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Chromatographic purification of an insoluble histidine tag recombinant Ykt6p SNARE from *Arabidopsis thaliana* over-expressed in *E. coli*

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Abstract

In order to undertake in plant cell the study of the endoplasmic reticulum (ER)-Golgi apparatus (GA) protein and/or lipid vesicular transport pathway, expressed sequence tag (EST) coding for a homologue to the yeast soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) Ykt6p has been cloned in *Arabidopsis thaliana* by reverse transcription polymerase chain reaction (RT-PCR). The corresponding protein was over-expressed as a recombinant histidine-tag (his-tag) protein in *E. coli*. Starting from one litter of culture, an ultrasonic homogenization was performed for cell disruption and after centrifugation the *Arabidopsis* Ykt6p SNARE present in inclusion bodies in the pellet was solubilized. After centrifugation, the clarified feedstock obtained was injected onto an immobilized metal affinity chromatography (IMAC) in presence of 6 M guanidine and on-column refolding was performed. Folded and subsequently purified (94% purity) recombinant protein was obtained with 82% of recovery.

Keywords: Protein purification; Histidine tag; *Arabidopsis* SNARE Ykt6p

1. Introduction

In plant cells, the molecular machinery involved in the first step of secretion (endoplasmic reticulum (ER) to the Golgi apparatus (GA)) has begun to be investigated. Vesicle coat proteins (COP) GTPases [1] and nucleotide exchange proteins [2–4] have been shown to be involved. Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are membrane proteins required in the targeting and fusion of donor membrane-derived structures with their acceptor membranes [5]. The *Arabidopsis* genome shows families of SNAREs very similar to those known in the other eukaryotes [6]. Ykt6p is one of these proteins [7] which could be associated to trimeric and tetrameric SNARE complexes [8]. In order to determine the intracellular location and possible interactions that exist between Ykt6p and other SNAREs, we have cloned Ykt6 from *Arabidopsis thaliana* and over-expressed the corresponding recombinant protein as a histidine-tag (his-tag) protein in *E. coli* to facilitate purification [9,10]. The histidine tag allowed us to use immobilized metal affinity chromatography (IMAC) [11–16].

In order to optimize the expression of the recombinant protein, transformed bacteria were grown in a bio-reactor culture. At two different temperatures, 37 and 28 °C, the protein was expressed as inclusion bodies, leading to a complex purification process.

The insoluble nature of the protein as inclusion bodies requires a solubilization and refolding for the isolation of the protein in the native form [17]. The refolding process can be performed with different chromatographic methods, size exclusion chromatography [18–26], immobilization

Abbreviations: COP, coat proteins; ER, endoplasmic reticulum; EST, expressed sequence tag; GA, Golgi apparatus; His-tag, histidine tag; IMAC, immobilized metal affinity chromatography; LB, Lennox broth; RT-PCR, reverse transcription polymerase chain reaction; PET, plasmid expression tag; SNAREs, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors

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on gel matrices [27], ion exchange chromatography [28], hydrophobic interaction chromatography [29], immobilized metal affinity chromatography [30–35], affinity chromatography [36,37], immobilized liposome chromatography [38].

This paper described a one-step chromatographic purification involving IMAC in presence of 6 M guanidine followed by on-column procedure where exchange of 6 M guanidine by 8 M urea and 8 M urea by renaturation buffer was performed before elution.

The objective of this chromatographic process is to obtain a highly purified protein for the production of antibodies which will be used for immuno-precipitation studies to characterize putative complexes with Ykt6p and for intracellular localization.

2. Experimental

2.1. Instruments

The bio-reactor was from Incelltech (Toulouse, France).

The chromatographic systems used throughout this study were the fast protein liquid chromatography (FPLC) system from Amersham Biosciences (Saclay, France). The data were collected and evaluated using the FPLC director data system.

The ultrasonic homogenizer Vibracell 72412, 600 W model was from Bioblock (Illkirch, France).

The electrophoresis apparatus Mini-Protean II, electroporator Gene Pulser II and image analysis Gel Doc 2000 were from Bio-Rad (Ivry-sur-Seine, France).

For recovery studies, we used an Uvikon 930 spectrophotometer (Kontron, Montigny Lebretonneux, France) to measure absorbance at 595 and 600 nm.

ELISA plates (Nunc-Immuno Plate MaxiSorp Surface) were from Nunc Brand Products (Roskilde, Denmark).

Vivaspin 20 concentrator was from Sartorius (Palaiseau, France).

2.2. Chemicals

Plasmid expression tag (PET) 15b and *E. coli* BL21 (bacteria lysogenic for bacteriophage DE3) were from Novagen (Madison, WI, USA). Prostar First Strand RT-PCR kit was from Stratagene (La Jolla, USA), kit RNeasy plant was from Qiagen (Courtaboeuf, France). Original TA cloning kit with the pCR2.1 plasmid, Luria–Bertani (LB) culture media for the growth of *E. coli* and isopropyl β-D-thio-galactopyranoside (IPTG) were from Invitrogen (Groningen, The Netherlands). *E. coli* JM109 was from New England Biolabs (Beverly, USA).

Chelating Sepharose fast flow and the XK 16/20 and C10/10 columns were from Amersham Biosciences (Saclay, France). Imidazole extra pure was from BDH laboratory (Poole, England).

Protein G immobilized on Sepharose 4B fast flow, complete and incomplete Freund's adjuvant, all salts, metals and additive were from Sigma (L'Isle d'Abeau Chesnes, France). Peroxidase-labeled goat $(Fab')_2$ fragments anti-rabbit IgG was from Sanofi-Pasteur (Marnes la coquette, France). All salts were of HPLC grade, and the buffers were filtered through a 0.22 μm membrane filter.

The Western blot chemiluminescence reagent plus kit and the polyScreen PVDF transfer membrane were from NEN Life Science Products (Boston, MA, USA).

Autoradiography films were from Eastman Kodak Company (Rochester, NY, USA).

Keyhole limpet hemocyanine (KLH) was from Genosphere Biotechnologies (Paris, France).

2.3. Preparation of the cellular extract containing Atgp1

2.3.1. Cloning of Ykt6p coding sequence

A 628 pb fragment coding for Ykt6p was generated by reverse transcription polymerase chain reaction from total RNA extracted from *Arabidopsis*. The fragment was then subcloned into the pCR2.1 plasmid and used as a template in a PCR reaction with 5′ and 3′ primers containing 29 nucleotides with *Nde*1 and *Bam*H1 restriction sites, respectively. The 3′ primer contained also the procaryote codon stop TAA. The PCR product was then subcloned into the pCR2.1 plasmid. The Ykt6p coding sequence was then digested and inserted into the *Nde*1/*Bam*H1 linearized pET15b plasmid to create the Ykt6p his-tag construction. *E. coli* JM109 was then transformed with the construction by electroporation. The resulting recombinant Ykt6p his-tag protein was then expressed in *E. coli* BL21 (DE3) according to the manufacturer's instructions. The six histidine tag residues allowed binding to immobilized metal affinity chromatography column.

2.3.2. Expression of the recombinant protein in bio-reactor

A bio-reactor containing 1 l of LB medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) and 100 μg/ml ampicillin, was inoculated with an overnight culture of *E. coli* BL21 ($A_{600\,nm} = 0.35$) transfected with the recombinant plasmid Ykt6p-pET15b. The volume of the culture medium was 200-fold bigger than the inoculated volume. The culture was grown at 37 °C or 28 °C to 2.10^8 cells/ml ($A_{600\,nm} = 0.7$). IPTG was added to a final concentration of 0.3 mM and cells incubated at 37 °C for 5 h or at 28 °C for 12 h. After induction, bacteria were centrifugated and placed in an ice-water bath and disrupted by sonication in three short pulses of 30 s with 2 min in ice between each pulse. Samples were centrifuged in an CS 100 Hitachi microfuge at 25,000 × *g* for 30 min at 4 °C and supernatants removed.

2.3.3. Solubilization of the inclusion bodies

Pellets containing inclusion bodies in which the recombinant protein has been expressed at 37 °C, were resuspended

in 40 ml cold 2 M urea, 20 mM Tris–HCl, 0.5 M NaCl, 2% Triton X-100 pH 8.0, and sonicated as described above. After a centrifugation at 25,000 × *g* for 30 min at 4 °C, pellets were subjected to an urea wash.

Inclusion bodies were then solubilized in 5 ml 20 mM Tris–HCl, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine hydrochloride, 1 mM 2-mercaptoethanol, pH 8.0 by stirring for 30 min at room temperature. After a centrifugation at high speed at 4 °C, remaining particles were removed by passing the samples through a 0.22 μm filter and the filtrate injected onto the column.

2.4. Chromatographic procedure

2.4.1. Preparation of the IMAC

The chelating Sepharose fast flow was packed in an XK16/20 column. A slurry was prepared with 20 mM Tris–HCl, 0.5 M NaCl, pH 8.0, in a ratio of 75% settled gel to 25% buffer and was degassed.

The column was filled through the outlet with a few centimeters of buffer and was closed. The gel slurry was poured into the column in one continuous motion. The remainder of the column was filled up with buffer and the top piece mounted and connected to a pump. The bottom outlet of the column was opened and the pump set at 130% of the flow-rate to be used during chromatography (1 ml/min). The packing flow-rate was maintained for three bed volumes after a constant bed height was reached. The final volume of the gel was 3.41 ml. The column was wash with 10 ml distilled water and 1 ml of 0.3 M $NiSO_4$ solution was then loaded. The column continued to be washed with 10 ml distilled water and equilibrated with 5–10 ml of 20 mM Tris–HCl, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine hydrochloride, 1 mM 2-mercaptoethanol, pH 8.0.

2.4.2. Immobilized metal affinity chromatography procedure

Samples were loaded to the chelating Sepharose Fast Flow column followed by a wash with 80 ml of 20 mM Tris–HCl, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine hydrochloride, 1 mM 2-mercaptoethanol, pH 8.0. A second wash was performed with 5 ml of 20 mM Tris–HCl, 0.5 M NaCl, 20 mM imidazole, 6 M urea, 1 mM 2-mercaptoethanol, pH 8.0, at a flow-rate of 1 ml/min until UV baseline was reached. The bound protein was treated by 30 ml of a linear 6–0 M urea gradient, starting with the previous second wash buffer. The column was then washed with 7 ml of buffer without urea (20 mM Tris–HCl, 0.5 M NaCl, 1 mM 2-mercaptoethanol, pH 8.0).

The elution buffer was composed of 20 mM Tris–HCl, 0.5 M NaCl, 1 mM 2-mercaptoethanol, pH 8.0 and a linear gradient of imidazole from 20 mM to 0.5 M run at 0.5 ml/min. Fractions were collected, pooled and desalted onto a Sephadex G 25 packed in C 10/10 column. Eluates were then concentrated to 6 ml using ultrafiltration devices Vivaspin 20.

2.5. Analytical procedures

2.5.1. Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [39] using a Mini-protean II apparatus and a Tris–glycine–SDS buffer were used to monitor the purification during the chromatographic procedures.

Electrophoresis was performed for 1 h at 150 V using 12.5% polyacrylamide gels. Detection was done by Coomassie brilliant blue R250.

2.5.2. Rabbit polyclonal IgG anti-Ykt6p and Western blotting

Polyclonal antibodies against 17 amino acids specific to the N-terminal sequence of Ykt6p (NASDVSHFGYFQRSSVK) were obtained by immunizing two rabbits four time under the skin. Injections were performed every 15 days with 500 μg of a pure keyhole limpet hemocyanine (KLH)-conjugated peptide in the presence of complete (for the first injection) or incomplete Freund's adjuvant. A week after the latest injection, blood was collected under general anesthesia and immunserum (also non-immunserum before any injection as a control) were obtained by centrifugating the blood at 25,000 × *g* in a CS 100 Hitachi microfuge for 5 min.

Total IgG were then purified from the immunserum by affinity chromatography on a Protein-G Sepharose 4B and their titre determined by ELISA.

For Western blots, the chromatographic fraction containing purified Ykt6p was separated by electrophoresis as described above, with a 10% polyacrylamide gel, and transferred overnight at 4 °C onto a polyvinyllidone difluoride membrane in a Tris–glycine buffer. Briefly, this membrane was then incubated 1 h at room temperature in 1% BSA in 0.01 M phosphate-buffered saline (PBS), 0.138 M NaCl, 2.7 mM KCl, 0.05% Tween 20, pH 7.4. The membrane was then incubated in the same buffer containing 1/2000 of specific polyclonal IgG against the 17 amino acids of Ykt6p, for 1 h at 25 °C and washed five time in the same buffer as described below. The membrane was then incubated with peroxidase-labeled goat $(Fab')_2$ fragments anti-rabbit IgG (1/20,000) for 1 h at room temperature. After five washes, the bound antibodies were revealed using the Western blot chemiluminescence reagent plus kit and autoradiography films according to the manufacturer's instructions.

2.5.3. Protein concentration

The Ykt6p SNARE concentration was estimated by determining the total protein concentration using Coomassie blue [40] with bovine serum albumin as standard and by measuring the percentage of Ykt6p SNARE by gel scanning.

3. Results and discussion

3.1. Solubilization of the recombinant Ykt6p over-expressed in E. coli at 37 and 28 °C

In order to compare the amount of recombinant Ykt6p (recYkt6p) over-expressed in *E. coli* cultured in a bio-reactor at 37 °C or 28 °C, cells were centrifuged, concentrated in a small volume of 20 mM Tris–HCl buffer solution, pH8, and sonicated. Total proteins from cell lysate were then fractionated by SDS–PAGE (Fig. 1A). At both temperatures, the similar amount of total proteins was recovered (Table 1). To estimate the amount of soluble recYkt6p in bacteria, cell lysates were submitted to centrifugation and pellets and

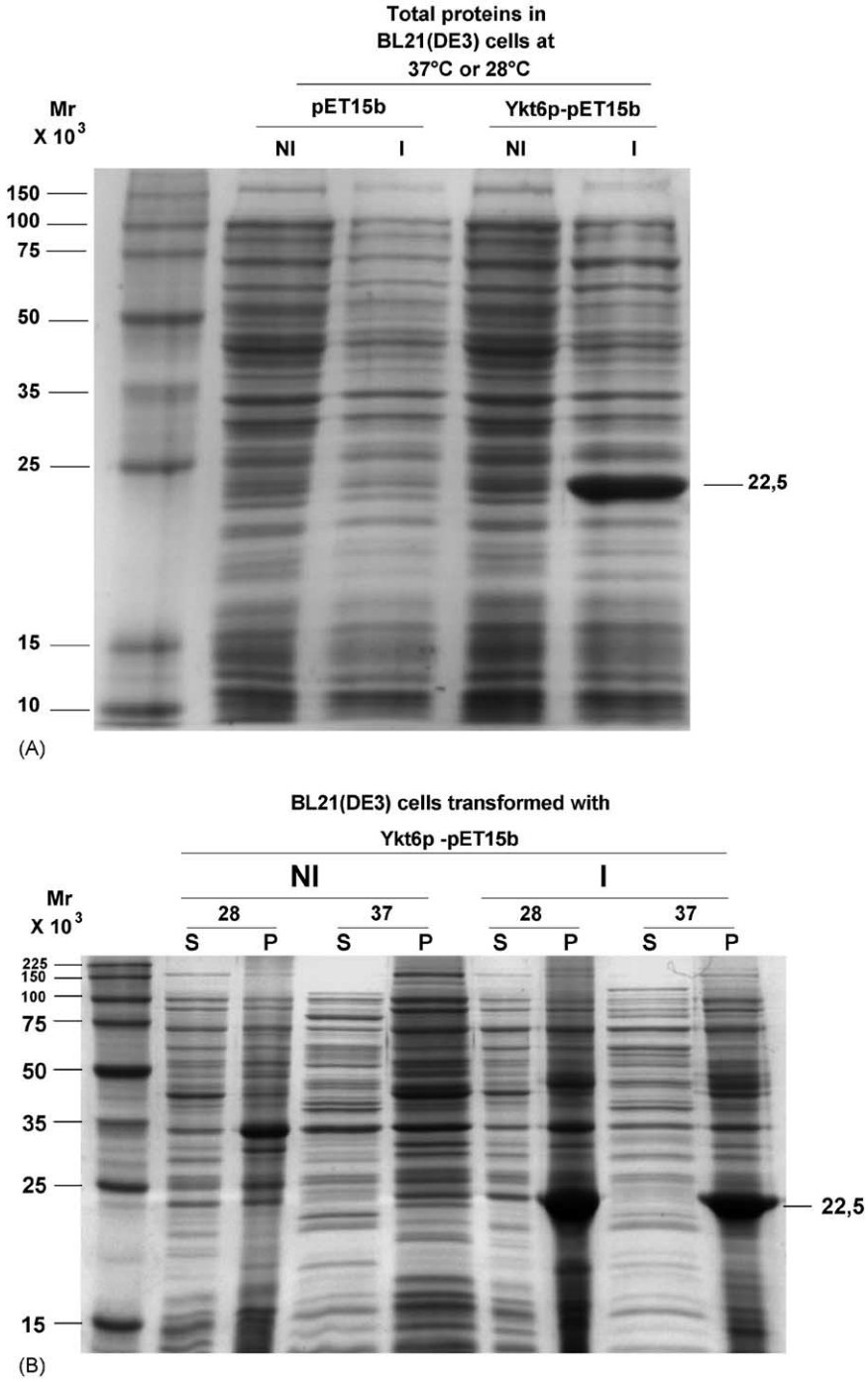

Fig. 1. Over-expression of recombinant Ykt6p in *E. coli* cultured in a bio-reactor at 37 and 28 °C: (A) 5 μg of total proteins from cell lysate were loaded on 12.5% SDS–PAGE system and Coomassie blue-stained. pET15b: control expression with the empty vector, *Ykt6p*-pET15b: His-tag Ykt6p, I: IPTG-induced culture, NI: non-induced culture. (B) Cell lysates from 37 and 28 °C bio-reactor cultures were centrifugated, and pellets and supernatants fractionated and proteins (5 μg) stained as described above. S: supernatant, P: pellet.

Table 1
Purification of recombinant histidine tag Ykt6p SNARE from one liter of bio-reactor culture medium

	Volume (ml)	Total proteins (mg)	recYkt6p (mg)	[recYkt6p] (μg/ml)	Step recovery (%)	Fold purification
Start (37 °C or 28 °C)	1000	128	19.6	19.6		
Cell lysate	40	120	18.5	463	100	
Solubilized inclusion bodies	5	106	17	3400	92	1.07
IMAC through flow	80	86.5	0.4			
IMAC 20 mM imidazole wash	5	2.1	n.d.			
IMAC urea descendant gradient	30	n.d.	n.d.			
IMAC eluate	17	17.3	16.3	959	88.1	6.28
Eluate concentrated (Vivaspin column)	6	17	16	2670	86.5	

RecYkt6p was highly expressed in the cytosol of *E. coli* as inclusion bodies at 37 °C or 28 °C. More than 88% of the over-expressed protein was recovered with more than 94% purity. IMAC eluate fraction was then desalted by group size exclusion chromatography and concentrated three times for further analysis.
n.d.: no detected.

supernatants fractionated by SDS–PAGE (Fig. 1B). Most of the recYkt6p was recovered in the pellet at both temperatures, indicating that the secreted protein was aggregated, leading to a complex purification process. As expected, recYkt6p was found at a relative molecular mass of 22,500.

Insoluble proteins were then solubilized in the presence of guanidine, and used as a source of recombinant protein in the subsequent chromatographic step.

3.2. Purification of the His-Tag Ykt6p

Solubilized pellets obtained from *E. coli* culture medium grown at 37 °C were loaded onto a chelating Sepharose fast flow column previously loaded with Ni^{2+} and equilibrated with 20 mM Tris–HCl, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine hydrochloride, 1 mM 2-mercaptoethanol, pH 8.0.

After adsorption of the protein of interest, the gel was washed with the equilibration buffer followed by a quick wash containing 20 mM imidazole and 6 M urea instead of 5 mM imidazole and 6 M guanidine hydrochloride. The contaminants passed into the through flow.

Bound recYkt6p was then treated by a linear 6–0 M urea gradient, and desorption of the protein performed by a second linear gradient up to 0.5 M imidazole (Fig. 2). The fraction was eluted at 0.12 M imidazole.

The fraction containing the His-tag Ykt6p was analyzed by SDS–PAGE followed by a Coomassie blue-staining and Western blotting (Fig. 3A and B). Proteins solutions were then desalted on a Sephadex G25 and concentrated in 6 ml PBS. The purification process and its efficiency are summarized in Table 1. Purified anti-Ykt6p IgG was then used to detect the protein in ER/Golgi membranes isolated from leek seedlings [41,42]. Fig. 4 shows that the antibody recognized a protein at a relative mass of around 25,000, slightly at a higher value than the recombinant protein (Figs. 1 and 3). This is understandable keeping in mind that the

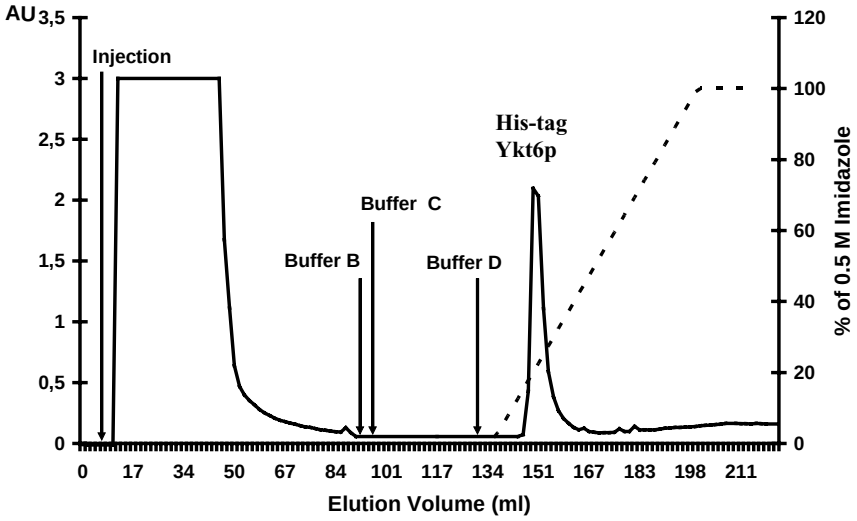

Fig. 2. Capture of His-tag Ykt6p with immobilized metal affinity chromatography (IMAC). Column: chelating Sepharose fast flow (3.41 ml of gel) loaded with 1 ml of 0.3 M $NiSO_4$ solution. Sample: solubilized inclusion bodies; buffer A: 20 mM Tris–HCl, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine hydrochloride, 1 mM 2-mercaptoethanol, pH 8.0; buffer B: 20 mM Tris–HCl, 0.5 M NaCl, 20 mM imidazole, 6 M urea, 1 mM 2-mercaptoethanol, pH 8.0; buffer C: 6–0 M urea gradient in buffer B; buffer D: 20 mM Tris–HCl, 0.5 M NaCl, 1 mM 2-mercaptoethanol, pH 8.0, and a linear gradient of imidazole from 20 mM to 0.5 M. Detection at 280 nm; flow-rate: 1 ml/min (buffers A–C) and 0.5 ml/min (buffer D).

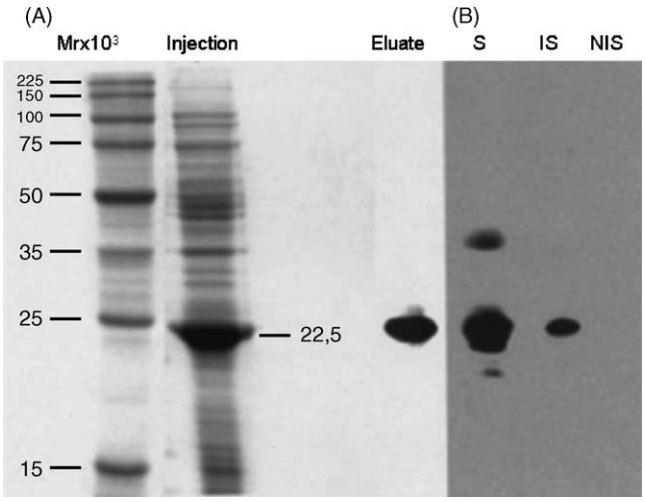

Fig. 3. Analysis of fractions eluted from IMAC, by Coomassie blue-staining (A) and Western blotting (B). (A) Five micrograms of total proteins were loaded on 12.5% SDS–PAGE system and Coomassie blue-stained. EL: eluate of IMAC; NR: not retained by IMAC. (B) 1.5 μg of total proteins from starting material and IMAC eluate fraction were separated by electrophoresis with a 10% polyacrylamide gel, and analyzed by Western blotting. Overnight transferred proteins onto PVDF membrane were incubated with 1/2000 diluted polyclonal IgG against 17 amino acids specific to Ykt6p N-terminal primary sequence. Revelation was performed using a goat antirabbit peroxidase conjugated antibody (1/20,000). S: starting material, corresponding to total proteins from solubilized inclusion bodies; IS: immunserum; NIS: non-immunserum.

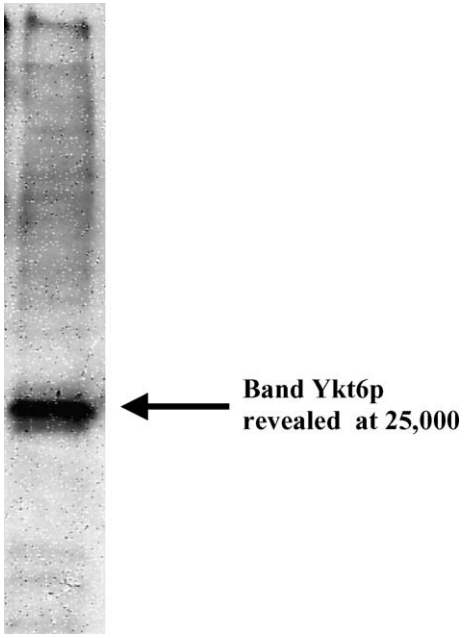

Fig. 4. Western blotting of ER/Golgi membrane proteins from leek seedlings with purified anti-Ykt6p IgG. Five micrograms of total proteins from membrane fractions were loaded on 12.5% SDS–PAGE. Overnight transferred proteins onto PVDF membrane were incubated with 1/5000 diluted polyclonal IgG against Ykt6p N-terminal primary sequence (17 amino acids). Revelation was performed using a goat antirabbit peroxidase conjugated antibody at 1/20,000. A relative mass of 25,000 from membrane fractions shows that the protein is isoprenylated in plants and that it cannot be for the recombinant one in *E. coli*.

protein is isoprenylated in plants and that it cannot be for the recombinant one in *E. coli*. No labeling was detected for purified plasma membranes (not shown).

These results show that the purified protein corresponds to the *A. thaliana* expected his-tag protein since it is recognized at a relative molecular mass of 22,500 by polyclonal antibodies against 17 amino acids specific to Ykt6p, in the N-terminal primary sequence (NASDVSHFGYFQRSSVK).

4. Conclusion

The process described in this paper allows the over-expression in a bio-reactor and the purification of the plant SNARE *A. thaliana* Ykt6p. Starting from 1 l culture media, the protein was found highly expressed in the cytosol of *E. coli* as inclusion bodies at different temperatures leading to a complex purification process. The His-tag allowed easy purification using a one-step chromatography by immobilized metal affinity chromatography.

These purification conditions allowed us to recover more then 88% of the over-expressed Ykt6p with more than 94% purity. Biochemical and structural studies can be now considered with the purified protein.

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