Expression of a prokaryotic P-type ATPase in *E. coli* Plasma Membranes and Purification by Ni²⁺-affinity chromatography

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ABSTRACT

In order to characterize the P-type ATPase from *Synechocystis* 6803 [Geisler (1993) *et al.* J. Mol. Biol. 234, 1284] and to facilitate its purification, we expressed an N-terminal 6xHis-tagged version of the ATPase in an ATPase deficient *E. coli* strain. The expressed ATPase was immunodetected as a dominant band of about 97 kDa localized to the *E. coli* plasma membranes representing about 20-25% of the membrane protein. The purification of the *Synechocystis* 6xHis-ATPase by single-step Ni-affinity chromatography under native and denaturating conditions is described. ATPase activity and the formation of phosphointermediates verify the full function of the enzyme: the ATPase is inhibited by vanadate (IC₅₀ = 119 µM) and the formation of phosphorylated enzyme intermediates shown by acidic PAGE depends on calcium, indicating that the *Synechocystis* P-ATPase functions as a calcium pump.

ABBREVIATIONS

EP, phosphoenzyme; IPTG, isopropyl-β-D-thiogalactopyranoside; Ni-NTA, Ni²⁺-nitrilotriacetic acid; PM, plasma membrane; PMCA, plasma membrane Ca²⁺-ATPase(s); SER, sarco(endo)plasmic reticulum; SERCA, sarco(endo)plasmic reticulum Ca²⁺-ATPase(s), 6xHis, 6 x histidine affinity tag.

INTRODUCTION

The *Synechocystis* PCC 6803 P-ATPase (sll1614 (13)) belongs to the superfamily of P-type ATPases catalyzing the transport of various cations (1) and aminophospholipids (2). All the members contain one large subunit with several highly conserved domains related to ATP-binding, phosphorylation and ATP-hydrolysis. During their reaction cycle, a high energy aspartyl-phosphate intermediate (EP) appears (16), further, all of them exhibit sensitivity toward vanadate.

Structurally, the *Synechocystis* P-ATPase described in (12) belongs to the subfamily of eukaryotic Ca²⁺-
ATPases of the SER-type (12). These SERCA (16-18) differ from the Ca-ATPases of the PM-type (19-21) in a number of biochemical and functional properties. The ATPase is most similar to the \textit{Synechococcus} 7942 Ca\textsuperscript{2+}-ATPase (5). Further, the enzyme has been shown to be cotranscribed with a GTPase gene located downstream from the ATPase gene (15).

Kinetic and immunological investigations of cytoplasmic membranes from \textit{Synechocystis} 6803 have shown that this P-type ATPase is present in very small amounts. Further, the investigation of this enzyme is limited by the existence of nine P-ATPase genes revealed during the analysis of the entire genome of \textit{Synechocystis} 6803 (13). Beside the one described here, three more putative Ca\textsuperscript{2+}-ATPase genes were identified. The other five ATPase genes resemble typical prokaryotic P-ATPases; two of them show striking homologies to cadmium pumps, and one to the \textit{kdpB} subunit of the \textit{E. coli} potassium pump (14).

Therefore, we developed a heterologous expression system of the 6xHis-tagged \textit{Synechocystis} ATPase in \textit{E. coli} to prove the assumed calcium specificity of this P-ATPase. The purification of the engineered enzyme under native conditions by Ni\textsuperscript{2+}-affinity chromatography (11) after detergent solubilization is described. This method has been proven efficient for a number of soluble proteins but has not been used routinely for membrane-embedded proteins. The expressed enzyme is active both in the delipidated and the membrane-bound form as shown by phosphoenzyme formation and ATPase activity. In the original article (3) special emphasis is laid on functional differences to eukaryotic Ca\textsuperscript{2+}-ATPases. Differing from this, we describe here how despite its toxicity in prokaryotic systems, \textit{E. coli} can alternatively be used as a suitable host for the heterologous expression of members of this superfamily.

MATERIALS AND METHODS

Oligopeptide Synthesis and Antiserum Production - Rabbits were immunized s.c. with 500 µg of a 15-mer oligopeptide (amino acid residues 651-664 of the \textit{Synechocystis} enzyme (12)) coupled to keyhole limpet hemocyanin in an equal volume of Freund’s complete adjuvants (Difco Labs., Detroit). Injections were repeated after 3 weeks in Freund's incomplete adjuvants and monovalent antisera were purified by caprylic acid and ammonium sulfate precipitation.

Expression of the \textit{Synechocystis} 6803 P-ATPase in \textit{E. coli} - \textit{E. coli} strains TKR2000 (14) and M15 (Qiagen) carrying the repressor plasmid pREP4 (Qiagen) were transformed with the engineered plasmid pQE32-8 constructed as described elsewhere (3). The first 5 aa of the native enzyme (MDFPT) were, substituted by the sequence MRGSHHHHHHHRIMRYP. Large-scale expression cultures (400 or 800 ml SB (SB: 25g bacto-tryptone, 15g yeast extract, 10g KCl per liter)) supplemented with 2% glucose (w/v), ampicillin (200 µg/ml) and kanamycin (25 µg/ml) were inoculated 1:50 with mid-log precultures. At a cell density of 0.4 (550 nm), 10 µM IPTG was added; after 3 - 4 h, cells were harvested by centrifugation, washed with washing buffer (1 mM EDTA, 50 mM Tris/Cl pH 8.0) and stored frozen.

Purification of the expressed \textit{Synechocystis} 6xHis-ATPase - Cells were resuspended in glycerol-buffer (5 ml/g wet weight; 300 mM NaCl, 30% (v/v) glycerol, 1 mM β-mercaptoethanol, 10 mM Tris/Cl pH 8.0), and n-octylglucoside (46 mM) or TX-100 (1%) was added. After freezing in N\textsubscript{2} and thawing...
on ice, cells were sonicated and the expressed 6xHis-ATPase was solubilized by stirring for 1h at 0°C. After addition of Ni-NTA agarose (5 ml/g wet weight; Qiagen) and 2-5h agitation, the resin was packed into a column. For analytical chromatography, the column was washed with glycerol buffer containing 0 - 40 mM imidazole and 4.6 mM n-octylglucoside until the A280 of the flow-through was less than 0.01. The 6xHis-ATPase was eluted by a FPLC-mediated 0 - 500 mM imidazole gradient containing 0.46 mM n-octylglucoside. For preparative chromatographies, a third washing step with 60 mM imidazole was added and the protein was eluted by 5 - 10 ml of glycerol buffer containing 250 mM imidazole.

Slot-Blot and Western Blot Analysis - Aliquots of collected fractions from the Ni-chelate affinity chromatography were vacuum blotted onto nitrocellulose using the slot-blot system from Schleicher & Schuell. Total cell extracts, recombinant plasma membranes from *E. coli* TKR2000/pQE32-8 and purified *Synechocystis* 6xHis-ATPase were separated by 7.5% PAGE of the Laemmli-type (24), electroblotted to nitrocellulose and immunodetected using the Amersham ECL system.

**Phosphorylated intermediates of the *Synechocystis* Ca²⁺-ATPase** - Phosphoenzyme formation was performed in 50 µl of ice-cold medium containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl₂ with [γ³²P]ATP and the specified supplements for 20 s. Purified *Synechocystis* 6xHis-ATPase (2 µg protein) was added and after 10 min phosphorylation was terminated by the addition of 0.5 ml 10% TCA. After 15 min at 0°C, the pellets were washed once with ice-cold distilled water. For SDS-gel electrophoreses, samples were resuspended in 10 µl of Papp loading buffer (23) for 10 min at room temperature using a Hamilton syringe. Phosphorylated membranes were separated by 7.5% PAGE of the Laemmli-type (24); phosphorylated intermediates of the purified 6xHis-ATPase were run on acidic 7.5% SDS-polyacrylamide gels of the Sarkadi-type (18) at 4°C. Gels were Coomassie stained, dried and autoradiographed.

**ATP hydrolysis assays** - ATPase activity of the membrane-bound (plasma membrane vesicles from *E. coli* TKR2000/pQE32-8 (30 - 100 µg of protein)) or purified 6xHis-ATPase (2 - 7.5 µg of protein) was measured at 37°C in a volume of 1 ml. After preincubation for 10 min in hydrolysis buffer (50 mM KCl, 50 mM MgCl₂, 50 mM HEPES/KOH pH7.5) with the specified supplements the reaction was started by the addition of 2 mM ATP including [γ³²P]ATP. Aliquots of 300 µl were drawn after 5, 10 and 15 min, stopped with 1 M HClO₄ on ice and radioactivity of inorganic phosphate was measured by liquid scintillation counting. Plasma membrane vesicles from *E. coli* TKR2000/pQE32 (without ATPase insert) show ATP hydrolysis rates in the same order as the blank values (no enzyme addition); the experimental data were corrected by these blank values.

**RESULTS AND DISCUSSION**

**Expression of the *Synechocystis* 6xHis-ATPase** - Membrane proteins overexpressed in *E. coli* can be toxic for the host cells (25), most likely due to the association of the protein with or incorporation into vital membrane systems (26). To control expression strictly, we used the expression vector pQE32 in combination with the repressor plasmid pREP4 (Qiagen). The expression vector contains two lac operator elements which permits in combination with high levels of lac repressor provided from the pREP4 plasmid, some control over the expression on the transcriptional level. For cloning, *E. coli* XL1 Blue (Stratagene)—harboring the laq⁹ mutation and therefore producing enough lac repressor—was
suitable. But this strain was less efficient for expression most probably due to its leaky transcription before induction. For the production of higher yields and subsequent purification, the strain M15[pREP4] (Qiagen) was used.

Further, it became obvious that for reasonable expression levels, freshly transformed cells, mid-log precultures, and high levels of ampicillin (200 µg/ml) were essential to maintain construct stability. Highest production of heterologous protein was obtained using the rich SB medium supplemented with 2% glucose to maximize repression of expression and early inductions at an A550 of 0.4. Various IPTG concentrations were tested; mild induction of E. coli M15/pQE32-8 using 10 µM concentrations of IPTG leading to only partial clearing of the promoter turned out to be a good compromise between toxicity and reasonable expression yields.

Fig. 1: Expression of the *Synechocystis* 6xHis-ATPase in *E. coli*. (A) *E. coli* TKR2000/pQE32-8 was grown in SB medium, and total cell extracts were taken before (lane 0) and 1, 2, 3 and 4h (lane 1 - 4) after induction with 10 µM IPTG. (B) Cells were harvested after 3h, lysed by sonication and cellular debris was removed by centrifugation at 10,000 x g (P1). The supernatant (S1) was centrifuged at 100,000 x g and the pellet was resuspended and washed by centrifugation as before (supernatant = S2, pellet = P2). (C) Aliquots of fractions 12, 16 and 18 obtained from an analytical FPLC-mediated purification (see Fig. 2) were taken for separation. All samples were subjected to 7.5% PAGE, blotted onto nitrocellulose and immunostained with antisera raised against the synthesized peptide.
The *Synechocystis* P-ATPase was immunodetected as a dominant band of about 97 kDa by immunoblotting of total cell extracts from TKR2000/pQE32-8 cells (Fig. 1A lane 1). Most of the recombinant ATPase was found in the membrane fractions (Fig. 1B, lane P2) while only small amounts were found to be soluble (lane S1 and S2). Using the genetically engineered strain *E. coli* TKR2000 (14) lacking the ATPase activities of both the plasma membrane-bound K⁺-ATPase of the P-type (ΔkdpABCDE81(9)) and the F-ATPase (ΔIBEFHA) we were able to use recombinant plasma membranes directly for the investigation of the membrane-bound enzyme (see *ATP hydrolysis assay*). The yield of total plasma membrane protein obtained was up to 5 mg from 1g (wet weight) of cells. Immunological quantitation using isolated 6xHis-ATPase as a standard revealed that the content of the *Synechocystis* ATPase on the total plasma membrane protein was between 20 and 25%.

**Purification of the *Synechocystis* 6xHis-ATPase by Ni-affinity Chromatography** - After solubilization of plasma membrane proteins of transformed *E. coli* by non-ionic detergents, the 6xHis-tagged ATPase was purified by Ni-chelate affinity chromatography (11). Beside TX-100 used under denaturing conditions, n-octylglucoside turned out to be more efficient than dodecyl-maltoside or Tween-20. To avoid elution of contaminating proteins due to disulfide cross-linkages, hydrophobic or ionic interactions, 1 mM β-mercaptoethanol, 30% glycerol and 300 mM NaCl were added. Binding in a batch procedure and subsequent packing of the resin-protein complex into a column seemed to be much more efficient compared to the column procedure. To receive higher yields, the binding time was elongated up to 4h.
In analytical preparations, most of the 6xHis-ATPase is eluted between 70 and 200 mM by a linear 0 - 500 mM FPLC-mediated purification imidazole gradient (see Fig.2). For large-scale preparations, a step gradient was applied with a third extensive washing step containing 60 mM imidazole and an elution step with 250 mM imidazole yielding in 300 µg of highly purified ATPase protein (from 1g (wet weight) of cells).

Under denaturating conditions (8 M Urea/ 1% TX-100) the yield was three-fold higher due to a more efficient solubilization and binding of the 6xHis-ATPase to the affinity matrix. Efficient purification of the engineered ATPase under native and denaturative conditions show that the N-terminal His-tag of the ATPase is exposed and easy accessible for the Ni-NTA agarose.

**Phosphoenzyme formation of the Synechocystis 6xHis-ATPase** - To investigate whether the expressed enzyme is active, the isolated enzyme was exposed to [$\gamma^3$P]-ATP under different conditions in order to form phosphorylated enzyme intermediates (E1P (16)). For phospho-intermediate separation, LiDS-PAGE at pH 2.4 (6), acidic SDS-PAGE of the Sarkadi-type (18) and PAGE of the Laemmli-type (24) were tested. While LiDS-PAGE resulted in only limited separation, acidic SDS-PAGE at an actual pH of 5.5-7 (18) resembled a good compromise between separation and conservation of the acyl-phosphate linkage. However, Laemmli gels at an actual pH of 9.5 (18) can also be used to separate phosphorylated intermediates; loss of radiolabelling could be compensated by longer exposition times.

![Fig. 2: Ni-chelate affinity chromatography of the expressed Synechocystis 6xHis-ATPase.](image)
In the presence of $[\gamma^3P]$ATP, the ATPase forms a 97 kDa phosphointermediate (Fig. 3, lane 1) which is strongly calcium dependent: chelation of calcium by EGTA (5 mM) reduces phosphorylation strongly (Fig. 3, lane 2) supporting the assumption that the enzyme acts as a Ca$^{2+}$-ATPase in vivo. The phosphointermediate is highly sensitive to higher temperatures and to hydroxylamine (see Fig. 2B in the original article) indicating an acylphosphate linkage.

Furthermore, E$_1$P formation is strongly enhanced by addition of the dephosphorylation blocking La$^{3+}$ (Fig. 3, lane 3) increasing the steady state concentration of phosphorylated intermediate (18). The stabilization by lanthanum has been described so far only for mammalian PM Ca$^{2+}$-ATPases (18) and the SERCA3 isoform of rat kidney (23).

**ATP hydrolysis by the Synechocystis 6xHis-ATPase**

Determination of ATPase activity showed that the isolated as well as the membrane-bound *Synechocystis* 6xHis-ATPase is active. In the presence of calcium, the ATPase activity of membrane vesicles show a linear rate of 3.16 ± 0.30 µmol/h x mg of protein; the rates of the isolated enzyme are about ten times higher. Considering an inside-out orientation of membrane-vesicles of about 50% and an ATPase proportion of 20-25% on total membrane protein, the ATPase activities of isolated and of membrane-bound enzymes are therefore equivalent. pH and temperature dependencies of the *Synechocystis* ATPase activity show broad optima (Fig. 4) with an alkaline pH optimum as reported for the SERCA3 isoform (10).

![Fig. 3: Phosphorylated intermediates of the purified *Synechocystis* 6xHis-ATPase.](image)

2 µg of the isolated enzyme expressed in *E. coli* and purified by Ni-chelate chromatography were incubated with $[\gamma^3P]$ATP in MOPS/KCl/MgCl$_2$/500 µM Ca$^{2+}$ medium (lane 1) supplemented with either 5 mM EGTA (lane 2) or 100 µM La$^{3+}$ (lane 3). Phosphorylated intermediates of the isolated were separated on 7.5% PAGE of the Sarkadi-type as described under Materials and Methods.
Fig. 4: pH and temperature dependence of ATPase activity of the expressed Synechocystis 6xHis-ATPase. ATPase activity of recombinant plasma membranes from E. coli TKR2000/pQE32-8 (50 µg protein) was assayed at 37°C at various pH values (p). Temperature dependent ATP hydrolysis (●) was determined at pH 7.5 using 2 µg of isolated enzyme in hydrolysis buffer plus 100 µM Ca²⁺. Rates are calculated in percent of the maximum activity.

Both preparations showed only a calcium stimulation of ATPase activity between 10% and 20% while a strong Ca²⁺-stimulation was found in the EP formation experiments (Fig. 3). On the other hand, addition of the calcium ionophor A23187 to recombinant membrane vesicles resulted in a 15% stimulation of ATP hydrolysis indicating a more stringent calcium dependence of the membrane-bound enzyme. Similar results have been reported for a microsomal Ca²⁺-ATPase of yeast (8) and for the ER-localized Ca²⁺-ATPase of carrot cells (7). So far, we have no indications for a negative effect of the N-terminal 6xHis tag on the folding and activity of the enzyme in respect to its calcium specificity. The physiological role of the hydrophilic N-terminal stretch is unknown, however, a negative effect caused by the insertion of the 6 histidine residues is unlikely but cannot be excluded.
The expressed ATPase is sensitive to vanadate - the inhibitor of all P-ATPases - with an IC\textsubscript{50} of 119 µM while total inhibition of the enzyme is obtained by mM concentrations (see Fig. 5). The apparent vanadate affinity is in the same range as found for SER Ca\textsuperscript{2+}-ATPase isoforms (10, 8), but is much higher than IC\textsubscript{50} values for PM Ca\textsuperscript{2+}-ATPases (2-3 µM, (19)). The Ca\textsuperscript{2+}-ATPase from \textit{Synechococcus} 7942 seems to be more sensitive to vanadate; its calcium transport is totally blocked by 250 µM vanadate (5).

To our knowledge with the \textit{Arabidopsis} H\textsuperscript{+}-ATPase isoform AHA2 expressed in yeast only one further example describing the expression of a His-tagged P-ATPase exists (22). Recently, the turgor sensor subunit kdpD of \textit{E. coli} was expressed as His-tagged version (4). However, our results demonstrate that \textit{E. coli} can be used as host for the expression of a functional P-ATPase if several precautions regarding its toxicity are undertaken. Further, the here described method might also be useful for achieving purification of other P-type ATPases. Compared to other expression systems like yeast, baculovirus or mammalian cell cultures, the \textit{E. coli} host has many advantages in respect to its easy handling, short reproduction time, and low costs.

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REFERENCES

1. Møller J.V., Juul B., le Maire M. 1996. Structural organization, ion transport, and energy transduction of P-type ATPases. *Biochim. Biophys. Acta* **1286**, 1-51.

2. Tang X., Halleck M.S., Schlegel R.A., Williamson P. 1996. A subfamily of P-type ATPases with aminophospholipid transporting activity. *Science* **272**, 1595-1497.

3. Geisler M., Koenen W., Richter J., Schumann J. 1998. Expression and characterization of a *Synechocystis* PCC 6803 P-type ATPase in *E. coli* plasma membranes. *Biochim. Biophys. Acta* **1368**, 267-275.

4. Jung K., Tjaden B., Altendorf K. (1997) Purification, Reconstitution, and Characterization of kdpD, the Turgor Sensor of Escherichia coli. *J. Biol. Chem.* **272**, 10847-10852.

5. Berkelman T., Garret-Engele P., Hoffman N.E. 1994. The pacL gene of *Synechococcus* sp. Strain PCC 7942 encodes a Ca$^{2+}$-Transporting ATPase. *J. Bacteriol.* **176**, 4430-4436.

6. Lichtner R., Wolf H.U. 1980. Phosphorylation of the isolated high-affinity (Ca$^{2+}$ + Mg$^{2+}$) ATPase of the human erythrocyte membrane. *Biochim Biophys Acta* **598**, 472-85.

7. Hsieh W., Pierce W.S., Sze H. 1991. Calcium-pumping ATPases in vesicles from carrot cells. Stimulation by calmodulin or phosphatidylserine, and formation of a 120 kilodalton phosphoenzyme. *Plant Physiol* **97**, 1535-1544.

8. Okorokov L.A., Tanner W., Lehle L. 1993. A novel primary Ca$^{2+}$-transport system from *Saccharomyces cerevisiae*. *FEBS Lett* **216**, 573-577.

9. Bowman E.M., Siebers A., Altendorf K. 1988. Bafilomycins: A class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proc. Natl. Acad. Sci. USA* **85**, 7972-7976.

10. Lytton J., Westlin M., Burk S.E., Shull G.E., MacLennan D.H. 1992. Functional comparisons between isoforms of the sarcoplasmic or endoplasmic reticulum family of calcium pumps. *J. Biol. Chem.* **267**, 14483-14489.

11. Hochuli E., Döbeli H., Schacher A. 1987. New metal chelate adsorbents selective for proteins and peptide containing neighbouring histidine residues. *J. Chromatography* **411**, 177-184.

12. Geisler M., Richter J., Schumann J. (1993) Molecular Cloning of a P-type ATPase Gene from the Cyanobacterium *Synechocystis* sp. PCC 6803. Homology to Eukaryotic Ca$^{2+}$-ATPases. *J. Mol. Biol* **243**, 1284-128913.

13. Kaneko T., Sato S., Kotani H., Tanaka A., Azamizu E., Nakamura Y., Miyajima N., Hirosawa M., Sugiura M., Sasamoto S., Kimura T., Hosouchi T., Matsuno A., Muraki A., Nakazaki N., Naruo K., Okumura S., Shimpo S., Takeuchi C., Wada T., Watanabe A., Yamada M., Yasuda M., Tabata S. 1996. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. *DNA Res.* **3**, 109-13614.

14. Siebers A., Altendorf K. 1988. The K$^{+}$-translocating Kdp-ATPase from *Escherichia coli*. *Eur. J. Biochem* **178**, 131-140.

15. Geisler M., Jakobs. B., Richter J., Schumann J. 1996. Expression and Characterization of a *Synechocystis* PCC 6803 P-type ATPase in *E. coli* Plasma Membranes. *Biochim. Biophys. Acta* **1309**, 189-193.
16. Clarke D.M., Loo T.W., MacLennan D.H. 1990. Functional Consequences of Mutations of Conserved Amino Acids in the β-Strand Domain of the Ca^{2+}-ATPase of Sarcoplasmic Reticulum. *J. Biol. Chem.* **265**, 14088-14092.

17. Inesi G., Zhang Z., Sagara Y., Kirtley M.E. 1994. Intracellular signaling through long-range linked functions in the Ca^{2+} transport ATPase. *Biophys. Chemistry* **50**, 129-138.

18. Sarkadi B., Enyedi A., Földes-Papp Z., Gárdos G. 1986. Molecular Characterization of the *in Situ* Red Cell Membrane Calcium Pump by Limited Proteolysis. *J. Biol. Chem.* **261**, 9552-9557.

19. Carafoli E. 1991. Calcium Pump of the Plasma Membrane. *Phys. Rev.* **71** 129-153.

20. Carafoli E. 1995. Biogenesis: Plasma membrane calcium ATPase: 15 years of work on the purified enzyme. *FASEB* **8**, 993-1002.

21. Heim R., Iwata T., Zvaritch E., Adamo H.P., Rutishauer B., Strehler E.E., Guerini D., Carafoli E. 1992. Expression, Purification, and Properties of the Plasma Membrane Ca^{2+} Pump and of Its N-terminally Truncated 105-kDa Fragment. *J. Biol. Chem.* **267**, 24476-24484.

22. Lanfermeijer F.C., Venema K., Palmgren M.G. 1997. Purification of heterologously expressed plant plasma membrane H^{+}-ATPase by Ni^{2+} affinity chromatography. *Ann. NY Acad. Sci.* **834**, 139-141.

23. Papp B., Enyedi A., Kovács T., Sarkadi B., Wuytack F., Thastrup O., Gárdos G., Bredoux R., Levy-Toledano S., Enouf J. 1991. Demonstration of Two Forms of Calcium Pumps by Thapsigargin Inhibition and Radioimmunoblotting in Platelet Membrane Vesicles. *J. Biol. Chem.* **266**, 14593-14596.

24. Laemmli U.K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

25. Villalba J.M., Palmgren M.G., Berberian G.E., Ferguson C., Serrano R. 1992. Functional Expression of Plant Plasma Membrane H^{+}-ATPase in Yeast Endoplasmic Reticulum. *J. Biol. Chem.* **267**, 12341-12349.

26. Miroux B, Walker J.E. 1996. Over-production of proteins in Escherichia coli: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J. Mol. Biol.* **260**, 289-298.