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# Large-scale purification of the proton pumping pyrophosphatase from *Thermotoga maritima*: A "Hot-Solve" method for isolation of recombinant thermophilic membrane proteins

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**Abstract**

Although several proton-pumping pyrophosphatases ($H^+$-PPases) have been overexpressed in heterologous systems, purification of these recombinant integral membrane proteins in large amounts in order to study their structure–function relationships has proven to be a very difficult task. In this study we report a new method for large-scale production of pure and stable thermophilic $H^+$-PPase from *Thermotoga maritima*. Following overexpression in yeast, a "Hot-Solve" procedure based on high-temperature solubilization and metal-affinity chromatography was used to obtain a highly purified detergent-solubilized TVP fraction with a yield around 1.5 mg of protein per litre of yeast culture. Electron microscopy showed the monodispersity of the purified protein and single particle analysis provided the first direct evidence of a dimeric structure for $H^+$-PPases. We propose that the method developed could be useful for large-scale purification of other recombinant thermophilic membrane proteins.


*Keywords:* $H^+$-PPase; *Thermotoga maritima*; Ni-NTA affinity chromatography; Detergent solubilization; Single particle analysis

## 1. Introduction

Membrane-bound proton-translocating inorganic pyrophosphatases ($H^+$-PPases, EC 3.6.1.1) are integral membrane proteins that couple pyrophosphate (PPi) hydrolysis to $H^+$ movement across biological membranes [1]. $H^+$-PPases have been shown to occur in higher plants, parasitic protists, photosynthetic and thermophilic bacteria, archaea [2] and, more recently, in eggs and ovaries of the insect *Rhodnius prolixus* [3]. Several $H^+$-PPases have been isolated from the native membranes [4], but due to their low levels of expression the yield of purified protein has been very low. Heterologous expression in *E. coli* or *S. cerevisiae* has been repeatedly used as a tool for biochemical characterization, as both organisms lack an endogenous $H^+$-PPase [4–7]. Nevertheless, to our knowledge, no successful purification procedure has been reported so far for isolation of recombinant $H^+$-PPases. Indeed, integral membrane proteins pose particular challenges for

*Abbreviations:* PPi, inorganic pyrophosphate; $H^+$-PPase, proton-pumping pyrophosphatase; DTT, ditiothreitol; DDM, n-dodecyl-β-D-maltopyranoside; TVP, *Thermotoga maritima* membrane-bound $H^+$-PPase; PMSF, phenyl-methyl-sulphonyl-fluoride; MES, 2-morpholinoethansulfonic acid; NTA, nitriloacetic acid; Tris, Tris-(hydroxylmethyl) aminomethane; EM, electron microscopy; SOM, self-organizing mapping

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expression, purification and structural characterization, and their thermophilic counterparts are becoming the proteins of choice due to their higher stability. Still a high-throughput structural genomics project currently in process for the hyperthermophilic bacterium *Thermotoga maritima* failed to systematically express and purify integral membrane proteins [8]. Therefore, development and/or improvement of systems for the heterologous expression and isolation of large amounts of these thermophilic proteins would be of considerable interest.

The thermophilic $H^+$-PPase from *Thermotoga maritima* (TVP) was the first eubacterial $K^+$-stimulated membrane-bound pyrophosphate to be described [7]. A partial purification procedure for TVP expressed in *E. coli* inner-membrane vesicles (IMV) has been recently reported [9]. In this work, we report the purification of a hexahistidine-tagged version of TVP after overexpression in yeast, making use of the hyperthermophilic properties of the protein. To our knowledge, this is the first $H^+$-PPase purified to electrophoretical homogeneity from a heterologous source. The homogeneity of the purified detergent-solubilized protein was analyzed by electron microscopy and a single particle analysis showed a dimeric organization. This study demonstrates that TVP provides new possibilities for the functional and structural analysis of $H^+$-PPases. Moreover, the original two-step purification procedure described herein, which takes advantage of a high-temperature solubilization strategy not previously attempted, could be used for easy and fast purification of large amounts of other thermophilic membrane proteins expressed in heterologous systems.

## 2. Materials and methods

All chemicals used in this work were of analytical grade.

### 2.1. Plasmid construction

For overexpression of TVP in yeast, the corresponding full-length *vppa* gene was amplified by PCR using plasmid pTVP [7] as a template. The primers used for this amplification – TVP1: 5′-TTT**GTCGAC**ATG*CGAGGATCACA TCATCATCATCATCAT*TAGTGGCTGCTCTTTTC-3′ and TVP2: 5′-AAA**TC-TAGA**T CAGAACAGGTGAAC-3′ – contained artificial restriction sites *Xho*I and *Xba*I (bold), respectively, to allow cloning in two different multicopy plasmids: pJR1 [7], containing the *URA-3* gene and the inducible *GAL-1* promoter, and a plasmid bearing the *LEU-2* selectable marker and the strong constitutive promoter of the *PMA1* gene. The plasmids thus obtained were named pRLTVP1 and pRLTVP2, respectively. Primer TVP1 also contained a nucleotide sequence coding for the amino acid sequence $RGSH_6$ (italicized). Thus, a hexahistidine tag was introduced after the starting methionine at the N-terminal end of TVP.

### 2.2. Yeast transformation and culture

Wild type *S. cerevisiae* strain W303-1A (MATa, *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) was used as a heterologous host for overexpression. Cells were transformed using the LiOAc/PEG method [10] and selected by growth on 2% agar plates in culture medium without uracil or leucine, as convenient.

For purification trials, yeast transformants bearing plasmid pRLTVP1 were grown in selective liquid media with glucose as the sole carbon source up to the stationary phase. Aliquots of 50 ml were then washed twice with water, resuspended in 1 l of pre-warmed rich galactose medium, and allowed to grow for 16 h at 30 °C with shaking (160 r.p.m.) before collection.

For large-scale purification, yeast expressing TVP from plasmid pRLTVP2 was grown in 50 ml of selective liquid medium with glucose up to the stationary phase. Several 1-l aliquots of pre-warmed rich glucose medium were then inoculated with these cultures and incubated for 18 h at 30 °C with shaking (160 r.p.m.).

### 2.3. Crude membrane fraction isolation

Cells were harvested by centrifugation at 3000 rpm (Beckman rotor JA14) for 5 min and washed subsequently with 1/2 and 1/10 of the original culture volume with ice-cold water. The cell pellet was resuspended in 1 ml of homogenization buffer (213 mM Tris–HCl pH 7.5, 42.5 mM EDTA pH 8.0, 42.5% [v/v] glycerol, 0.5 mM PMSF, 1 mM pepstatin A, 5 mM DTT) per 2 g fresh weight.

For purification trials, four 8-ml aliquots of cell suspension were transferred to 30-ml plastic tubes and disrupted by vigorous shaking in the presence of 12 g of glass beads (ca. 0.5 mm diameter). Cell debris and glass beads were removed by centrifugation at 4000×*g* for 10 min and the supernatant was ultracentrifuged at 100,000×*g* for 40 min. The pellet was homogenized in resuspension buffer (50 mM Tris–HCl pH 7.5, 20% [v/v] glycerol, 1.34 mM DTT, 1.34 mM $MgCl_2$, 1.5 mM PMSF, 1 mM pepstatin A). Total membrane fraction samples at a concentration of 6 mg protein/ml were used directly for solubilization or frozen in liquid nitrogen and kept at −80 °C.

For large-scale purification, a total volume of 120 ml of cell suspension and 165 g of ice-cold glass beads were taken to a Bead-Beater (Biospec Products, Bartlesville, OK) breaking chamber. The chamber was filled with GTED20 buffer (10 mM Tris–HCl pH 7.6, 1 mM EDTA pH 8.0, 20% [v/v] glycerol, 1 mM DTT) and cells disrupted by shaking 10 times for 1 min under ice with a 1-min rest between shakings. Chamber contents were then transferred to 200-ml tubes and centrifuged at 4000×*g* for 10 min to separate unbroken cells and glass beads. After ultracentrifugation of the supernatant, total membranes were homogenized in a modified resuspension buffer containing 20 mM MES at pH 6.5 instead of Tris. Immediately before solubilization, samples were supplemented with $Na_2PPi$ 0.25 mM and KCl 1.34 mM.

### 2.4. Solubilization of membrane proteins

For test-solubilization of TVP from crude yeast membranes, 600 μg of total protein were solubilized with n-dodecyl-β-D-maltopyranoside (DDM) (Anatrace Inc., Maumee, OH, catalogue number D310S) at a detergent to protein ratio 3:1 (w/w). Samples were thoroughly mixed and incubated for 30 min at the desired temperature. After this time, they were taken to an air-driven ultracentrifuge (Beckman Airfuge model) in 500-μl tubes and centrifuged at 100,000×*g* for 10 min. Supernatants and pellets were collected separately and analyzed immediately or frozen in liquid nitrogen and kept at −80 °C.

For large-scale solubilization, 45-mg aliquots of total membrane protein in a volume of 30 ml were taken to 50-ml plastic tubes and heated at 75 °C for 20 min before addition of 10 ml of solubilization buffer (50 mM MES pH 6.5, 20% [v/v] glycerol, 1.35% DDM) at the same temperature. Samples were mixed by inversion and incubated at 75 °C for 2 h followed by centrifugation at 4500×*g* for 5 min. Supernatant was then transferred to fresh 50-ml tubes, placed on ice and allowed to cool for 10 min before repeating the centrifugation step. The supernatant of this last centrifugation was immediately used for Ni-NTA affinity purification of TVP.

### 2.5. Purification by Ni-NTA affinity binding chromatography

20-ml aliquots of solubilized fraction from the previous step were supplemented with 250 mM KCl and taken to 40 ml with resuspension buffer. These samples were incubated with 1 ml of Ni-NTA matrix (QIAGEN, catalogue number 30230) under gentle shaking at 40 °C for 1–2 h. Samples were cooled 10 min on ice before centrifugation at 3000×*g* for 3 min. Pellets were resuspended by gentle shaking in 10 ml ice-cold wash