Rapid purification of a functionally active plant sucrose carrier from transgenic yeast using a bacterial biotin acceptor domain

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Abstract A rapid and efficient method has been used for the purification of a *Plantago major* sucrose carrier from *Saccharomyces cerevisiae*. The C-terminal fusion of a bacterial biotin acceptor domain to the carrier protein did not interfere with the targeting to the yeast plasma membrane nor with the catalytic activity of the sucrose carrier. The chimeric construct is biotinylated by yeast cells in vivo and represents the only biotinylated protein in yeast membranes. Solubilized biotinylated carrier protein binds selectively to immobilized monomeric avidin and can be eluted as pure protein with free biotin. The purified protein is functionally active and catalyzes the energy-dependent transport of sucrose into proteoliposomes.

Key words: Biotinylation; Heterologous expression; Protein purification; Reconstitution; Sucrose transporter

1. Introduction

Plant sucrose carriers belong to a large family of membrane transporters including uni-, sym-, and antiporters with transport capacities for different substrates [1]. Despite the still increasing number of transporter sequences belonging to this family (genes or cDNAs of 60–70 family members have been cloned) only little structural information on this group of proteins is available. Information on the secondary structure of these proteins has been obtained primarily from studies of the *Escherichia coli* lactose permease and of different mammalian monosaccharide uniporters. A topological model with 12 putative transmembrane helices was first suggested for a human glucose uniporter [2] and later confirmed by others [3–5].

Hardly any information is available on the tertiary structure. The limited data which are currently available have been obtained mainly using site-directed mutagenesis (especially with the *E. coli* lactose permease: [6,7]) or random mutagenesis (with the *Chlorella kessleri* HUP1 glucose-H^+ symporter [8]). All deduced predictions on putative intramolecular interactions, however, are circumstantial and describe only tiny portions of the respective protein.

The only way to obtain a clear picture of the structure of these proteins is the generation of 2-dimensional or 3-dimensional crystals or the structural analysis of purified protein by NMR. For most membrane proteins, however, already the first steps towards any of these techniques are hindered by the low expression levels of these proteins, by their extreme hydrophobicity and the concomitant difficulties during purification.

The attachment of hydrophilic tags to such proteins has become an important tool to overcome at least part of these problems [9–12]. The present paper describes the advantages of a bacterial biotin acceptor domain for the one-step purification of the recombinant *P. major* sucrose carrier PmSUC2 [13] from transgenic yeast.

2. Materials and methods

2.1. Chemicals

D-[U-^{14}C]Sucrose (677 mCi/mmol) was purchased from Amersham Buchler (Braunschweig, Germany). Octyl-β-D-glucoside was purchased from Calbiochem (Bad Soden, Germany) immobilized monomeric avidin from Pierce (Rockford, IL, USA) and L-α-phosphatidylethanolamine from Sigma (Deisenhofen, Germany).

2.2. Strains

The proteinase-deficient *S. cerevisiae* strain c13-ABYS-86 [14] was used for all expression studies to avoid potential problems with yeast inherent proteinase activities. For cloning in *E. coli* the strain DH5α was used.

2.3. Construction of PmSUC2-Bio-His6

The full-length *PmSUC2* cDNA clone pTP18 [13] was used as template for a polymerase chain reaction (PCR) with an universal sequencing primer and the oligonucleotide PTP1XH6 (5′-GAA GGA ATT CTA GTG GTG GTG GTG GTG GTG GCT CGA GTG ACC TCC AGC CAC AC-3′) to add the sequence Ser-Ser-His-His-His-His-His-His to the C-terminus of the PmSUC2 protein [15]. The two serine residues were encoded by a unique *Xho*I restriction site, the last histidine codon was followed by a TAG stop codon and an *Eco*RI site. The PCR product was digested with *Eco*RI and ligated into pUC19 yielding plasmid pSH13.

The biotin acceptor domain from the α-subunit of the *Klebsiella pneumoniae* oxaloacetate decarboxylase [16] was excised as a 306-bp *Xho*I fragment from the vector pTC57 (provided by Dr. Ronald Kaback, Howard Hughes Medical Institute, University of California Los Angeles, USA), cloned into the unique *Xho*I site of pSH13, and the *Eco*RI insert was cloned into the yeast/*E. coli* shuttle vector NEV-E [17] yielding pSH213.

2.4. Transformation of yeast and transport tests

S. cerevisiae strain c13-ABYS-86 was transformed as described [18]. Strain GDY2133 contains the construct with the added biotin acceptor domain (PmSUC2-Bio-His6), strains GDY2 (NEV-E with unmodified PmSUC2) and GDY1000 (empty NEV-E vector) served as controls. Transformants were grown on CAA-medium (0.67% yeast nitrogen base without amino acids, 1% bacto casamino acids, 2% glucose).

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; cytochrome-*c*-oxidase, ferrocytochrome *c*:oxygen oxidoreductase (EC 1.9.3.1); OG, octyl-β-D-glucoside; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

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Transport of sucrose into yeast cells was tested in the presence of 10 mM D-glucose as previously described [13].

2.5. SDS-polyacrylamide gel electrophoresis and Western blots

Solubilized proteins were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose filters as described [19,20]. Binding of anti-PmSUC2 antibodies [21] or streptavidin-peroxidase on Western blots was detected with a chemiluminescence Western blot detection kit. Protein concentrations were determined as in [22].

2.6. Isolation of yeast total membranes or plasma membranes

Plasma membranes and total mebranes from yeast cells were isolated as previously described [12].

2.7. Purification of PmSUC2-Bio-His6 with immobilized monomeric avidin

The yeast strain GDY2133 was grown in CAA medium that was supplemented with D-(+)-biotin (2 mg/l) and total membranes were prepared. A fraction that contained 2 mg of protein was solubilized in 1 ml of SK-buffer (50 mM KPO_4, pH 6.3 with 500 mM KCl, 1.25% OG, 50 mM sucrose, 2 mg/ml L-α-phosphatidylethanolamine) and centrifuged at 150,000 × *g* for 30 min. The supernatant was applied to an avidin-sepharose-column (bed volume 150 μl) that had been prepared as published [9] and equilibrated in SK-buffer. Unspecifically bound proteins were removed by washes with 2 ml of SK-buffer and 2 ml of S-buffer (= SK-buffer without KCl). The PmSUC2-Bio-His6 protein was finally eluted from the column with 2 ml of S-buffer that contained 2 mM D-(+)-biotin.

2.8. Preparation of proteoliposomes and reconstitution of purified protein

Cytochrome-*c*-oxidase was incorporated into liposomes as described [23] and 660 μl of this preparation were mixed with 1 ml of the column-purified protein. Sucrose and OG were added from stock solutions to yield the initial concentrations of 50 mM and 1.25%, respectively. After a 20 min incubation on ice the reconstitution was induced by rapid dilution of this mixture into 80 ml of 50 mM KPO_4-buffer pH 6.3. After stirring at RT for 5 min, vesicles were collected by ultracentrifugation (100,000 × *g*, 1 h, 4°C), resuspended in 360 μl 50 mM KPO_4-buffer pH 6.3, containing 1 mM $MgSO_4$, frozen in liquid nitrogen, and stored at −80°C. Transport of sucrose into reconstituted vesicles was measured at 30°C and at a sucrose concentration of 112 μM (specific activity 78.6 mCi/mmol) as described [12].

3. Results

3.1. Identification of modified PmSUC2 proteins in plasma membranes of transgenic baker's yeast

The C-terminal modification of the PmSUC2 protein was confirmed by nucleic acid sequencing, the amino acid sequence of the modified C-terminus is given in Fig. 1. Expression levels of the modified and unmodified carrier proteins were studied on Western blots of SDS-solubilized plasma membrane proteins from transformed yeast cells. Signals of comparable intensities were obtained with anti-PmSUC2 antibodies in Fig. 2A for PmSUC2 (lane 1) and PmSUC2-Bio-His6 (lane 3), indicating that the modified protein is equally well expressed and targeted to the plasma membrane as the unmodified PmSUC2 protein. The fusion of the 96 amino acid biotin accepting domain (110 amino acids with the protease site and the histidine tail) caused a shift in the apparent molecular weight from 35 kDa to 50 kDa. This 50 kDa derivative is the only protein which is detected by streptavidin in yeast plasma membranes (Fig. 2B) or total membranes (data not shown), which indicates not only that the *K. pneumoniae* biotin acceptor domain is biotinylated by the yeast biotin ligase, but also that yeast membranes do not contain inherent biotinylated proteins. Formation of aggregates amongst the overexpressed, lipophilic transport proteins varies with the preparation and yields additional signals at higher apparent molecular weights (Fig. 2A).

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501  IKNLSVAGGH SSIEGRTAAA PAPAPAPAPA SAPAAAAPAG AGTPVTAPLA  550
551  GTIWKVLASE GQTVAAGEVL LILEAMKMET EIRAAQAGIV RGIAVKAGDA  600
601  VAVGDTMLTL VDSSHHHHHH *                                 620
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Fig. 1. C-Terminal sequence of the PmSUC2-Bio-His6 protein. Numbers give the position of amino acid residues in the PmSUC2-Bio-His6 protein. Amino acid residues 501–510 = C-terminus of the unmodified PmSUC2 protein; amino acid residues 513–516 (IEGR) = factor Xa restriction-protease site; amino acids 517–612 (underlined) = residues 501 to 594 from the α-subunit of the *Klebsiella pneumoniae* oxalacetate decarboxylase [16]; amino acids 615–620 = polyhistidine tail. The boxed double-serine residues were generated during the introduction of the *Xho*I cloning site.

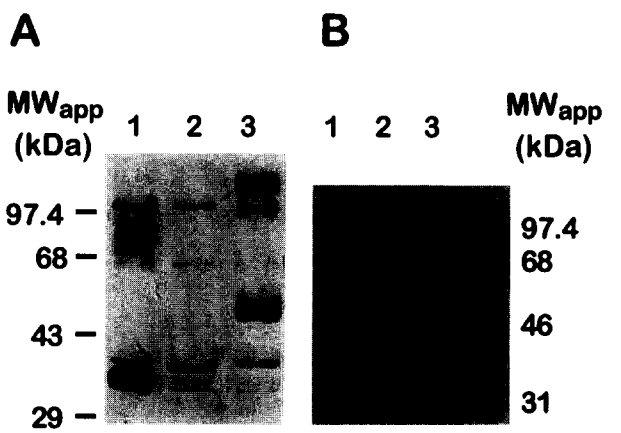


Fig. 2. Identification of modified PmSUC2 proteins in SDS-extracts from yeast plasma membranes. SDS-extracts from plasma membranes of yeast strains GDY2 (unmodified PmSUC2; lanes 1), GDY1000 (control; lanes 2) and GDY2133 (PmSUC2-Bio-His6; lanes 3) were separated on SDS-polyacrylamide gels (3 μg/lane) and blotted. (A) Blots were treated with affinity purified anti-PmSUC2 antibodies. (B) Blots were treated with streptavidin-horseradish peroxidase conjugate.

Fig. 3 shows that the strains expressing PmSUC2 or PmSUC2-Bio-His6 transport sucrose with similar rates; sucrose transport into *S. cerevisiae* control cells (GDY1000) is negligible.

3.2. In vivo biotinylation of PmSUC2-Bio-His6 protein in S. cerevisiae

Initial attempts to purify PmSUC2-Bio-His6 on avidin-columns showed that only a small fraction of the protein was biotinylated in yeast (2–5%) and that the majority of the protein was in the flow-through fraction. This protein was detected on Western blots with anti-PmSUC2 antiserum but not with streptavidin-peroxidase conjugate (data not shown). Since *S. cerevisiae* is a natural auxotroph for biotin the usual concentrations of biotin in the growth medium (3 μg/l in CAA-medium) may become limiting during extensive overexpression of biotinylated proteins. An inherent high affinity biotin transport system [24] could be used to import sufficient biotin into the respective yeast cells. Strain GDY2133 was therefore grown at different biotin concentrations, total membranes were prepared, and solubilized proteins were separated and blotted. The Western blot presented in Fig. 4 shows a drastic increase in PmSUC2-Bio-His6 biotinylation with increasing biotin concentrations in the medium, and maximum labelling is seen in lanes 5 and 6

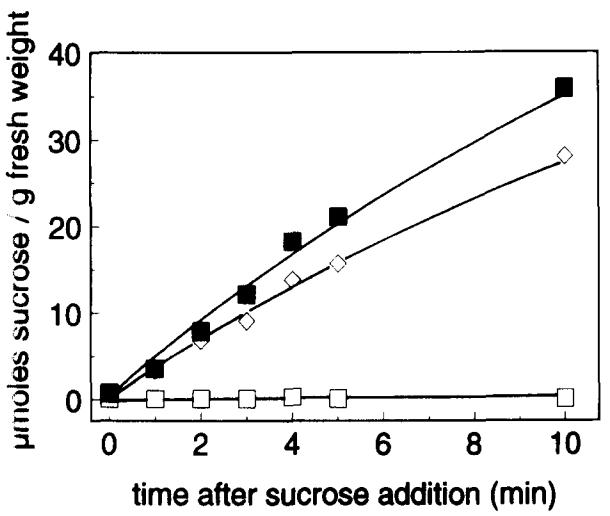


Fig. 3. Transport activities for sucrose by wild type and modified PmSUC2 transport proteins in transgenic yeast cells. □ = strain GDY1000 (control); ◇ = strain GDY2 (PmSUC2); ■ = strain GDY2133 (PmSUC2-Bio-His6).

(2 and 20 mg/l, respectively). For this reason the yeast medium was supplemented with 2 mg/l biotin for all further experiments.

3.5. *Purification of PmSUC2-Bio-His6 with immobilized monomeric avidin and reconstituition of purified protein into proteoliposomes*

Total membranes were isolated from GDY2133 cells which were grown on CAA-medium supplemented with biotin. After solubilization with OG and removal of insoluble material by centrifugation solubilized proteins were applied to an avidin-Sepharose-column. The vast majority of the loaded protein did not bind to the column under the selected conditions (Fig. 5A; lane 2). Washes at high salt (500 mM) removed unspecifically bound proteins (Fig. 5A; lanes 3 and 4) and additional washes with no KCl in the buffer had no further effect. Addition of 2 mM biotin caused an immediate and complete efflux of one single protein from the column (Fig. 5A; lanes 7 and 8). With anti-PmSUC2 antiserum this protein could be identified as PmSUC2-Bio-His6 protein on Western blots (Fig. 5B). Varying amounts of aggregated PmSUC2-Bio-His6 protein were detected on the top of the gel in different experiments.

The functional integrity of the purified PmSUC2-Bio-His6 protein was demonstrated by reconstitution into proteoliposomes which were energized with beef heart cytochrome-*c*-oxidase. Fig. 5C shows that the purified PmSUC2-Bio-His6 protein catalyzes the proton-motif-force-dependent sucrose uptake into these vesicles.

4. Discussion

Since the functional expression of the first plant sugar transporter in *S. pombe* [25] yeast cells became increasingly important for the expression cloning of many other carrier proteins [26] and recently also for the patch-clamp analysis of recombinant channel proteins [27]. Large scale purification of proteins from yeast, however, was assumed to be limited in this organism [26]. Here we describe a simple, rapid and efficient technique for the purification of recombinant membrane proteins from baker's yeast.

After addition of the large hydrophilic domain of the biotin acceptor domain from *K. pneumoniae* [16] PmSUC2-Bio-His6 bound quantitatively to avidin-Sepharose-columns and was released only in the presence of free biotin in the elution buffer (Fig. 5A and B). No contaminations with other proteins could be detected even when large amounts of purified protein were loaded on SDS-gels (data not shown). PmSUC2-Bio-His6 protein purified with immobilized monomeric avidin showed no loss in transport activity when tested in proteoliposomes with an artificial proton-motif-force generator (Fig. 5C).

For a direct comparison of the usefulness of two C-terminal tags for protein purification the 6 C-terminal histidine residues of PmSUC2-Bio-His6 were used to enrich PmSUC2-Bio-His6 from *S. cerevisiae* GDY2133 with a Ni^{2+}-nitrilo acidic acid-column (data not shown) [15]. Due to the permanent loss of PmSUC2-Bio-His6 during the various washes and due to the co-purification of an inherent, histidine-rich yeast protein less PmSUC2-Bio-His6 protein of lower purity was obtained. This problem has been described previously [12].

It had been shown previously that a bacterial biotinylation domain from *Propionibacterium shermanii* [28,29] is accepted by the yeast biotin ligase [30]. The presented paper demonstrates (i) that the biotin acceptor domain from *K. pneumoniae* is a substrate for the yeast biotin ligase, (ii) that no biotinylated yeast protein is membrane localized and that the simple preparation of crude membranes eliminates co-purification of inherent yeast proteins, (iii) that the C-terminal addition of this domain has no influence on the expression and targeting of the studied protein, and (iv) that this C-terminal fusion can be used for the one-step purification of recombinant membrane proteins from yeast.

From the presented data it can be calculated that 2–5 mg of functionally active PmSUC2-Bio-His6 protein can be isolated from 10 liters of yeast cells. The purification of biotin-tagged

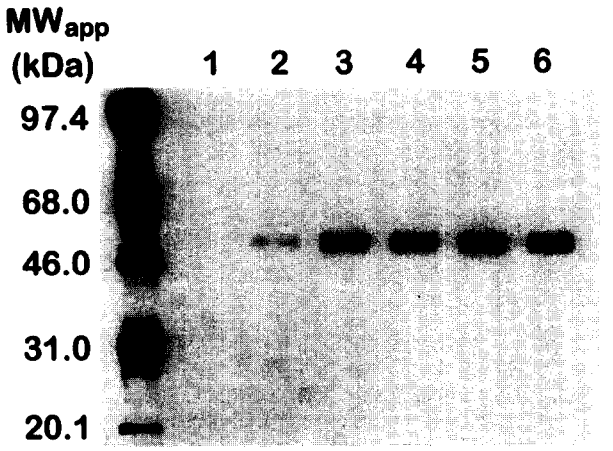
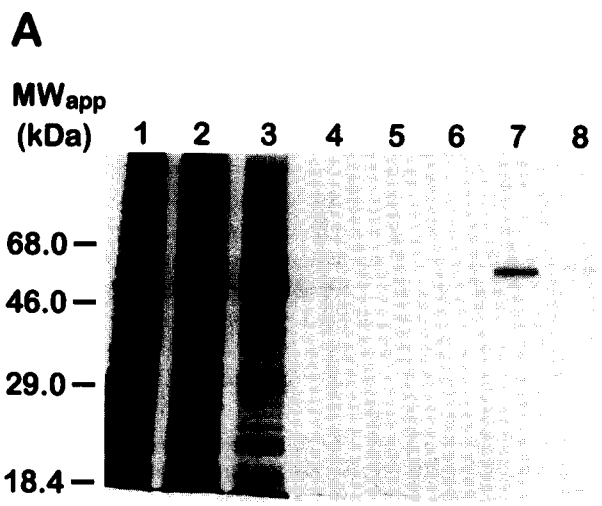
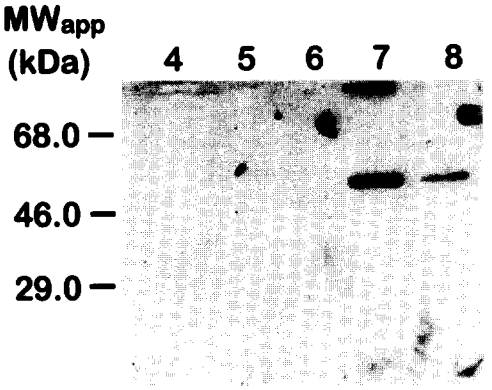


Fig. 4. In vivo biotinylation of PmSUC2-Bio-His6 by *Saccharomyces cerevisiae*. Yeast strain GDY2133 was grown in CAA-medium containing different concentrations of biotin (lane 1 = 3.0 µg/l (no extra biotin added), lane 2 = 5.4 µg/l, lane 3 = 23 µg/l, lane 4 = 200 µg/l, lane 5 = 2 mg/l, lane 6 = 20 mg/l). Total membrane proteins (30 ng) were separated on polyacrylamide gels, Western blotted and treated with streptavidin-horseradish peroxidase conjugate. Biotinylated molecular weight markers were used.





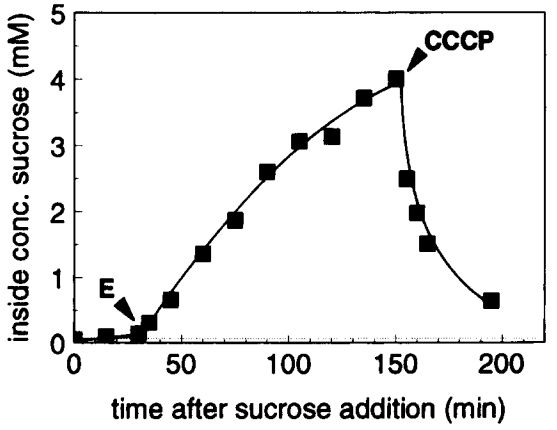


Fig. 5. Purification of PmSUC2-Bio-His6 protein from yeast strain GDY2133 with monomeric avidin, detection on Western blots, and reconstitution into proteoliposomes. (A) Lane 1 = OG solubilized total membranes; lane 2 = flow through; sequential washes with 1 ml of the following buffers: lane 3 = SK-buffer; lane 4 = SK-buffer; lane 5 = S-buffer; lane 6 = S-buffer; lane 7 = S-buffer with 2 mM biotin; lane 8 = S-buffer with 2 mM biotin. See section 2 for description of the buffers. (B) Same protein separation as shown in (A). Lanes 4–8 correspond to lanes 4–8 in (A). Proteins were Western blotted and detected with anti-PmSUC2 antiserum. (C) Uptake of [^{14}C]sucrose into proteoliposomes, that were prepared from a 1 ml fraction of PmSUC2-Bio-His6 (lane 7 of A and B). E indicates the addition of ascorbate, TMPD and cytochrome-*c* to the transport test, CCCP indicates the addition of uncoupler.
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plant membrane proteins from recombinant yeast cells will thus be a tool to obtain sufficient amounts of functionally active protein for further structural analyses.

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