V I
		. . . . . .
E.hirae	481	TATGACCGTTGTTTTCTAACCGAGCAACGTCGCTTAGGGGAACAGCTTGGTTACCCTGAA
	161	Y D R C F L T E Q R R L G E Q L G Y P E
optimal codon	481	TATGATCGCTGCTTCCTGACCGAACAGCGTCGTCTGGGTGAACAGCTGGGTTATCCGGAA
	161	Y D R C F L T E Q R R L G E Q L G Y P E
		. . . . . .
E.hirae	541	CTATTAGAAGAGATCATTGCTTTTATCGATTTAACGAATATCACCACGACAGCAAGAGGG
	181	L L E E I I A F I D L T N I T T T A R G
optimal codon	541	CTGCTGGAAGAAATTATCGCGTTCATCGATCTGACCAACATTACCACCACCGCGCGTGGT
	181	L L E E I I A F I D L T N I T T T A R G
		. . . . . .
E.hirae	601	ATCTTGCAGCATCGTTCTGCAGGTTTTATGACAACAGTTATTTCAAGTTCAGGAAGTATT
	201	I L Q H R S A G F M T T V I S S S G S I
optimal codon	601	ATTCTGCAACATCGTAGCGCGGGTTTTATGACCACCGTGATTAGCAGCAGCGGTAGCATT
	201	I L Q H R S A G F M T T V I S S S G S I
		. . . . . .
E.hirae	661	CCGAAAGACACATTGCTTTCCTTTGTTCGTGGGGAAATGGCATCTTTTACTCAGTTTTTA
	221	P K D T L L S F V R G E M A S F T Q F L
optimal codon	661	CCGAAAGATACCCTGCTGTCTTTTGTTCGTGGTGAAATGGCGAGCTTTACCCAGTTTCTG
	221	P K D T L L S F V R G E M A S F T Q F L
		. . . . . .
E.hirae	721	CTGACAACAGATTACAGTGAGCTATTAAAGCAAGTCATCCATGAAGAACAGATTGATTTA
	241	L T T D Y S E L L K Q V I H E E Q I D L
optimal codon	721	CTGACCACCGATTATAGCGAACTGCTGAAACAGGTGATCCACGAAGAACAAATCGATCTG
	241	L T T D Y S E L L K Q V I H E E Q I D L
		. . . . . .
E.hirae	781	GTTAGCTTGGAACAATTGAAAGATGATTATTTAAGTTCTTTTTATCAAGTAGCACAGACA
	261	V S L E Q L K D D Y L S S F Y Q V A Q T
optimal codon	781	GTTAGCCTGGAGCAACTGAAAGATGATTATCTGAGCAGCTTCTATCAGGTTGCGCAGACC
	261	V S L E Q L K D D Y L S S F Y Q V A Q T
		. . . . . .
E.hirae	841	CAAGCGTTTGGCCCGTTACCATTACTAGCTTTTTTGAACGCAAAAGAAGTCGAAAGTAAA
	281	Q A F G P L P L L A F L N A K E V E S K
optimal codon	841	CAGGCGTTTGGTCCGCTGCCGCTGCTGGCGTTTCTGAATGCGAAAGAAGTGGAAAGCAAA
	281	Q A F G P L P L L A F L N A K E V E S K
		. . . . . .
E.hirae	901	AATCTGCGCCTTTTGATCATTGGCAAACGAAATCACTTTTCACTGGAACAACTAAAAGAA
	301	N L R L L I I G K R N H F S L E Q L K E
optimal codon	901	AACCTGCGCCTGCTGATTATTGGCAAACGCAACCACTTCAGCCTGGAACAGCTGAAAGAA
	301	N L R L L I I G K R N H F S L E Q L K E
		. . .
E.hirae	961	AGGATGAGACAGGTCTATGACTTATAA
	321	R M R Q V Y D L *
optimal codon	961	CGTATGCGCCAGGTTTATGATCTGTAA
	321	R M R Q V Y D L *

b

		. . . . . .
E.hirae	1	ATGCGATTAAACGTCAATCCTACGAGAATGGAGCTAACTCGTTTAAAGAAACAATTAACG
	1	M R L N V N P T R M E L T R L K K Q L T
optimal codon	1	ATGCGTCTGAATGTGAATCCGACCCGTATGGAACTGACCCGTCTGAAAAAACAGCTGACC
	1	M R L N V N P T R M E L T R L K K Q L T
		. . . . . .
E.hirae	61	ACAGCGACAAGGGGACACAAACTTTTAAAGGACAAACAAGATGAATTGATGCGTCAATTT
	21	T A T R G H K L L K D K Q D E L M R Q F
optimal codon	61	ACCGCGACCCGTGGTCATAAACTGCTGAAAGATAAACAGGATGAACTGATGCGCCAGTTT
	21	T A T R G H K L L K D K Q D E L M R Q F
		. . . . . .
E.hirae	121	ATTTTACTGATCCGTAAAAATAATGAGTTACGCCAAGCAATAGAAAAAGAAACCCAAACA
	41	I L L I R K N N E L R Q A I E K E T Q T
optimal codon	121	ATTCTGCTGATCCGTAAAAACAACGAACTGCGCCAGGCGATTGAAAAAGAAACCCAGACC
	41	I L L I R K N N E L R Q A I E K E T Q T
		. . . . . .
E.hirae	181	GCAATGAAAGATTTTGTCTTAGCAAAGTCAACAGTCGAAGAAGCTTTTATTGACGAACTT
	61	A M K D F V L A K S T V E E A F I D E L
optimal codon	181	GCGATGAAAGATTTTGTGCTGGCGAAAAGCACCGTTGAAGAAGCGTTCATTGACGAACTG
	61	A M K D F V L A K S T V E E A F I D E L
		. . . . . .
E.hirae	241	TTGGCATTACCAGCGGAAAACGTCTCAATTTCTGTAGTTGAGAAAAATATTATGAGTGTC
	81	L A L P A E N V S I S V V E K N I M S V
optimal codon	241	CTGGCGCTGCCGGCGGAAAATGTTAGCATCAGCGTTGTGGAAAAAAACATCATGAGCGTT
	81	L A L P A E N V S I S V V E K N I M S V
		. . . . . .
E.hirae	301	AAAGTTCCCCTCATGAATTTTCAATACGATGAAACATTGAATGAGACACCATTAGAGTAT
	101	K V P L M N F Q Y D E T L N E T P L E Y
optimal codon	301	AAAGTGCCGCTGATGAACTTCCAGTACGATGAAACCCTGAATGAAACCCCGCTGGAATAT
	101	K V P L M N F Q Y D E T L N E T P L E Y
		. . . . . .
E.hirae	361	GGCTATCTTCATTCTAATGCAGAGTTGGATCGTTCGATCGATGGTTTTACGCAGCTCTTA
	121	G Y L H S N A E L D R S I D G F T Q L L
optimal codon	361	GGCTATCTGCATAGCAACGCGGAACTGGATCGTAGCATTGATGGCTTTACCCAGCTGCTG
	121	G Y L H S N A E L D R S I D G F T Q L L
		. . . . . .
E.hirae	421	CCAAAGCTTTTGAAGCTGGCAGAAGTTGAAAAAACATGTCAACTTATGGCTGAAGAGATC
	141	P K L L K L A E V E K T C Q L M A E E I
optimal codon	421	CCGAAACTGCTGAAACTGGCGGAAGTGGAAAAAACCTGTCAGCTGATGGCGGAAGAAATT
	141	P K L L K L A E V E K T C Q L M A E E I
		. . . . . .
E.hirae	481	GAGAAAACCAGAAGAAGGGTCAATGCGTTGGAATATATGACGATTCCTCAATTGGAAGAA
	161	E K T R R R V N A L E Y M T I P Q L E E
optimal codon	481	GAAAAAACCCGTCGTCGTGTGAATGCGCTGGAATATATGACCATTCCGCAGCTGGAAGAA
	161	E K T R R R V N A L E Y M T I P Q L E E
		. . . . . .
E.hirae	541	ACGATTTATTATATTAAAATGAAGCTGGAAGAAAACGAACGAGCAGAAGTAACTCGCCTG
	181	T I Y Y I K M K L E E N E R A E V T R L
optimal codon	541	ACCATCTATTATATCAAAATGAAACTGGAAGAAAATGAACGCGCGGAAGTTACCCGTCTG
	181	T I Y Y I K M K L E E N E R A E V T R L
		. . . .
E.hirae	601	ATCAAAGTGAAAAATATGGGAACAGAAGAGTAA
	201	I K V K N M G T E E *
optimal codon	601	ATCAAAGTTAAAAACATGGGCACCGAAGAATAA
	201	I K V K N M G T E E *

C

		. . . . . .
E.hirae	1	ATGACTTATAAAATCGGAGTAGTAGGTGACAAGGATTCTGTCTCGCCTTTTCGATTATTT
	1	M T Y K I G V V G D K D S V S P F R L F
optimal codon	1	ATGACCTATAAAATTGGCGTGGTGGGCGATAAAGATAGCGTTAGCCCGTTTCGTCTGTTT
	1	M T Y K I G V V G D K D S V S P F R L F
		. . . . . .
E.hirae	61	GGCTTTGATGTACAGCATGGTACGACAAAGACTGAAATAAGAAAAACAATCGATGAGATG
	21	G F D V Q H G T T K T E I R K T I D E M
optimal codon	61	GGCTTTGATGTGCAGCATGGCACCACCAAAACCGAAATCCGCAAAACCATCGATGAAATG
	21	G F D V Q H G T T K T E I R K T I D E M
		. . . . . .
E.hirae	121	GCTAAGAATGAATATGGTGTGATCTATATCACCGAACAATGTGCAAATCTGGTCCCTGAA
	41	A K N E Y G V I Y I T E Q C A N L V P E
optimal codon	121	GCGAAAAACGAATATGGCGTGATCTACATCACCGAACAGTGTGCGAATCTGGTGCCGGAA
	41	A K N E Y G V I Y I T E Q C A N L V P E
		. . . . . .
E.hirae	181	ACGATTGAGCGCTATAAAGGACAATTGACACCTGCGATCATTTTGATTCCTAGTCATCAA
	61	T I E R Y K G Q L T P A I I L I P S H Q
optimal codon	181	ACCATCGAACGTTATAAAGGTCAGCTGACCCCGGCCATTATTCTGATTCCGAGCCATCAG
	61	T I E R Y K G Q L T P A I I L I P S H Q
		. . . . . .
E.hirae	241	GGAACCCTTGGTATCGGTTTAGAAGAGATCCAAAATAGTGTGGAAAAAGCTGTTGGACAA
	81	G T L G I G L E E I Q N S V E K A V G Q
optimal codon	241	GGCACCCTGGGTATTGGTCTGGAAGAAATCCAGAACAGCGTTGAAAAAGCGGTGGGCCAG
	81	G T L G I G L E E I Q N S V E K A V G Q
		. .
E.hirae	301	AATATTTTATAA
	101	N I L *
optimal codon	301	AACATCCTGTAA
	101	N I L *

Figure 2. Nucleotide sequences and amino acid sequences of *ntpC*, *ntpD* and *ntpG genes* from *E. hirae* and cloned DNA. (a) *ntpC* (P43456), (b) *ntpD* (Arai, unpublished) and (c) *ntpG* (P43455). The nucleotides and amino acids are numbered at the left of the sequence, respectively. The replaced codons are underlined.

materials and methods. First, we tried to express *ntpC*, *ntpD* and *ntpG* genes in *E. coli* BL21(DE3) cells at 30°C. Expression was successful for *ntpC* and *ntpG*, whereas expressed *ntpD* has been precipitated as inclusion bodies at 30°C. It has been reported that, the expression of *ntpD* alone was unstable and did not give high yield (Arai et al., 2009). Finally, we could express *ntpD* gene *in vivo* by reducing the bacterial culture temperature from 30 to 20°C. First, we tried to express *ntpC*, *ntpD* and *ntpG* genes in *E. coli* BL21(DE3) cells without IPTG induction and got low level of expression. Next, we tried to express *ntpC*, *ntpD* and *ntpG* genes with IPTG induction and checked the expression level at 2 h intervals started from 0 to 10 h. NtpC and NtpG subunit proteins were expressed at the highest level as soluble proteins after 6 h of IPTG induction. Whereas, NtpD subunit protein was expressed at the highest level as soluble protein after 8 h of IPTG induction. This time variation for the highest level of expression of NtpC, NtpD and NtpG as soluble proteins after IPTG induction may be due to the variation of bacterial culture temperature. NtpC, NtpD and NtpG proteins were expressed separately as His-tagged soluble proteins in *E. coli* BL21(DE3) cells with induction of IPTG. Expressed NtpC, NtpD and NtpG proteins were confirmed as assessed by SDS-PAGE (Figure 3) before purification. Findings of our study indicated that NtpC, NtpD and NtpG subunit proteins are stably expressed in *E. coli* BL21(DE3) cells.

Purification of the central stalk subunit proteins

NtpC, NtpD and NtpG proteins were individually purified by using Ni Sepharose 6 fast flow column. Amount of purified NtpC, NtpD and NtpG proteins were 14, 17 and 15 mg/1 liter culture, respectively. Purified NtpC and NtpG proteins were stable at 4°C for long time and NtpD was not at high concentration. But at low concentration, NtpD was stably maintained at 4°C for at least one week as checked by analytical gel filtration assay. The molecular weights of these purified proteins were estimated as 38 kDa for NtpC, 27 kDa for NtpD and 11 kDa for NtpG

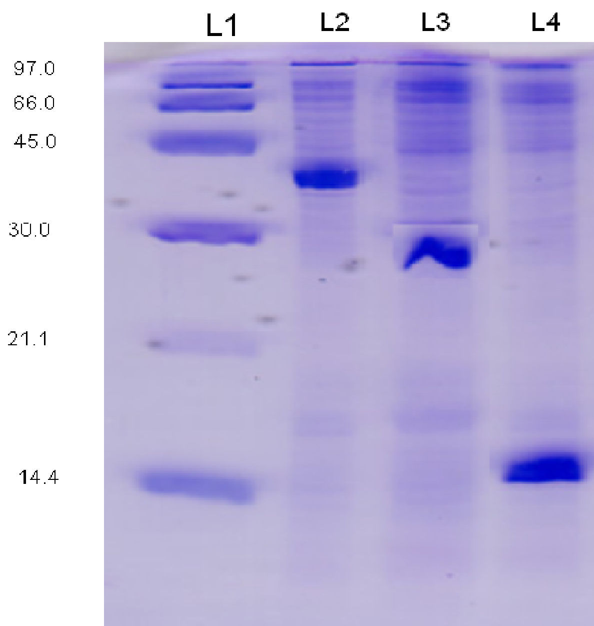

Figure 3. SDS-PAGE profile of expressed NtpC, NtpD, and NtpG subunit proteins. (L1, L2, L3 and L4 indicate LMW marker, expressed NtpC, NtpD and NtpG, respectively).

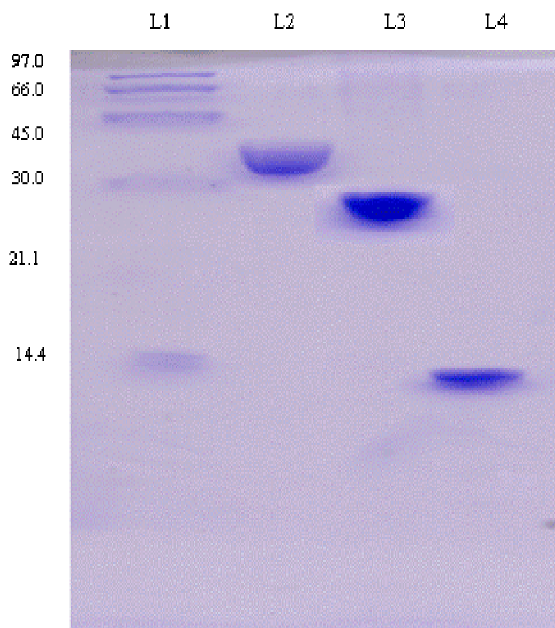

Figure 4. SDS-PAGE profile of purified NtpC, NtpD, and NtpG subunit proteins. (L1, L2, L3 and L4 indicate LMW marker, purified NtpC, NtpD and NtpG, respectively).

(Figure 4), being identical with corresponding subunits of *E. hirae* V-ATPase. From our study it was concluded that, central stalk subunits of Na^+-translocating V-type ATPase from *E. hirae* can be expressed and purified in *E. coli*.

ACKNOWLEDGEMENTS

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

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