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# Expression and purification of human and *Saccharomyces cerevisiae* equilibrative nucleoside transporters☆

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ABSTRACT

Nucleosides play an essential role in the physiology of eukaryotes by acting as metabolic precursors in *de novo* nucleic acid synthesis and energy metabolism. Nucleosides also act as ligands for purinergic receptors. Equilibrative nucleoside transporters (ENTs) are polytopic integral membrane proteins that aid in regulating plasmalemmal flux of purine and pyrimidine nucleosides and nucleobases. ENTs exhibit broad substrate selectivity across different isoforms and utilize diverse mechanisms to drive substrate flux across membranes. However, the molecular mechanisms and chemical determinants of ENT-mediated substrate recognition, binding, inhibition, and transport are poorly understood. To determine how ENT-mediated transport occurs at the molecular level, greater chemical insight and assays employing purified protein are essential. This article focuses on the expression and purification of human ENT1, human ENT2, and *Saccharomyces cerevisiae* ScENT1 using novel expression and purification strategies to isolate recombinant ENTs. ScENT1, hENT1, and hENT2 were expressed in W303 *Saccharomyces cerevisiae* cells and detergent solubilized from the membrane. After detergent extraction, these ENTs were further purified using immobilized metal affinity chromatography and size exclusion chromatography. This effort resulted in obtaining quantities of purified protein sufficient for future biophysical analysis.



Equilibrative nucleoside transporters (ENTs) are polytopic integral membrane proteins (IMPs) found only in eukaryotic organisms that regulate the flux of purine and pyrimidine nucleosides and nucleobases, but not nucleotides across cellular membranes [1]. In addition to controlling the flux of endogenous ligands, ENTs are clinically relevant drug transporters that modulate the effectiveness for a variety of FDA/EMA approved medications (e.g., antineoplastic, antiarrhythmic, antihypertensive, and antiviral medications), and ENTs are known biomarkers for therapeutic efficacy in the treatment of certain cancers [2,3]. ENTs have also been associated with regulating seizure activity by regulating adenosine transport [4], and in seizures related to alcohol withdrawal syndrome [5,6]. Another important functional consideration was ENT-mediated adenosine transport. Acting through distinctive GPCRs, adenosine signaling facilitates an array of physiological responses such as vasodilation, coronary blood flow, myocardial oxygen supply-demand balance, inflammation, neurotransmission, hypoxia, trauma, and ischemia [7–14]. Inhibitors of ENTs also offer a

*The abbreviations used are:* mAU, milli-absorbance units; β-Me, β-mercaptoethanol; DDM, n-docecyl-β-D-maltoside; ENT, equilibrative nucleoside transporter; IMP, integral membrane protein; MWCO, molecular weight cutoff; SC-HIS, synthetic complete histidine dropout media; SEC, size exclusion chromatography; TMD, *trans*-membrane domain; YPG, yeast extract-peptone-galactose; GPCR, G-protein coupled receptor; MFS, major facilitator superfamily; IMAC, immobilized metal affinity chromatography; MBP, maltose-binding protein; LMNPG, lauryl maltose neopentyl glycol; OG/OCTG, octylglucoside; DM/DCM, decylmaltoside; CYMAL-4, cyclohexylbutylmaltoside; CPM, counts per minute; FDA, Food and Drug Administration; EMA, European Medicines Agency.

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protective advantage in ischemia, trauma, hypoxia, and certain types of seizure disorders by impeding adenosine uptake [4,7,8,11–13,15].

There are four human ENT isoforms (hENT 1–4, SLC29A1-4), each having 11 predicted transmembrane domains (TMDs) and large hydrophilic loops at either the N-terminus (hENT3-4) or in the region between TMD5 and TMD7 (hENT1-4) [16] – an anticipated topology that was comparable to other representatives of the Major Facilitator Superfamily (MFS) [17]. ENTs are ubiquitously expressed across most tissue types. ENT1 and ENT2 are primarily located in the plasma membrane. ENT3 contains an N-terminal dileucine motif (DE)XXXL(LI) [18] (characteristic motif for endosomal/lysosomal targeting) in the hydrophilic region of the sequence that precedes the first transmembrane domain (TMD) leading to an enrichment of ENT3 in intracellular membranes of the endosome/lysosome and mitochondria.

Functionally, ENTs are bidirectional, facilitative, transporters that employ concentration gradients of transportable permeants to regulate efflux and influx across cellular membranes. Indeed, passive transport was a hallmark of the ENT family but active proton-linked transport has been identified in protozoa [19], plants [20], and the transport activity of human ENT3 and ENT4 are stimulated at acidic pH [21,22]. A definitive transport mechanism has not been explicated for the ENT family, yet the working theory was that they function through an alternating access model consistent with other members of the MFS family [23,24]. The presence of active co-transport suggests the molecular transport mechanism can vary for members within the ENT family. However, functional characterization of ENTs at the molecular level has been largely hampered by struggles associated with large-scale heterologous overexpression and production of purified ENTs as a foundation for driving detailed biochemical analyses.

Initial characterization of ENTs occurred in erythrocytes [25,26], followed by mammalian tumor cell lines [27,28], then *Xenopus laevis* oocytes [1], and this ultimately led to attempts of characterizing purified transporters [29–33] – but ENTs were impervious to characterization in a purified state. Characterization of purified ENTs has faced several hurdles: 1) finding detergents that adequately solubilize and stabilize the proteins [32], 2) determining the lipid composition of artificial membranes [30,33], and 3) obtaining functionally active protein [29–32]. The instability of detergent solubilized protein has been a major barrier associated with the molecular characterization of purified ENTs. Subsequently, there are currently no molecular structures for any member of the ENT family.

In an effort to thwart the difficulties associated with human ENTs, recent purification efforts have focused on orthologs from lower order species. ScENT1 from *Saccharomyces cerevisiae* [34] (previously referred to as Function Unknown Now 26) is the only known ENT in yeast. ScENT1 is ~19% identical to hENT1-3 and has a predicted topology and cellular localization (vacuolar membrane) [35,36] similar to hENT3. Unlike hENT3, ScENT1-mediated transport does not appear to be impacted by membrane potential [34]. Given ScENT1's broad transport profile [34] and cellular localization, it is presumably involved in nucleoside and nucleobase recycling between the vacuole and cytoplasm in an effort to establish and maintain metabolic balance. ENTs have been identified as the sole mediators of nucleoside transport in plants, however, most plant ENTs function as substrate-proton symporters [20]. *Arabidopsis thaliana* has had 5 of its 8 ENT isoforms functionally characterized. AtEN7 is ~19% identical to hENT1-2 and ScENT1, and localizes to the plasma membrane [37]. Thus far, AtENT7 is the only isoform not stimulated by external pH [38]. Therefore, it may be functioning as an archetypical ENT.

Recombinant ScENT1 utilized homologous overexpression and was detergent extracted, purified using membrane isolation, Immobilized Metal Affinity Chromatography (IMAC), and Size Exclusion Chromatography (SEC), and reconstituted into liposomes with non-native lipids for functional characterization [34]. AtENT7 was heterologously expressed in *Pichia pastoris* followed by detergent extraction, purification using IMAC and fluorescence SEC, and functionally assessed using microscale thermophoresis [39]. ScENT1 and AtENT7 are the only ENT family members to undergo detergent extraction from the lipid bilayer and retain functional characteristics in purified form - a major advancement in the path to structure determination. In the current work, we present protocols for the overexpression and purification of functional ScENT1, human ENT1, and human ENT2 proteins and compare our approaches to those previously published.

## 1. Experimental procedures

The composition of all buffers can be found in Table 1. The pH of Tris-HCl was determined at room temperature and proteins were kept on ice or at 4 °C unless indicated otherwise.

### 1.1. Molecular cloning of ScENT1, hENT1, and hENT2

Native ScENT1 open reading frame was PCR amplified from S288C *Saccharomyces cerevisiae* genomic DNA (Novagen #69240-3) using ScENT1-FOR (GGTAGCGGCGGAGGAGGCAGCGGACTAGTCCCAAGAGGTAGTAGTACTAGTGCGGACACT) and ScENT1-REV (AATGTAAGCGTGACATAACTAATTACATGACTCGAGTCACCCCCTGATAATAAAGTCAAT) primers. The resulting PCR product was cloned into the modified yeast 2μ plasmid "83χ" [40,41], by homologous recombination in yeast, resulting in an N-terminal 10-histidine-thrombin-GSS-ScENT1 protein expression construct. Native hENT1 and hENT2 were PCR amplified from a human cDNA library (Invitrogen) using LIC primers FOR (CAAGGACCGAGCAGCCCCTCAACAACCAGTCACCAG) and REV (ACCACGGGGAACCAACCCTCCAGGACAAGGCCCGTTAACACACT), where the underlined nucleotides are target gene sequence without a start codon. The resulting PCR products were cloned into the modified yeast 2μ plasmid termed "83β2" (Membrane Protein Expression Center, UCSF, San Francisco, CA), which contains an N-terminal 10X histidine-MBP (maltose binding protein) tag followed by a 3C protease cleavage site. All plasmid maintenance and propagation was conducted in XL2Blue Ultracompetent *Escherichia coli* cells (Stratagene #200150).

### 1.2. Expression of ScENT1, hENT1, and hENT2 in Saccharomyces cerevisiae

Expression efforts utilized W303-Δpep4 (*leu2-3112 trp1-1 can1-100 ura3-1 ade2-1 hwas3-11,15 Δpep4 MATα*) *S. cerevisiae* cells transformed with sheared salmon sperm DNA (Invitrogen #15632-011) using a lithium acetate transformation protocol [42]. Positive yeast transformants were selected in 1x synthetic complete histidine dropout media ("SC-HIS") to select for episomal HIS3 expression. 1x SC-HIS growth media contains 1x CSM-HIS (Sunrise Science #1023), 0.67% (*w/v*) yeast nitrogen base without amino acids (RPI #Y20040), and a final carbohydrate concentration of 2% (*w/v*) [ScENT1, 1% (*w/v*) D-glucose (Sigma-Aldrich #G8270), and 1% (*w/v*) D-raffinose (Carbosynth #OR06197); hENT1 and hENT2 2% (*w/v*) D-glucose]. ScENT1 cultures were grown in 10 L working volumes using a 12 L fermenter at 30 °C with 500 μL of 50% antifoam 204 (Sigma-Aldrich #A6426). Agitation was set from 200 to 350 rpm based on a dissolved oxygen range of 90%–20% with an airflow rate of 2.5 L/min. Each round of protein expression contained 7.125 L of 1X SC-His medium and 375 ml of overnight culture. Following a 24 h growth period, protein expression was induced by adding 2.5 L