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Cell-Free Synthesis Optimization of Barley Vacuolar Na^+/H^+-Antiporter, a Highly Hydrophobic Protein

Anna S. Nizkorodova • Bulat K. Iskakov*

Ajtkhozhin Institute of Molecular Biology and Biochemistry, Committee of Science,
Ministry of Education and Science of the Republic of Kazakhstan, 86, Dosmukhamedov Str., 050012, Almaty, Kazakhstan

Corresponding author: * **bulat.iskakov@mail.ru**

ABSTRACT

Membrane proteins (MPs) play a fundamental role in living cells as receptors, ion channels, carriers and adhesion molecules. In contrast to soluble proteins, MPs frequently aggregate in aqueous systems and are prone to incomplete synthesis. In this work we have optimized conditions for synthesis of full-length hydrophobic protein – barley vacuolar Na^+/H^+-antiporter (HvNHX2), in a cell-free translation system based on wheat germ extract. To achieve the full-length HvNHX2 synthesis we have tested different modifications of the cell-free translation system, such as the addition of different detergents and hydrophobic L-amino acids. Addition into the translation system of the amino acids valine (23.5 mM) and isoleucine (15 mM) significantly improved synthesis of full-length protein. We also investigated the influence of 5'-untranslated region (5'UTR) of *Potato virus Y* (PVY) genomic (g)RNA on cell-free translation of *HvNHX2*-open reading frame. The 5'UTR of PVY gRNA enhanced the translation efficiency of uncapped *HvNHX2* mRNA 4 times more than control mRNA which possessed an arbitrary polylinker-derived 5'UTR.

Keywords: *in vitro* expression, membrane proteins, mRNA 5'UTR, translation enhancer
Abbreviations: 5'UTR, 5'-untranslated region; **GUS**, β-glucuronidase; **HvNHX2**, barley vacuolar Na^+/H^+-antiporter; **MP**, membrane protein; **PVY**, *Potato virus Y*; **gRNA**, genomic RNA; **TCA**, trichloroacetic acid; **WG**, wheat germ

INTRODUCTION

Membrane proteins (MPs) perform many critical functions in living systems such as transporting materials across cell boundaries, participating in cell adhesion and signaling. Their importance makes them popular targets for research and drug development (Robelek *et al.* 2007). However, the study of MPs has been hampered by generally low yields obtained from *in vivo* expression systems due to cell toxicity (Sinner *et al.* 2004; Maurer *et al*. 2005; Robelek *et al.* 2007; Shirouzu *et al.* 2007). In contrast to soluble proteins, MPs frequently aggregate in aqueous systems and are prone to incomplete synthesis. To overcome this obstacle, an *in vitro* approach for producing MPs is usually used (Maurer *et al*. 2005).

Preparative scale cell-free expression has now become an emerging alternative tool for the high level production of integral MPs. Many toxic effects attributed to the overproduction of recombinant proteins are eliminated by cell-free expression as viable host cells are no longer required. A unique characteristic is the open nature of cell-free systems that offers a variety of options to manipulate the reaction conditions in order to protect or to stabilize the synthesized recombinant proteins (Klammt *et al.* 2006). Detergents or lipids can easily be supplemented and MPs can therefore be synthesized directly into a defined hydrophobic environment of choice that permits solubility and allows the functional folding of the proteins (Park *et al.* 2007). Alternatively, cell-free produced precipitates of MPs can efficiently be solubilized in mild detergents after expression.

In this work we have determined optimal conditions for synthesis of full-length hydrophobic protein – vacuolar Na^+/H^+-antiporter (HvNHX2) of barley (Vasekina *et al.* 2005), in a cell-free translation system from wheat germ (WG). Vacuolar Na^+/H^+-antiporters are integral MPs residing in the vacuolar membrane and providing coupled downhill movement of H^+ with the uphill movement of Na^+. These proteins have been shown to play important roles in cellular ion homeostasis, including the sequestration of Na^+ ions into the vacuole and vacuolar pH regulation (Yamaguchi *et al.* 2005).

To achieve the full-length synthesis of HvNHX2 we have tested different modifications of the cell-free translation system, such as the addition of ionic and non-ionic detergents, as well as of hydrophobic L-amino acids. Modification of a cell-free translation system by the addition of hydrophobic L-amino acids for full-length synthesis of MP was used for the first time. Addition into the translation system of valine and isoleucine amino acids at 23.5 and 15 mM, respectively significantly promoted the synthesis of full-length protein. We also investigated the influence of *Potato virus Y* (PVY) genomic (g)RNA 5'-untranslated region (5'UTR) on translation of *HvNHX2* open reading frame (ORF). This 5'UTR of PVY gRNA is well known as a translation enhancer however was previously tested only with ORFs coding for soluble proteins (Levis and Astier-Manifacier 1993; Akbergenov *et al.* 2004). In this work we have shown that the 5'UTR of PVY gRNA enhances the translation efficiency of *HvNHX2* mRNA about 4-fold more than control mRNA with an arbitrary polylinker-derived 5'UTR sequence.

MATERIALS AND METHODS

Plasmid construction

The sequence of the *Hordeum vulgare* cDNA-gene *NHX2* was cut with *Nco*I and *Sal*I (Fermentas Inc., Hanover, MD, USA) from plasmid "pmRNA" (kindly provided by Dr. A.V. Babakov, see Acknowledgments) and introduced between the same restriction sites into plasmid "pY-GUS" encoding bacterial β-glucuronidase (GUS) that was described earlier (Gallie *et al.* 1991; Akbergenov

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et al. 2004), instead of the GUS-coding segment. In the resulting construct the ORF *HvNHX2* was placed under the control of bacteriophage T7 promoter and translational enhancer (5'UTR) of PVY gRNA. Before transcription *in vitro* the plasmid "pY-NHX2" was linearized by *Eco*RI (Fermentas Inc.).

In vitro transcription

Synthesis of uncapped mRNAs was carried out using bacteriophage T7 RNA polymerase (Fermentas Inc.) as described previously (Gurevich *et al.* 1991). Integrity of mRNAs was assessed by agarose gel electrophoresis.

In vitro translation

Translation *in vitro* was carried out in a WG cell-free system as described previously (Madin *et al.* 2000) with slight modifications. The standard reaction mixture in 25 µl contained: 20 mM Tris-acetate pH 7.6; 90 mM potassium acetate; 2 mM magnesium acetate; 2 mM DTT; 0.1 mM spermine; 1 mM ATP; 0.1 mM GTP; 10 mM creatine phosphate; 0.12 mg/ml creatine kinase (all reagents of Sigma-Aldrich, St. Louis, MI, USA); 0.2 mM of each amino acid (L-[^{35}S]methionine, 2 µCi, was added separately); 2 µg of mRNA and 11 µl of WG extract. Incubation was for 60 min at 26°C. Translation efficiency was estimated by fluorometric measurement of GUS activity as described by Gallie *et al.* (1991).

Assessment of [^{35}S] radioactivity in polypeptides synthesized *in vitro*

Total incorporation of [^{35}S] methionine into translation products was determined as follows: 5 µl of translation mixture was added to 5 ml of 10% trichloroacetic acid (TCA), mixed and passed through a GF/C filter (Whatman International Ltd., Maidstone, England). Filters were washed three times with 5 ml of 10% TCA (Sigma-Aldrich) and twice with 5 ml of 70% ethanol and finally dried at 60°C for 20 min. Radioactivity was measured in a counter LS-100C (Beckman) in scintillation liquid (1% PPO, 0.02% POPOP) as described by Sambrook *et al.* (1989). Also the [^{35}S]-labeled translation products (8 µl of reaction) were analyzed by electrophoresis in a 12% polyacrylamide gel (PAG) in the presence of sodium dodecylsulfate (SDS) as described by Laemmli (1970). After electrophoresis gels were dried and exposed to X-ray film (Agfa HealthCare NV, Mortsel, Belgium) at -70°C as described by Sambrook *et al.* (1989). Densitometry of HvNHX2-bands was performed using ImageJ 1.42 analysis software (NIH, USA).

Fluorometric GUS quantitation assay

GUS-enzyme quantitation assay of *in vitro* translation samples was performed as described earlier (Cervera 2008) on a "Hoefer Scientific Instruments" Mini-Fluorometer TKO 100 model. Enzyme activity was measured in standard units.

Statistical analysis

Microsoft Excel 2003 software was used for data processing. The comparison of categorical data variables were tested by a paired *t*-test ($P < 0.05$).

RESULTS AND DISCUSSION

Barley vacuolar Na^+/H^+-antiporter (cds accession No: AY247791) is a protein of 546 amino acids (predicted size 56 kDa) which consists of 12 membrane-spanning domains, short N-terminal (22 amino acids) and C-terminal (108 amino acids) domains directed into the cytoplasm (Vasekina *et al.* 2005). Most abundant are non-polar amino acids Leu (14.1%), Val (8.79%), Ile (8.24%), Ala (7.33%), Phe (6.96%), etc. Since HvNHX2 is a highly hydrophobic protein (60.8% of non-polar and only 19.05% of charged amino acids) its synthesis *in vitro* was expected to be quite problematic. To increase the production of HvNHX2 in a WG cell-free system we tried to use the translation enhancer – 5'UTR of PVY gRNA (Robaglia 1989). When substituted, the 5'UTR of reporter mRNA coding for GUS, this sequence was shown to enhance translation efficiency several times (Levis *et al.* 1993; Akbergenov *et al.* 2004). To investigate whether this sequence would increase translation of mRNA coding for the hydrophobic protein HvNHX2 we prepared three types of mRNA: [5'pl-HvNHX2]; [5'PVY-HvNHX2]; [5'PVY-GUS] (5'pl – 5'UTR derived from plasmid polylinker; 5'PVY – 5'UTR of PVY gRNA). The construct [5'PVY-GUS] served as a positive standard to check the efficient functioning of the WG system; construct [5'pl-HvNHX2] served as a negative control since the polylinker-derived 5'UTR is unable to stimulate efficient translation of an adjacent ORF (Gallie *et al.* 1991; Akbergenov *et al.* 2004). All three mRNAs were synthesized *in vitro*, isolated, checked for integrity and equal amounts of them were translated in the WG cell-free system in the presence of [^{35}S]-methionine. The radioactivity incorporated into TCA-precipitable products of translation was determined as presented in **Fig. 1**. As expected, translation levels of mRNAs with 5'PVY-leader were higher (~ 4 times) than mRNA [5'pl-HvNHX2], indicating that 5'PVY-leader indeed may increase the frequency of translation initiation on uncapped mRNAs. Incorporation levels of [^{35}S] methionine into GUS and HvNHX2 synthesized on mRNAs with 5'PVY-leader were approximately equal.

Then we analyzed the polypeptide composition of *in vitro* translation reactions by SDS-PAG electrophoresis. Newly synthesized [^{35}S]-labeled polypeptides were detected by subsequent autoradiography of dried PAG and the results are presented in **Fig. 2**.

From an autoradiogram, most of [^{35}S]-labeled polypeptides synthesized on HvNHX2- coding mRNAs (**Fig. 2**, lanes 2-4) were located in a broad zone of 35-40 kDa and were most likely represented by incompletely synthesized

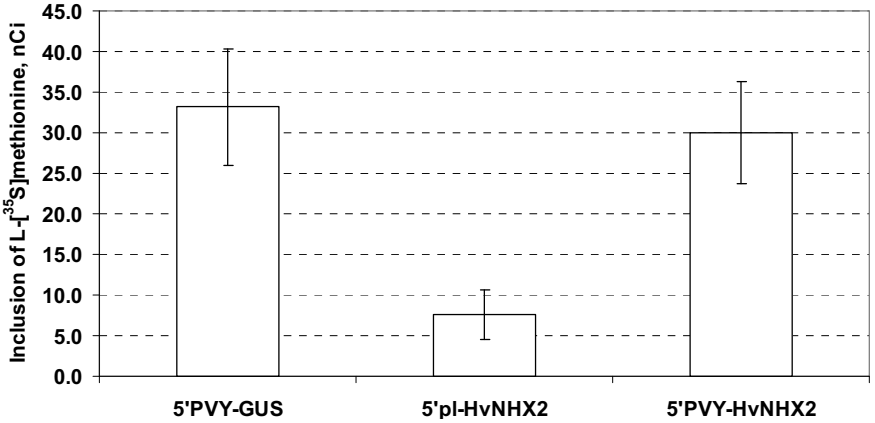

Fig. 1 Levels of L-[^{35}S]methionine incorporation into TCA-precipitable products synthesized in WG cell-free systems programmed by equal amounts of different mRNAs. Values represent mean ± Standard Error (SE).

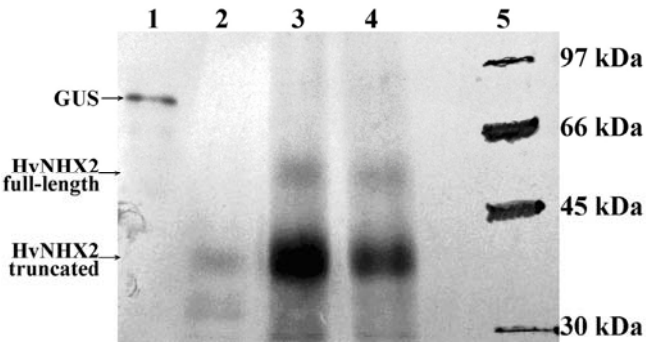

Fig. 2 Autoradiography after electrophoresis in 12.5% SDS-PAG of [^{35}S]-labeled polypeptides synthesized in WG cell-free system when translating different mRNAs. Lane 1 – translation products of [5'PVY-GUS] mRNA (2 μg); Lane 2 – translation products of [5'pl-HvNHX2] mRNA (2 μg); Lane 3 – translation products of [5'PVY-HvNHX2] mRNA (2 μg); Lane 4 – translation products of [5'PVY-HvNHX2] mRNA (1 μg); Lane 5 – marker proteins with known molecular masses indicated on the right in kDa. Positions of GUS (M_r 68000), full-length HvNHX2 (M_r 52000-54000) and truncated HvNHX2 (M_r 35000-40000) are indicated on the left.

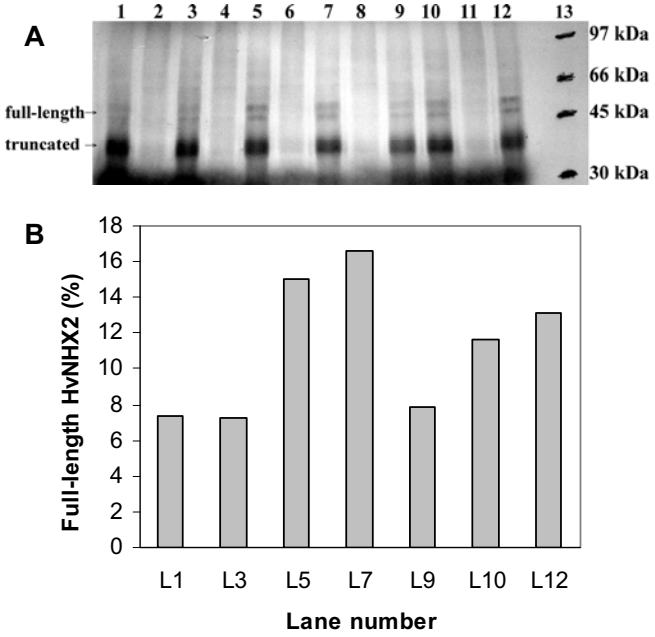

Fig. 3 Autoradiography after electrophoresis in 15% SDS-PAG (**A**) and optical density analysis (**B**) of [^{35}S]-labeled polypeptides synthesized in WG cell-free system programmed by mRNA [5'PVY-HvNHX2] and with different additions as indicated below: Lane 1 – no additions; Lane 2 – 1% Triton X-100; Lane 3 – 0.5 mM sarcosine; Lane 4 – 1% Triton X-100 and 0.5 mM sarcosine; Lane 5 – 15 mM Ile and 23.5 mM Val; Lane 6 – 15 mM Ile and 23.5 mM Val + 1% Triton X-100; Lane 7 – 15 mM Ile and 23.5 mM Val + 0.5 mM sarcosine; Lane 8 – 15 mM Ile and 23.5 mM Val + 1% Triton X-100 + 1 mM sarcosine; Lane 9 – 15 mM Ile and 23.5 mM Val + 1 mM sarcosine; Lane 10 – 20 mM Ile and 23.5 mM Val + 0.5 mM sarcosine; Lane 11 – 20 mM Ile and 23.5 mM Val + 1% Triton X-100; Lane 12 – 20 mM Ile and 23.5 mM Val; Lane 13 – marker proteins with known molecular masses indicated on the right in kDa (RPN756 Rainbow, Bio-Rad). Positions of full-length HvNHX2 (M_r 52000-54000) and truncated HvNHX2 (M_r 35000-40000) are indicated on the left.

(truncated) HvNHX2 molecules. Only a minor amount of newly synthesized polypeptides were found in the 52-54 kDa zone that corresponds to full-length HvNHX2. Incomplete synthesis of HvNHX2 may be caused by the hydrophobic property of this protein rather than the incapability of the WG cell-free system to synthesize large proteins because much longer soluble protein GUS with molecular weight of 68.2 kDa (Gallagher 1992) was completely synthesized under the same conditions (**Fig. 2**, lane 1). In contrast to 5'pl-, the 5'PVY-leader was able to synthesise some full-length HvNHX2 (**Fig. 2**, lanes 3, 4 vs. lane 2).

Incomplete synthesis of hydrophobic proteins *in vitro* may be due to several reasons including the shortage of hydrophobic amino acids and the absence of "hydrophobic environment" like the deficiency in the membrane structures (endoplasmic reticulum), which could accept and accommodate hydrophobic polypeptides in the course of their synthesis (Maurer *et al.* 2005; Klammt *et al.* 2006).

To improve the yield of full-length HvNHX2 we made a number of additions to the WG cell-free system, such as detergents (Maurer *et al.* 2005) and hydrophobic amino acids.

The following ionic detergents at a final concentration of 0.5-1 mM were tested: cetyl trimethyl ammonium bromide (CTAB), cholic acid, sarcosine, sodium hexabromide and lithium dodecylsulphate. We also used non-ionic detergent Triton X-100 at a final concentration of 1%. Hydrophobic amino acids L-leucine (Leu), L-isoleucine (Ile), L-phenylalanine (Phe), and L-valine (Val) were added to the translation system at final concentrations of 8.5-23.5 mM.

Table 1 Influence of different additions to WG cell-free system on synthesis of GUS protein.

Additions to WG cell-free translation system	Final concentration of added agent	Comparative expression level, % (mean ± SE)
No additions	-	100 ± 1.9 (n = 4)
CTAB	0.5 mM	28 ± 5.3 (n = 3)
Cholic acid	0.5 mM	57 ± 2.0 (n = 3)
Sarcosine	0.5 mM	95 ± 2.1 (n = 3)
Sodium hexabromide	0.5 mM	71 ± 3.9 (n = 3)
Lithium dodecylsulphate	0.5 mM	80 ± 3.9 (n = 3)
Triton X-100	1%	52 ± 2.0 (n = 4)
L-Leu	8.5 mM	114 ± 1.0 (n = 3)
L-Ile	8.5 mM	111 ± 2.3 (n = 3)
L-Phe	8.5 mM	74 ± 2.5 (n = 3)
L-Val	8.5 mM	117 ± 1.6 (n = 3)

Values are presented in percents relative to system without additions, taken as 100%.

The influence of these additions was tested using reporter mRNA [5'PVY-GUS] and the levels of GUS protein synthesis were measured by β-glucuronidase activity. Results shown in **Table 1** suggest that most detergents were inhibitory while excess hydrophobic amino acids were not harmful and could even slightly stimulate protein synthesis. Based on these results we performed cell-free translation of mRNA [5'PVY-HvNHX2] in the presence of [^{35}S]-methionine as well as several combinations of additions mentioned above. Translation reactions were analyzed by SDS-PAG electrophoresis, [^{35}S]-labeled polypeptides were detected by subsequent autoradiography and the results are presented in **Fig. 3A**. For some lanes densitometric analysis was performed using ImageJ software as follows. On each indicated lane the equal rectangles were scanned covering zones from 30 to 60 kDA and the square under the 52-54 kDa zone was calculated as a percent from the sum of that under the 52-54 plus 35-40 kDa zones. The densitometry results are shown in **Fig. 3B**.

From **Fig. 3A** it can be seen that newly synthesized full-length HvNHX2 is presented on an autoradiogram by two bands differing in 2-3 kDa (52 and 54 kDa), equally in non-modified control reactions (lane 1) and in translation systems with modifications. Thus two bands of HvNHX2 could not be the result of the additions of detergents and amino acids. We also observed these two bands on a western-blot (not shown) that was developed with antibodies produced against the C-terminal domain of HvNHX2 (Vasekina *et al.* 2005). Similarly, in trout red blood cells the Na^+/H^+-antiporter (βNHE) is also presented on an SDS-electrophoregram by two bands differing in 2-4 kDa that were shown to be the result of N-glycosylation of the protein (Malapert *et al.* 1997). Possibly the same reason caused the

difference in electrophoretic mobility of HvNHX2.

Addition of lithium dodecylsulphate as well as L-leucine and L-phenylalanine (not shown) caused no changes in the amount of full-length HvNHX2 protein synthesized in the WG system. Triton X-100 inhibited the synthesis of HvNHX2 most in any combination. Two of the amino acids used, namely Ile and Val, significantly improved synthesis of full-length HvNHX2. The most significant increase of full-length protein synthesis (~2-fold more than the control, **Fig. 3B**) was caused by a combination of Ile (15 mM) and Val (23.5 mM), whereas the addition into the translation system of these amino acids separately (not shown) caused no statistically significant improvement in HvNHX2 synthesis. Increasing the concentration of Ile (**Fig. 3B**, lane 12) and Val (not shown) over 15 and 23.5 mM, respectively somewhat decreased the quantity of full-length protein. It is possible that such concentrations of amino acids, which are amphoteric and hydrophobic at the same time, are required for maintenance of a favorable environment for correct protein folding and thus full-length synthesis of HvNHX2.

CONCLUSIONS

Synthesis of large and highly hydrophobic proteins in a WG cell-free system is severely hampered and most of such polypeptides remain unfinished (truncated at the C-terminus). We have found combinations of agents, whose addition to the WG cell-free system significantly increased the proportion of full-length polypeptides, namely by supplying hydrophobic L-amino acids Ile (15 mM) and Val (23.5 mM), significantly improved full-length synthesis of HvNHX2 (M_r 52000-54000), a highly hydrophobic Na^+/H^+-antiporter from the barley vacuolar membrane.

We also have shown that 5'UTR of PVY gRNA may serve as translational enhancer for mRNAs encoding hydrophobic proteins since this sequence provides ~ 4 times increase in yield of target protein.

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