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A native Zn/Cd pumping P_{1B} ATPase from natural overexpression in a hyperaccumulator plant

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Abstract

We report here the first purification of a P_{1B} type ATPase, a group of transporters that occurs in bacteria, plants and animals incl. humans, from a eukaryotic organism in native state. TcHMA4 is a P_{1B} type ATPase that is highly expressed in the Cd/Zn hyperaccumulator plant *Thlaspi caerulescens* and contains a C-terminal 9-histidine repeat. After isolation from roots, we purified TcHMA4 protein via metal affinity chromatography. The purified protein exhibited Cd- and Zn-activated ATPase activity after reconstitution into lipid vesicles, showing that it was in its native state. Gels of crude root extract and of the purified protein revealed TcHMA4-specific bands of about 50 and 60 kDa, respectively, while the *TcHMA4* mRNA predicts a single protein with a size of 128 kDa. This indicates the occurrence of post-translational processing; the properties of the two bands were characterised by their activity and binding properties.

Keywords: Cadmium; Heavy metals; Metal activation; P_{1B} type ATPase; TcHMA4; Zinc

Metal hyperaccumulating plant species not only tolerate high amounts of heavy metals like zinc or cadmium in the environment, but also take them up actively and accumulate them up to several percent of their shoots dry mass. This ability makes them attractive for cleaning up metal-contaminated soils (phytoremediation: [1,2]). Many studies have been conducted to reveal mechanisms of heavy metal hyperaccumulation. Nonetheless, very little is known about mechanisms and proteins involved in transporting hyperaccumulated heavy metals from soil via the roots and stems into their storage sites. These are mainly large epidermal storage cells in the leaves [3,4]. The transport proteins involved in hyperaccumulation may belong to different families such as the CPx-type ATPases (P_{1B} ATPases), ZIPs, Nramps, CDFs and CAXs [5–7]. P_{1B} ATPases are a subfamily of the P-type ATPase superfamily, a group of ubiquitous membrane proteins that use ATP to pump cations across membranes against their electrochemical gradient. They are found in a wide range of organisms including bacteria, yeast, plants and animals including humans [8]. Plant P-type ATPases have eight to twelve transmembrane (TM) domains, N and C termini exposed to the cytoplasm, and a central cytoplasmic domain which harbours phosphorylation and ATP binding sites [9,10]. Surprisingly, while many genes encoding heavy metal transporters have been

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identified, very few of the proteins encoded by these genes have been isolated, purified and biochemically characterised, although this is necessary to understand their mechanisms of metal transport. No crystal structures for any heavy metal transporter of the ZIP, CDF and CPx-ATPase families in eukaryotes have been described, and only peptide subdomains of any of these transport proteins have been expressed and characterised in bacteria [11,12]. One reason for the lack of such studies are most likely the well-known difficulties with heterologous overexpression of membrane proteins, in particular those that transport potentially toxic substances like heavy metals. Membrane proteins expressed in bacteria or yeast systems often exhibit incorrect folding, and furthermore it is difficult to judge if the target protein is post-translationally modified as it would be if expressed in its natural system. Expression systems like bacteria and yeast are not able to tolerate the high heavy metal levels which can be reached in the cells when a metal transporter is overexpressed in such systems.

Hyperaccumulators offer a way to circumvent those problems. First, these plants have a strongly elevated expression of metal transporters [13–17]). Second, the protein studied here, TcHMA4, is a CPx-type Cd/Zn-ATPase (1185 amino acids in length). It has a natural C-terminal sequence of 9 His residues [16,18] which can be used as a natural tag for its purification by metal affinity chromatography. Finally, isolating the protein in this way will reveal possible post-translational modifications. Therefore, we applied this strategy and systematically developed a protocol for purifying TcHMA4 to homogeneity in active state.

Materials and methods

Plant material, culture media and culture conditions. Thlaspi caerulescens J.&C. Presl (Ganges population from southern France) was germinated and grown as described in detail in [19], using 100 μM $ZnSO_4$ in the nutrient solution for Zn-replete but non-inhibitory conditions. The nutrient solution was aerated and was exchanged continuously at a flow rate of 1700 ml/d^{-1} per pot (i.e., 250 ml d^{-1} per plant).

Chemicals. The chemicals were purchased from the following manufacturers:

- Chemicals used in the buffers for isolation and solubilisation: Merck, Germany.
- Detergents: *n*-dodecyl-β-maltoside (DDM): Carl Roth, Germany; *N*,*N*-dimethyldodecylamine *N*-oxide (LDAO): Fluka, Germany; *n*-octyl-β-D-glucopyranoside and *n*-dodecyl-*N*,*N*-dimethyl-ammonio-3-propane-sulphonate (zwittergent 3–12): Anatrace, USA).
- Protease inhibitors: PMSF and PefaBloc: Fluka, Germany; protease inhibitor cocktail VI: Merck, Germany; protease inhibitor cocktail tablets "complete", EDTA-free: Roche, Switzerland.
- Antioxidants and chemicals for column buffers: Merck, Germany.
- Chemicals used for ATPase activity test: Sigma–Aldrich, Germany.

Column materials. The following immobilised metal affinity column materials were compared:

- Precharged "Protino" Ni–IDA (Macherey–Nagel, Germany).
- Precharged "Protino" Ni–TED (Macherey–Nagel, Germany).
- "Talon" resin precharged with Co^{2+} (Takara Bio Europe, France).
- Precharged Ni–Sepharose "fast flow" (GE Healthcare, UK).
- Uncharged His-Bind Fractogel (www.novagen.com), charged before the run with Cd^{2+}, Cu^{2+} or Zn^{2+}.

Other materials. Centrifugal protein concentrators: Amicon Ultra-15, membrane with 10 kDa exclusion size (Millipore Corporation, USA).

Instruments. Instruments of the following manufacturers were used:

- Wheat mill: Jupiter 872 with Messerschmidt stainless steel grinding engine (Messerschmidt Hausgeräte GmbH, Germany).
- Ultracentrifuge: Beckmann LE-80 (Beckmann, Germany).

Methods for testing protein purity. SDS–PAGE was conducted as described in [20], and Western-blotting was done using the Protran BA 85 nitrocellulose membrane (Whatman, UK). The peptide sequence for the primary antibody was generated from the cDNA-deduced amino acid sequence of TcHMA4 by selecting a region of the protein that is unique to this member of the HMA4 family and has a high degree of probability of reacting with the antibody. The peptide sequence, DKEKAKETKL LLASC, is derived from the 3′ cytoplasmic tail of the TcHMA4 protein and was provided to Sigma Genosys for antibody production (Sigma–Aldrich, USA). The peptide was conjugated with keyhole limpet hemocyanin, the conjugated peptide was injected subcutaneously into two rabbits and after several boost injections, three bleeds were performed on days 49, 56 and 77 after the injection. A 1:1000 dilution of antiserum from the third bleed was successful in detecting a single band for the TcHMA4 protein in plasma membrane vesicles isolated from *Thlaspi caerulescens* roots and was used in subsequent experiments. The secondary antibody (Anti-rabbit IgG) was purchased from Sigma–Aldrich (Germany).

Protein was determined by the Bicinchoninic acid assay [21], and all chemicals for this assay were from Sigma–Aldrich (Germany).

ATPase activity was assayed according to a protocol that was developed as described in [22] and modified for the current study. The liberated phosphate was quantified against phosphate standards. In the first step the ATPase was reconstituted into lipid vesicles. For this purpose the ATPase was diluted to 0.18 mg ml^{-1} and then mixed in a ratio 1:5 with phosphatidylcholine from soybean. Reconstitution took place for 10 min at 37 °C. For the ATPase reaction, the reconstituted protein was transferred into a buffer containing 0.6 M Tris, 2 M NaCl and 0.1 M $MgCl_2$. The reaction was started with the addition of 5 mM ATP and took place for 40 min at 30 °C. It was stopped with a solution containing 0.5% SDS, 0.5% NH_4MoO_4 and 2% (V/V) H_2SO_4. The colour was developed with 10% ascorbic acid and the absorbance at 750 nm was read after 2 min.

Results and discussion

Development of the isolation and purification protocol

Isolation of TcHMA4 protein

Frozen roots were ground to powder using a wheat mill pre-cooled with dry ice, and further ground to a finer powder with liquid nitrogen using a mortar and pestle. Frozen isolation buffer was added to the frozen roots during the grinding and the whole extract was thawed at room temperature. After mixing the suspension, it was centrifuged (246,000*g*, 4 °C, 1 h). The supernatant containing the cytoplasmic proteins was discarded and the pellet was retained. It was resuspended in solubilisation buffer and continuously stirred at 4 °C for 14 h (standardised by tests) for optimal solubilisation of TcHMA4.

The successful solubilisation of the Zn/Cd ATPase from membranes in a native form was the key step leading to the

purification of this protein. Therefore, the composition of the isolation and solubilisation buffers was systematically optimised.

The efficiency of protein solubilisation was first tested with different buffer systems including MES–KOH, NaH_2PO_4–NaOH, sodium citrate–HCl ($C_6H_5NaO_7$–HCl) and sodium acetate–acetic acid (CH_3COONa–$C_2H_4O_2$), of which the phosphate buffer functioned best. Four different detergents were tested (Fig. 1). These included the non-ionic detergents *n*-octyl-β-D-glucopyranoside and *n*-dodecyl-β-D-maltoside (DDM), and the zwitterionic detergents *N*,*N*-dimethyldodecylamine *N*-oxide (LDAO) and *n*-dodecyl-*N*,*N*-dimethyl-ammonio-3-propane-sulphonate (zwittergent 3–12). As the best results were obtained with DDM, different concentrations of this detergent were tested, showing that >5 mM DDM were needed for solubilisation. Saturation of the amount of isolated protein was reached at about 10 mM (Fig. 2). Further tests were conducted to identify the optimal concentrations of NaH_2PO_4 (0–320 mM) and NaCl (0–3.2 M). It could be observed that increasing the NaCl concentration up to 2 M proved to be beneficial for extracting a higher amount of the native membrane protein. However, an increase in the concentration of NaH_2PO_4 did not significantly affect the solubilisation. Variations in pH from 5.0 to 8.0 were tested for both the isolation and the solubilisation buffer. It was found that the optimal pH was 6.0, as it led to the highest amount and lowest degradation of isolated protein.

In initial tests, isolated TcHMA4 degraded into many small fragments within 1–2 days. Therefore, the effect of different protease inhibitors (phenylmethylsulphonylfluoride—PMSF, PeFa-Block and protease inhibitor cocktails), EDTA and –SH group protecting reductants (DTT, β-mercaptoethanol, Trihydroxypropylphosphine—THP and tris(2-carboxyethyl)phosphine—TCEP) was tested. Protease inhibitors did not lead to any improvement (TcHMA4 degradation bands on the SDS gel and Western blot). Antioxidants had a strong positive effect, which is understandable in view of the 58 cysteine residues in the TcHMA4 sequence (48 in the 60 kDa main band isolated here, see characterisation below). Ten millimolars TCEP was by far the best because it led to the smallest amount of degraded protein bands on SDS gels and Western blots.

The final composition of the buffers was:

- Isolation buffer: 50 mM Hepes, 250 mM KCl, 10 mM TCEP, pH 6.0.
- Solubilisation buffer: 180 mM NaH_2PO_4, 2.0 M NaCl, 10 mM TCEP and 10 mM DDM, pH 6.0.

Purification of TcHMA4

All column materials were tested for the binding of TcHMA4 at pH values of 5–9. Among the tested materials, the Ni–IDA column from Macherey–Nagel was the most effective in binding the TcHMA4 protein, leading to a strong band on SDS gel and Western blot in the elutions and hardly any TcHMA4 in the washes. Less binding was observed for the Ni–TED material. Cu-loaded fractogel yielded complete binding but lacked specificity, while Cd- or Zn-loaded fractogel, Talon-resin and Ni–Sepharose did not bind TcHMA4 protein at all. After these initial column binding tests, the binding conditions on the column were optimised for pH and salinity, resulting in the use

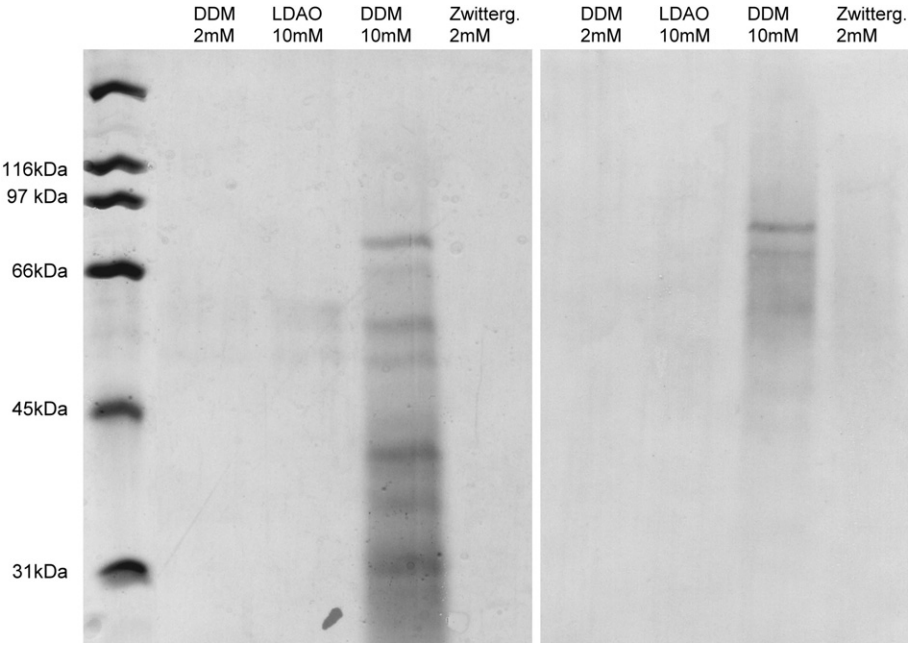

Fig. 1. Different detergents in the solubilisation buffer, gel and blot of crude extract. Lanes from left to right: 2 mM DDM, 10 mM LDAO, 10 mM DDM, 20 mM Zwittergent 3–12.

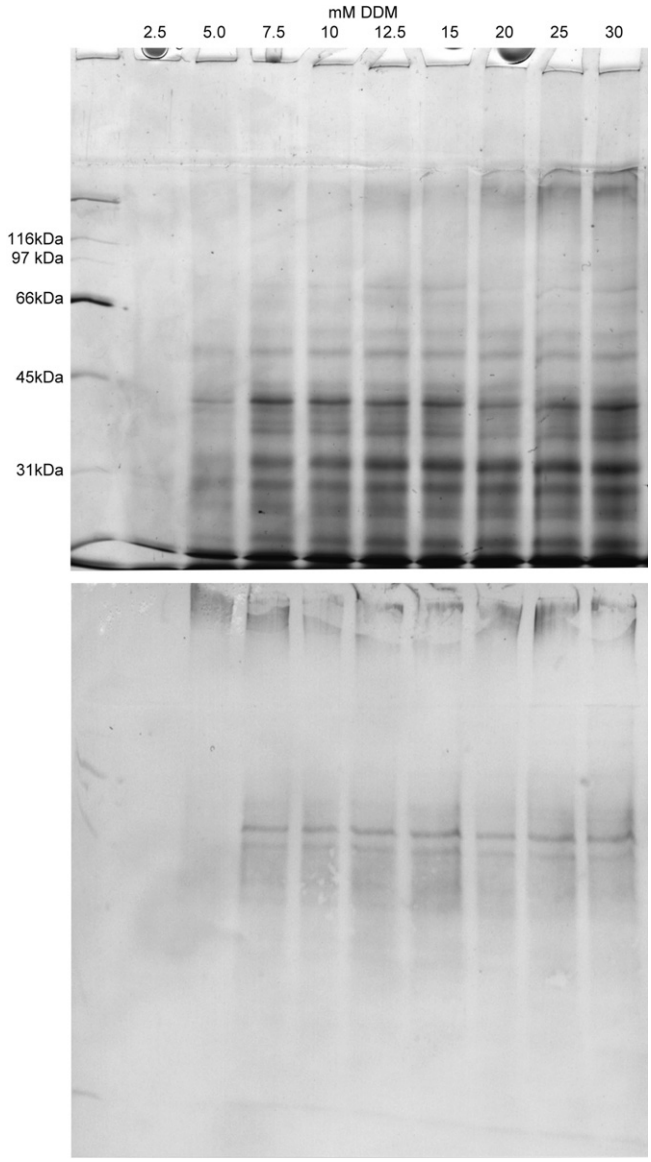

Fig. 2. Silver gel and Western blot of TcHMA4 solubilised with DDM in different concentrations. Lanes from left to right: Marker, 2.5, 5, 7.5, 10, 12.5, 15, 20, 25 and 30 mM DDM.

of pH 8.5 and 0.3 M NaCl on the column. Tests for separating TcHMA4 from weakly bound proteins resulted in the use of an exponential gradient of increasing imidazole and decreasing pH for elution.

The resulting protocol was as follows. First, the column was equilibrated with 10 bed volumes of a buffer containing 49.5 mM Hepes, 0.5 mM $NaH_2PO_4H_2O$ (pH 8.5), 300 mM NaCl, 2 mM TCEP and 2 mM DDM. The solubilised crude membrane protein was diluted 1:5 with the same buffer as used for equilibration and the pH was adjusted to pH 8.5. It was loaded onto the Ni-column at a flow rate of 0.1 bed volumes per minute. The unbound proteins were collected in the first wash fraction (designated as W_0). The column was then washed with 20 bed volumes of the same buffer. Subsequently, the bound protein was eluted with equilibration buffer, and an exponential gradient of imidazole increasing from 0 to 2 M and pH decreasing from 8.5 to 6.25. The elution fractions were collected, concentrated with centrifugal concentrators and stored on ice.

The purity of the eluted protein as well as the molecular weight was assessed using SDS–PAGE and Western blotting (Fig. 3). TcHMA4 was eluted in high purity at about 320 mM imidazole and pH 6.5. Protein estimation of this purified protein compared to the total amount of protein solubilised from the roots showed that we could isolate about 500 μg of TcHMA4 protein per gram dry weight (about 50 μg g^{-1} fresh weight) of root material. The purified TcHMA4 represented about 5% of the total protein solubilised from the roots.

Characterisation of purified TcHMA4

Gels of crude root extract and of purified protein showed two TcHMA4-specific bands of about 50 and 60 kDa, respectively (Figs. 2 and 3). In contrast, the mRNA for the *TcHMA4* gene, isolated from *T. caerulescens* plants grown under the same conditions as applied now, predicted a single protein with a size of 128 kDa [16]. This revealed that post-translational processing occurred. The binding of the 60 kDa band to the column and its recognition by the primary antibody (Fig. 3) showed that it contained the C-terminal ca. 550 amino acids of TcHMA4 including the polyhistidine repeat and the region containing 48 cysteine residues, but not the predicted phosphorylation site.

Nevertheless, the activity assay showed that the purified TcHMA4 is active as an ATPase with a turnover rate of

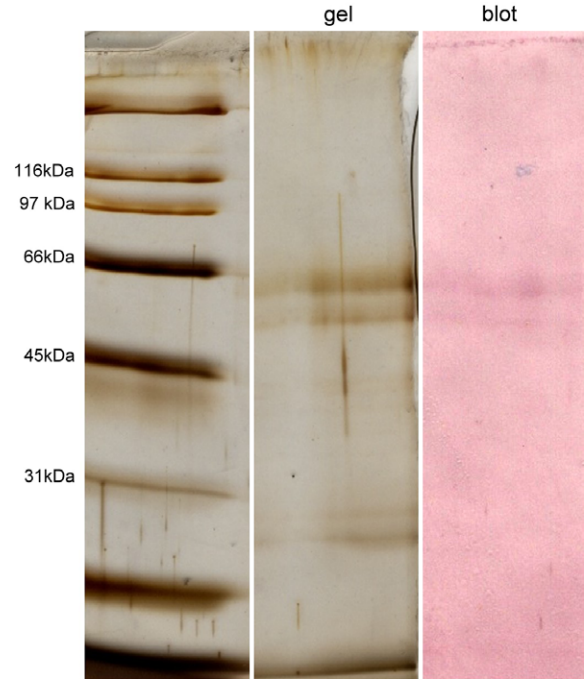

Fig. 3. Purified protein eluted from the Ni–IDA column: silver gel (left) and Western blot with TcHMA4 antibody (right).

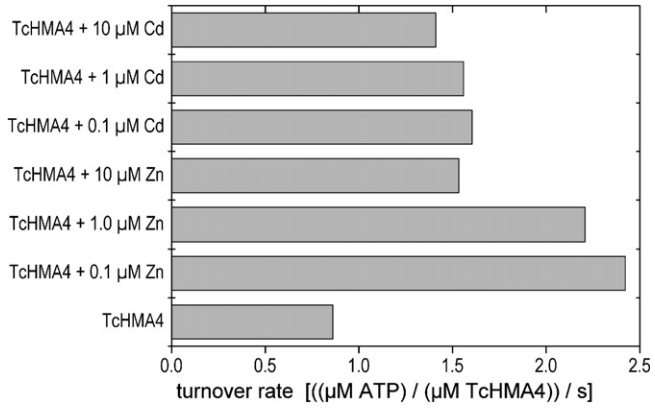

Fig. 4. Activity assay combined with metal activation test.

0.85 (µM ATP/µM TcHMA4)/s, verifying the intact state of the protein. This rate could be increased strongly by the addition of Zn^{2+} and Cd^{2+} to the reaction mix (Fig. 4), showing that TcHMA4 is activated by these two metals in concentrations of 0.1 µM. The activation by Zn^{2+} was much stronger compared to Cd^{2+}, indicating that this transporter is mainly responsible for zinc uptake. With increasing concentrations of both metal ions, the activity decreased slightly compared to the value at 0.1 µM, showing substrate inhibition.

The ATPase activity of the isolated protein, combined with the fact that the 50 kDa band was recognised by the primary antibody, therefore indicates that the 50 kDa band is a second subunit that contains the phosphorylation site and the antibody binding site. The finding that sometimes it was lost during purification while the 60 kDa band was always present indicates that the 50 kDa band may not contain the polyhistidine repeat, but is purified along with the 60 kDa band only if the two subunits are bound to each other.

Conclusions

In this project we succeeded to isolate and purify TcHMA4, a Cd/Zn transporting ATPase, from natural overexpression in the roots of *T. caerulescens*. This strategy, in contrast to heterologous overexpression, was chosen to obtain the protein in its native state without problems of misfolding and with all natural post-translational modifications. The latter was important, as the protein, which has a predicted size of 128 kDa based on the cDNA sequence, appears to be post-translationally split, resulting in a main band at ca. 60 kDa and a secondary band at ca. 50 kDa. Purification of TcHMA4 was achieved by systematic testing and optimisation of various parameters involved in solubilisation, column binding and elution. The resulting protocol allowed for isolation and purification of TcHMA4 in high yield, good purity and in its metal inducible active state. Future work will now focus on reconstitution into black membranes and will allow for a more detailed analysis of the transport characteristics of the protein. Spectroscopic and structural investigations are planned to analyse the binding environment of cadmium and zinc and the pumping mechanism.

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