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Expression, purification and characterization of human proton-coupled oligopeptide transporter 1 hPEPT1

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ABSTRACT

The human peptide transporter hPEPT1 (SLC15A1) is responsible for uptake of dietary di- and tripeptides and a number of drugs from the small intestine by utilizing the proton electrochemical gradient, and hence an important target for peptide-like drug design and drug delivery. hPEPT1 belongs to the ubiquitous major facilitator superfamily that all contain a 12TM core structure, with global conformational changes occurring during the transport cycle. Several bacterial homologues of these transporters have been characterized, providing valuable insight into the transport mechanism of this family. Here we report the overexpression and purification of recombinant hPEPT1 in a detergent-solubilized state. Thermostability profiling of hPEPT1 at different pH values revealed that hPEPT1 is more stable at pH 6 as compared to pH 7 and 8. Micro-scale thermophoresis (MST) confirmed that the purified hPEPT1 was able to bind di- and tripeptides respectively. To assess the in-solution oligomeric state of hPEPT1, negative stain electron microscopy was performed, demonstrating a predominantly monomeric state.

1. Introduction

The mammalian small intestine can be thought of as a sieve that allows uptake of dietary nutrients, many of them through specific transport proteins. Among these, the human proton-coupled oligopeptide transporter 1 (hPEPT1) accounts for cellular uptake of di- and tripeptides produced from digestion of dietary proteins in the small intestine [1]. hPEPT1 is a low-affinity, high-capacity transporter, suggesting that it does not saturate even in conditions of high peptide concentration [2]. It has a wide substrate specificity of di- and tripeptides and peptidomimetics [3]. Additionally, hPEPT1 is also involved in the absorption of several orally administered drugs, including angiotensin-converting enzyme (ACE) inhibitors, β-lactam antibiotics, an ionotropic glutamate receptor antagonist, Valacyclovir and L-DOPA-L-Phe [4–10]. Thus, the transporter also plays a significant role in enhancing the bioavailability of drugs that cannot or only poorly diffuse through the membrane [11–13].

hPEPT1 belongs to the solute carrier 15 (SLC15) family, which

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belongs to the large and well-characterized major facilitator superfamily [14]. The human genome encodes four SLC15 members; the prototypical and well-characterized hPEPT1 (SLC15A1) and hPEPT2 (SLC15A2), expressed primarily in the small intestine and kidneys, respectively, and, hPHT1 (SLC15A3) and hPHT2 (SLC15A4) that transport histidine in addition to some peptides and are expressed primarily in the spleen, skeletal muscles and lungs; placenta, leukocytes, and heart respectively [15,16].

hPEPT1 consists of 708 amino acid residues arranged in 12 transmembrane helices [17] and transports di- and tripeptides across the plasma membrane by utilizing an inward directed electrochemical H^+ gradient [18,19].

The most prominent differences between mammalian and bacterial PEPTs is the presence of a 200 amino acid extracellular domain between helices 9 and 10 [20,21] and the absence of two "extra" transmembrane helices in mammalian PEPTs [20]. A crystal structure of the extracellular domain showed that it consists of two Ig-domains [22]. Furthermore, one of these domains was shown to interact with intestinal trypsin, which implies that the domain is involved in forming a transporter metabolon, i.e., transporter-enzyme complex, that generates co-localized peptides [22].

To our knowledge, there are no reports on recombinant expression and purification of any eukaryotic POTs, that enable subsequent biophysical studies. In this work, we produced purified recombinant full length detergent solubilized hPEPT1, and subsequently performed biophysical studies. The produced protein was able to transport peptides into intact cells, and in membrane isolates respectively, and thus functionally active. The purified detergent solubilized protein was thermostable to 71 °C and able to bind di- and tripeptides.

2. Material methods

2.1. Generation of recombinant baculovirus

Expression of hPEPT1 was carried out in *Spodoptera frugiperda* (Sf9) insect cells using recombinant baculovirus expression vectors. Initially, hPEPT1-encoding transfer vectors were prepared using the pPEU5 vector suite (kindly made available from Nicholas Simon Berrow, (IRB), PECF, Barcelona, Spain (https://www.irbbarcelona.org/en/research/protein-expression), followed by co-transfection of transfer vector and linearized viral DNA into Sf9 insect cells for production of recombinant virus by homologous recombination. A pcDNA 3.1 plasmid comprising the human pept1 gene (Uniprot P46059) was kindly provided by Birger Brodin (University of Copenhagen). From this, the hPEPT1 encoding region was amplified by PCR using the primers F1b-hPEPT1 5′-AGGAGATATACCATGGGAATGTCCAAATCACACAGTTTC-3′ and R2a-hPEPT1 5′-CTTCCAGACCGCTTGACATCTGTTTCTGTGAATTGGC-3′ and the purified PCR product was inserted into the pPEU5 vector using the In-Fusion HD Cloning Kit from Clontech, following the procedures described by the manufacturer. The resulting transfer vector pPEU5-hPEPT1 encoded hPEPT1 with a C-terminal Human Rhinovirus HRV 3C-protease (3C) cleavage site followed by a mCherry fusion and a hexa-histidine-tag (***hPEPT1-3C-mCherry-His$_6$***). The plasmid was confirmed by DNA sequencing. A 2nd generation pPEU-hPEPT1-based construct was prepared using the *Multi*Bac^{Turbo} system [23]. The hPEPT1-3C-mCherry-His$_6$ encoding region of pPEU5-hPEPT1 was amplified by PCR using the primers F2b-hPEPT1 5′-ATTGCGGATCCATGGGAATGTCCAAATCACACAGTTTC-3′ and R10-hPEPT1 5′-GATCTGCGGCCGCTCATTTTTCGAACTGCGGGTGGCTCCAACCTT-GAAAATATAAATTTTCCCC GTGATGGTGATGGTGATGGTGATGTTTG-3'. The resulting construct encoded ***hPEPT1-3C-mCherry-His$_8$-TEV--strepII*** was flanked by a *Bam* HI site at the 5′-end and a *Not* I site at the 3′-end. For construction of the plasmid pACEBac1-hPEPT1, the insert was cloned into the transfer vector pACEBac1 (ATG:biosynthetics) via the *Bam* HI and *Not* I restriction sites. The plasmid was confirmed by DNA sequencing. The pACEBac1-hPEPT1 plasmid was transformed into DH10*Multi*Bac^{Turbo} cells (ATG:biosynthetics) by electroporation, resulting in integration of the hPEPT1-construct into the baculoviral genome by Tn7 transposition. The recombinant bacmid was amplified, purified using the Purelink HiPure Plasmid Miniprep Kit (Invitrogen Life Technologies) and used for FuGene®HD (Promega) based transfection of Sf9 insect cells for the generation of recombinant virus. Initial virus stocks (P0) were further amplified, and titers were quantified using either plaque assays [24] or a SYBR Green based qPCR method [25].

2.2. Expression

The recombinant hPEPT1-3C-mCherry-His8-TEV-StrepII was expressed in Sf9 insect cells by using the baculovirus systems. Sf9 insect cells were maintained at cell densities of 0.5×10^6 viable cells/ml in SF-900 III SFM (GIBCO) media containing antibiotics 100 U/ml penicillin and 100 mg/ml streptomycin and fetal bovine serum (4% V/V), incubated at 27 °C with continuous shaking at 150 r.p.m. Small-scale expressions of hPEPT1 were performed in 100 ml cell cultures. Recombinant baculovirus was added corresponding to different multiplicities of infection (MOI 0.05–10) and the cultures were harvested at time intervals 24 h, 48 h, 72 h and 96 h. For large-scale expression of hPEPT1, recombinant baculovirus was added at a MOI of 0.05 per 1l cell cultures at a density of 3–4 $\times$ 10^6 viable cells/ml. The cell viability was followed closely by Trypan blue staining and cell counting to determine the optimal harvest time; usually around 45–50 h post infection where the viability dropped below 80%. The cells were harvested by centrifugation at 1,500$\times$ g for 15 min at 4 °C.

2.3. Western blotting

Expression levels were quantified by Western blotting using antibodies targeting the histidine-tag according to procedures described previously [26]. Briefly, the cell pellet from 1 ml culture was resuspended in 150 μl of lysis buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 1% DDM, 5 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and supplemented with one cOmplete™ Protease Inhibitor Cocktail tablet (Roche)/10 ml buffer. The cell suspension was incubated on ice for 30 min and then centrifuged at 13,000$\times$ g at 4 °C for 15 min to remove any cell debris. The cleared cell lysates were separated by SDS-PAGE and MagicMark™ XP Western Protein Standard (Thermo Fisher Scientific) was included as reference. Blot transfer and immunodetection were performed according to the manufacturer's protocols.

2.4. Protein purification

The cell pellet from a 2 l culture was resuspended in 50 ml of resuspension buffer and protease inhibitor (PI) tablets (cOmplete™, Mini, EDTA-free, Roche), sonicated and centrifuged at 10,000$\times$ g for 10 min. The supernatant was ultracentrifuged at 100,000 g at 4 °C for 1 h. The supernatant was discarded, and the pellets were solubilized using a glass dounce tissue homogenizer in a solubilizing buffer with 1% LMNG at 4 °C for 2 h. Insoluble material was removed by ultracentrifugation at 100,000 g for 1h at 4 °C. For affinity purification via the *StrepII* tag, solubilized membranes were incubated with pre equilibrated Strep-Tactin high capacity superflow resin (IBA Life Sciences, 2 ml/l cell culture) for 2 h at 4 °C with gentle rotation. After 20 CV washing with a wash buffer (WB) containing 100 mM sodium phosphate pH 7.8, 150 mM NaCl, 5 mM imidazole and 0.01% LMNG, the purified proteins were eluted with an elution buffer (wash buffer containing 2.5 mM desthiobiotin). All fractions having proteins after analyzing on SDS-PAGE were pooled together and batch bound with pre-equilibrated Talon resin for 2 h. After washing with 10 CV of wash buffer containing 10 mM Imidazole, for on-column cleavage of the purification tags, 1 CV of wash buffer supplemented with a histidine-tagged 3C-protease at 0.125 mg/ml final concentration was mixed with the resin and incubated at 4 °C O/N. The resin was poured into a gravity column and the purified protein

was collected in the flow through and initial wash fractions. Fractions containing purified protein were concentrated using Amicon® Ultra-4 centrifugal filters with 100,000 MWCO. Finally, protein was stabilized at pH 6, 100 mM sodium phosphate, 150 mM NaCl, 0.01% LMNG by using PD10 desalting column. The stabilized purified hPEPT1 protein was concentrated by ultrafiltration (Amicon 100 kDa cutoff, Millipore).

2.5. Gel electrophoresis

The hPEPT1 samples were analyzed by SDS-PAGE using a 10% Bis-Tris gel (Novex®) following the protocols given by the manufacturer. Electrophoresis was performed using MES buffer and Mark12™ unstained protein standards (Thermo Fisher Scientific). The gels were stained using Instant blue.

2.6. Size exclusion chromatography

100 μl of 0.8 mg/ml concentrated hPEPT1 after PD10 desalting column was loaded on Superose 6 Increase 10/30 column in 100 mM sodium phosphate pH 6, 150 mM NaCl, without LMNG and eluted with a flow rate of 0.35 ml/min at 4 °C.

2.7. Detergent screening

Cell pellet from 2 l culture was resuspended in StrepII-lysis buffer (100 mM HEPES pH 7.8, 150 mM NaCl, 5% glycerol) supplemented with PI (1 tab/25 ml: cOmplete™, Mini, EDTA-free, Roche) + 1 mM Phenylmethanesulfonyl fluoride (PMSF), 1 μg/ml DNAse immediately before use. The cell suspension was sonicated on ice and ca 7 × 30 s (+30 s pause) at 70% power. Lysate was centrifuged at 10,000×*g* at for 10 min (SORVALL LYNC6000, rotor A27–8X50) to remove cell debris and insoluble material. Pre-spin was followed by ultracentrifugation in Beckman Coulter ultracentrifuge, Ti70, 35000 rpm, 1 h at 4 °C. Supernatant was discarded and the pellet was homogenized in a StrepII-lysis buffer. 0.9 ml of solubilize membranes was added to 0.1 ml of (10% stock solution in MQ) of 6 different detergent (OG, NM, DM, DDM, LMNG and DDM-CHS) and let it solubilized for 1 h at 4 °C. Insoluble material was removed by ultracentrifugation at 35,000 rpm, for 1 h at 4 °C. The cleared supernatant was used to run SDS-PAGE and Western blots were performed in the same way as reported earlier.

2.8. LC-MS analysis

The cleared filtered cell lysate (100 μl) was transferred to LC-MS glass vials with inserts. Next, 5 μl of sample was injected into the LC-MS (Agilent 1200 series HPLC connected to Esquire 3000 plus mass spectrometer, Bruker Daltonics). The analyte was separated on an Agilent poroshell C-18 column (5 mm, 2.1 × 75 mm, temperature 40 °C). The gradient applied for separation was kept at 5% acetonitrile for the first 3 min and then gradually increased to 100% acetonitrile for the next 6 min at a flow rate of 0.3 ml/min. Total run time was 12 min. The injected analyte after passing through the LC was sprayed using electrospray ionization (ESI) in a mass spectrometer, with a capillary voltage of 2750 V. The ion trap was set at 50,000 ions filling with a speed of 13,000 *m/z* per second. The gas temperature was set at 240 °C at a flow rate of 10 l/min and nebulizer gas pressure at 50 psi. Selected ion monitoring (SIM) methods were applied to monitor fragment ions from Gly-Sar. The parent ion/product used were *m/z* 147/*m/z* 129. The data analysis was done using Bruker Daltonics 3.3 software. All mass spectra were exported into cdf files and imported to Graphpad Prism 9 software (version 9.1.2; GraphPad Software Inc., La Jolla, CA, USA).

2.9. SSM electrophysiology assay

The cell pellet from 1l culture was resuspended in 20 ml of hypotonic buffer containing 10 mM HEPES pH 7.5, 1 mM EDTA, a protease inhibitor tablet (cOmplete™, Mini, EDTA-free, Roche), 1 mM PMSF and 1 mM TCEP, and then incubated on ice for 60 min with occasional swirling followed by cell disruption using a Dounce homogenizer. The cell lysate was centrifuged at 500× g at 4 °C for 10 min to remove cell debris. Subsequently, the supernatant was subjected to ultra-centrifugation at 100,000× g at 4 °C for 1 h to pellet the membranes. Isolated membranes were resuspended in a buffer containing 50 mM HEPES pH 7.5, 0.3 M sucrose, 0.1 M $CaCl_2$, 1 mM PMSF, 1 mM TCEP and further supplemented with a protease inhibitor tablet. Resuspended membranes were flash-frozen in liquid nitrogen and stored at −80 °C until use.

For the SURFE^{2}R assays either crude Sf9 membrane preparations (prepared as described above) or sucrose-gradient purified membrane fractions were used. For sucrose-gradient purification, isolated membrane pellets were carefully resuspended in 70% sucrose gradient buffer in 10 mM Tris pH 7.5 to a final concentration of 56% sucrose using first P1000 and P200 pipette tips and ultimately a syringe with a G21 needle. Up to 1 ml of resuspended membranes were transferred to ultra-clear ultracentrifugation tubes (Beckmann Coulter 344060) and gradients were gently built up with 5 ml 45% and 3 ml 16% sucrose buffers, respectively. The membranes were fractionated by ultracentrifugation in a Beckmann ultracentrifuge using a SW40Ti rotor at 100,000× g at 4 °C for 16 h. The membrane layer at the 16/45% interface was carefully collected and subjected to a wash step to remove sucrose. The membrane fraction was diluted with 5 vol of hPEPT1 assay buffer containing 140 mM KCl, 25 mM HEPES, 25 mM MES, 2 mM $MgCl_2$, pH 6.7, mixed well and centrifuged at 100,000× g at 4 °C for 30 min. Finally, the washed membrane pellets were resuspended thoroughly in hPEPT1 assay buffer supplemented with 5% glycerol and 0.2 mM dithiothreitol, flash-frozen in liquid nitrogen and stored at −80 °C until use.

Transport activity was measured with the hPEPT1 assay developed by IonGate Scientific Devices Heidelberg using a SURFE^{2}R N1 setup (Nanion). The assay is based on solid-supported membrane technology [27,28] that enables direct detection of moving charges. Sensors loaded with membrane preparations from Sf9 cells overexpressing hPEPT1 were prepared using gold sensors from Nanion following the protocols given by the manufacturer. Electrogenic dipeptide transport across the membranes was measured using a standard substrate concentration jump assay with consecutive exposure to non-activating (glycine) and activating (glycyl-glycine) buffers containing 140 mM KCl, 25 mM HEPES, 25 mM MES, 2 mM $MgCl_2$, pH 6.7 and supplemented with 20 mM glycine and 20 mM glycyl-glycine, respectively. Rapid exchange between the two solutions results in substrate (dipeptide) concentration gradients that induce transport across the membrane. As hPEPT1-mediated transport is proton-coupled, the transport is electrogenic and can be measured as currents running across the membrane (in either direction). Control measurements were performed using membranes from Sf9 cells infected with a pACEBac1-based recombinant baculovirus without any insert (empty-bac). Saturation curves were determined using sets of assay buffers with different concentrations of glycine and glycyl-glycine (0.625–50 mM). Similar experiments were performed using alanine and alanyl-alanine in the non-activating and activating buffers, respectively.

2.10. CPM thermostability assay

Thermostability assays were performed following a previously published protocol [29]. A 4 mg/ml stock concentration of 7-Diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM) dissolved in DMSO was diluted 20 folds with MQ. The working solution was well vortexed and allowed to equilibrate in the dark for 15 min. 10–20 μg of purified hPEPT1 at three different pH values (6, 7, 8) were added into a final volume of 45 μl of assay buffer in thin-walled 200 μl PCR tubes, and 5 μl CPM working solution was added. The solution was incubated further for 15 min at room temperature in the dark. Mx3005P qPCR (Agilent) was used to determine thermal shifts using filters 350 nm and

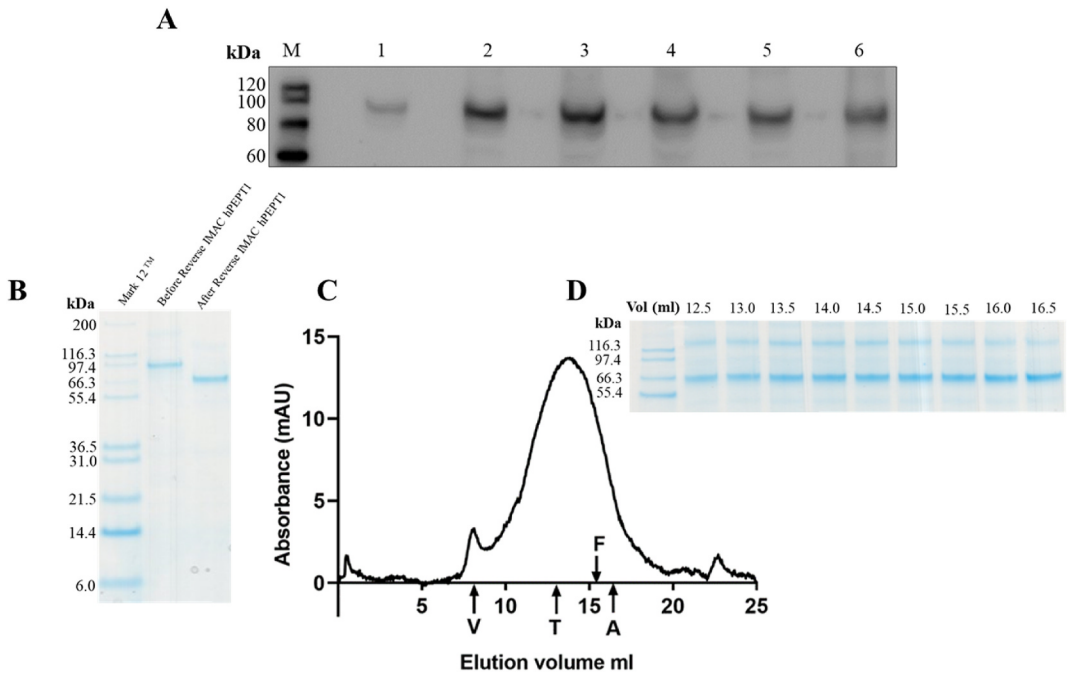

Fig. 1. A) Western blot showing efficiency of various detergents used for solubilization of tagged hPEPT1 expressed in Sf9 insect cells. Detergent used were numbered as follows: (lane 1) OG, NM (2), DM (3), DDM (4), LMNG (5), DDM-CHS (6). B) SDS-PAGE showing purified hPEPT1 before and after reverse IMAC. C) Size exclusion chromatogram of hPEPT1 purified in LMNG using a Superose 6 increase column 10/300 with a bed volume of 24 ml. The void volume and the standards Thyroglobulin (669 kDa), Ferritin (440 kDa) and Aldolase (158 kDa) elution volumes are marked. D) SDS-PAGE of SEC fractions.

440 nm with a temperature range of 25 °C to 95 °C. Graphpad Prism 9.0 was used to analyze data. Melting temperatures were calculated by fitting data to Boltzmann sigmoidal fit.

2.11. Circular dichroism (CD) spectroscopy

To analyze the secondary structural content of purified hPEPT1 in solution, CD spectroscopy was performed at pH values 6, 7 and 8 in 100 mM sodium phosphate and 50 mM NaF. Data were collected in the wavelength range 260–190 nm. Measurements were performed in a 1 mm quartz cuvette at 4 °C. The final spectrum was corrected by subtracting the spectrum of the buffer.

2.12. MST binding studies

MST experiments were performed using a NanoTemper Monolith NT.115 instrument (NanoTemper Technologies GmbH). Experiments were performed at 22 °C in 100 mM sodium phosphate (pH 6.0) and 100 mM NaCl. The concentration of dipeptide (Glycine-Sarcosine) and tripeptide (Ala-Ala-Ala) varied from 1 nM to 10 mM, while the concentration of hPEPT1 was kept constant at 10 nM. The sample was loaded into the NanoTemper Monolith NT.115 Premium Capillary and micro thermophoresis was carried out using medium MST power with 20% of Pico - RED excitation type. Dissociation constant K_d of hPEPT1 were calculated using Graphpad prism 9.0 software (version 9.1.2; GraphPad Software Inc., La Jolla, CA, USA).

2.13. Negative stain electron microscopy

LMNG-hPEPT1 from the SEC fraction around 14 ml was subjected to a series of dilutions (100× and 200×) with SEC buffer. Plastic and carbon coated 400 mesh copper TEM-grids were glow discharged at 25 mA using an PELCO easiGlow system (Ted Pella Inc.). A 3 μl aliquot of the diluted protein was applied to the glow discharged grid and incubated for 1 min, blotted, and washed/stained three times with 3 μl of 2% uranyl acetate. Micrographs were acquired using a 120 kV using Tecnai G2 Spirit TWIN transmission electron microscope (EMBION – the Danish National cryo-EM Facility, Aarhus node). The micrographs were recorded using a defocus range of 0.7–1.7 μm in underfocus. Images were automatically collected using a Tietz TemCam-F416 CMOS camera at a magnification of 67,000× (pixel size of 3.15 Å) employing Leginon [30]. Initial particles were picked manually and used for template based auto picking and 2D classification was carried using Cryosparc [31].

3. Results and discussion

3.1. Expression and purification of hPEPT1

We established recombinant expression of hPEPT1 (protein product MW 79.7 kDa) using the baculovirus system in Sf9 cells. Several different constructs were tested; however, the purest protein was obtained from a construct (***hPEPT1-3C-mCherry-His$_8$-TEV-strepII)*** consisting of the hPEPT1 gene, a 3C protease cleavage site, an 8xhistidine tag, a mCherry tag, a TEV protease cleavage site, and a Streptavidin tag II (Strep) (total protein product MW 89 kDa). Small scale expression suggested an optimal MOI of 0.05 and 48 h of incubation. Recombinant protein expression was indicated by a decrease in the Sf9 cell viability, and the appearance of a clear pink color attributed to the cherry tag. For solubilization and purification of hPEPT1 different detergents were tested and found to be equally efficient, as indicated the Western blots in (Fig. 1A). However, LMNG (lauryl maltose neopentyl glycol) was used for subsequent studies due to its low CMC. The protein purification procedure consisted tandem affinity chromatography, followed by cleavage of the tags and reverse IMAC. The expression of the recombinant protein was analyzed by SDS-PAGE (Fig. 1B) and showed efficient cleavage of the fusion protein; the cleaved hPEPT1 migrated as a 66 kDa band. Our final yields of pure hPEPT1 were up to 0.4 mg/l cell culture. Recombinant expression of hPEPT1 has been reported on several occasions previously in HeLa cells or in *X. laevis* oocytes [19], *P. pastoris* [32], Calu-3 cells [33] and HEK293 cells [34,35]. Collectively, these studies showed a band corresponding to a size of 60–70 kDa in Western blots and small-scale purification. To our knowledge purification of hPEPT1,

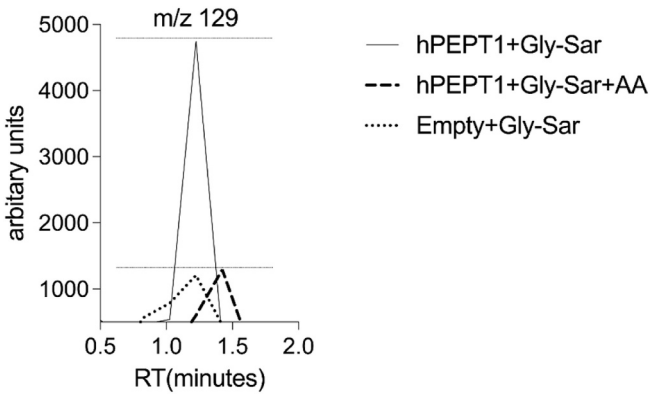

Fig. 2. Uptake of Gly-Sar measured by LC-MS/MS on Sf9 cells expressing hPEPT1 (solid line), and uptake in the presence of 10-fold excess of the dipeptide Ala-Ala (dotted line).

yielding mg quantities has not been reported previously.

3.2. Uptake assays

LC-MS based uptake assay on infected whole Sf9 cells was performed to measure uptake of the non-hydrolysable dipeptide Gly-Sar by hPEPT1, using uptake in uninfected cells as a control experiment (Fig. 2) The ion corresponding to *m/z* 129 $[(M+H)\text{-}H_2O]^+$ break down of Gly-Sar was detected in lysates of cells expressing hPEPT1 [36]. The intensity of ion corresponding to *m/z* 129 was lowered 4 folds in the presence of Ala-Ala as competitor and similar uptake was observed in uninfected Sf9 cells. This establishes that Gly-Sar is a substrate of PepT1 as observed previously [37]. The major peak at *m/z* 129 was observed, which corresponds to Gly-Sar ($[(M+H)\text{-}H_2O]^+$) [36].

We also performed electrophysiological studies using the SSM technology [27] on membranes isolated from Sf9 cells to investigate the functional activity of the expressed protein in the presence of tags (Fig. 3). Electrical responses of Sf9 membranes containing hPEPT1 were measured during dipeptide transport. The amplitude of the electrical response of sucrose purified membrane for dipeptide was 4-fold higher than the electrical response of crude membrane (Fig. 3A). The K_m value of 5.1 ± 0.3 mM was calculated from the saturation curve of hPEPT1-mediated transport of Gly-Gly (Fig. 3B). Our data present similar electrogenic transport characteristics, as demonstrated previously, where they used SSM technology for the electrophysiological characterization of hPEPT1 expressed in CHO cell line showed half-saturating concentrations (K_m) for Gly-Gln of 2.6 mM and for Gly-Gly of 5.4 mM [38]. Recently sucrose gradient purified plasma membrane vesicles gained from CHO cell lines overexpressing PepT1 showed EC_{50} = 6.8 ± 0.5 mM for hPEPT1-mediated Gly-Gly transport using SSM [39].

3.3. Thermostability and CD spectroscopy

To investigate the thermal stability of purified hPEPT1 at different pH values, we used a well-established assay, developed particularly for studying of membrane proteins using a thiol-specific probe, CPM [29]. This assay uses modification of cysteine residues as a sensor to determine overall protein stability. We examined the thermal stabilization of hPEPT1 at three different pH shown in (Fig. 4A.) Our results indicated that the stability of hPEPT1 is dependent on the pH; compared to pH values 7 and 8, pH value 6 significantly increased the T_m, showing that lower pH increased the hPEPT1 stability within the range of pH 6–8.

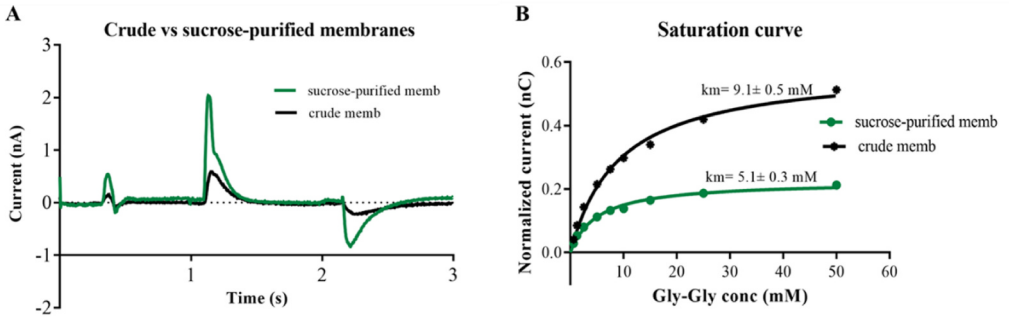

Fig. 3. SSM electrophysiology trace following uptake of Gly-Gly dipeptides by adsorbed hPEPT1. A) Transient current generated by hPEPT1-mediated proton-coupled dipeptide transport in crude membranes (black) and in sucrose purified membranes (green). B) Saturation curve of Gly-Gly uptake by hPEPT1 adsorbed on SSM.

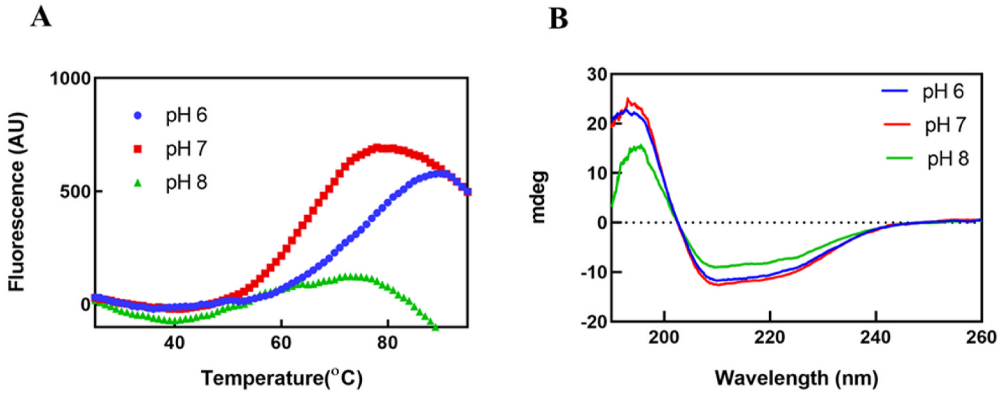

Fig. 4. A) Melting curves of hPEPT1 in solutions at different pH values. Derived T_m values: 71 °C at pH 6, 63 °C at pH 7 and 56 °C at pH 8. B) CD spectra of hPEPT1 at pH 6.0 (blue), 7.0 (red), 8.0 (green).

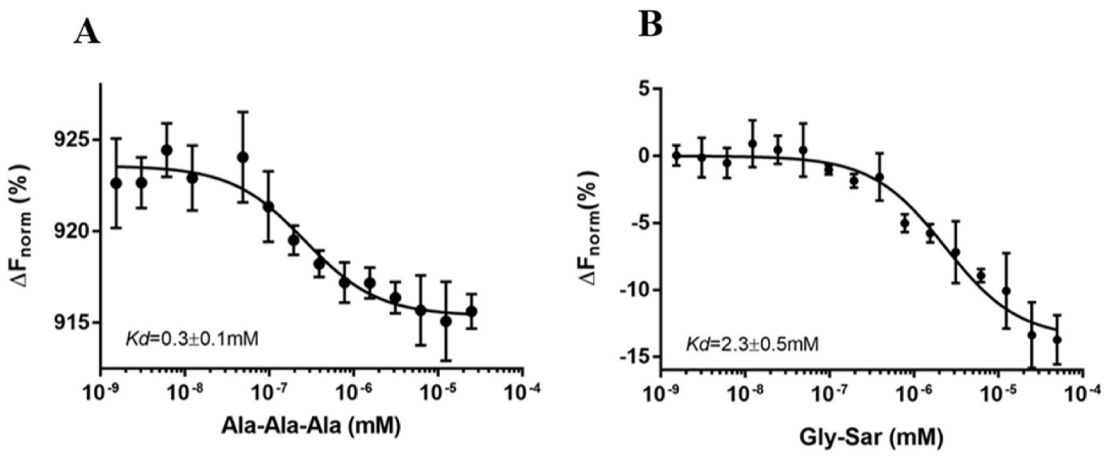

Fig. 5. Interactions of di and tri peptide with hPEPT1 measured by MST. (A) MST measurements showed that Dipeptide Gly-Sar bound to hPEPT1 with a dissociation constant (K_d) of 2.3 mM. (B) Tri peptide Ala-Ala-Ala interacts with hPEPT1 with a dissociation constant (K_d) of 0.3 mM.

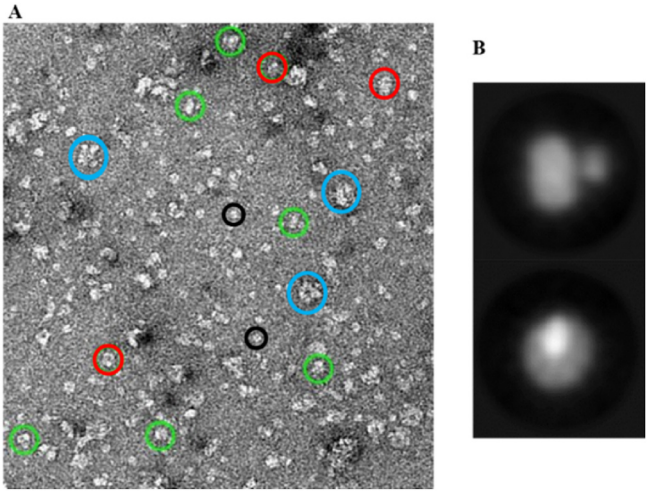

Fig. 6. A) A micrograph obtained by TEM after negative staining of LMNG solubilized hPEPT1 reveals the presence of both monomers and oligomers. Red and green circles showing two different views of monomeric particles. B) Representative 2D-classes showing side view and top view after 2D classification.

CD spectroscopic measurements on hPEPT1 were performed at concentrations of 1.0 μM (Fig. 4B). These results, at pH values 6.0, 7.0, 8.0 confirmed the alpha-helical content of this type of transport proteins, however they did not identify the presence of the beta-sheet containing Ig domains, as reported previously in their crystal structures [22]. This might be due to limitation of CD technique for some proteins.

3.4. Binding studies

To quantify binding affinities of Gly-Sar and Ala-Ala-Ala with hPEPT1 we used microscale thermophoresis (MST). Gly-Sar exhibited low millimolar binding affinity towards detergent solubilized hPEPT1 (K_d value of 2.3 ± 0.5 mM) (Fig. 5A) While Ala-Ala-Ala exhibited a K_d of 0.3 ± 0.1 mM (Fig. 5B). MST has been used to measure the binding affinities of several purified bacterial transporters [40–42]. These studies revealed that POTs in general exhibit a high micromolar to low millimolar binding affinities of different di and tri-peptides.

3.5. SEC and negative stain electron microscopy

The homogeneity hPEPT1 was assessed by size exclusion chromatography (SEC), which was also the final purification step (Fig. 1C). These analyses showed a broad peak ranging from 10 to 17 ml in elution volume peaking at 14 ml; all peak fractions contained hPEPT1 (Fig. 1D), apparently contain different oligoforms of hPEPT1.

To achieve further insight into the oligomeric state of hPEPT1, we performed negative stain EM studies on LMNG purified hPEPT1. Grids for negative stain EM were prepared using LMNG-hPEPT1 SEC fractions. The SEC was performed in the absence of LMNG to remove excess LMNG from sample. Owing to the very low critical micelle concentration of LMNG (CMC 11 nM) [43,44], excess micelles removal from sample is often challenging and some special techniques are required for it [45]. Separation of excess LMNG micelles from the protein-detergent complex by SEC was successful to some extent as empty micelles about 5 nm particles were still present (Fig. 6A) [46]. A total of 145 electron micrographs of negatively-stained sample were collected and 3,343 particles were picked manually. After a single round of 2D-class averaging and removal of blurred classes, good 2 D classes were selected for the calculation of template. This template picker picked 257,360 particles based on selected 2D classes. Particles were extracted and round of 2D-class averaging resulted into two final classes (Fig. 6). These classes had ellipsoidal shapes with an extra-membranous domains protruding out from the micelle. Our result provides useful information with respect to the dimension of the hPEPT1-surrounding micelle as well as ectodomain, which is evident from the 2D-classes.

Interestingly, rabbit PEPT1 has been reported to be a homomultimer of either four or five protomers [47]. hPEPT1 was mainly present in the form of monomers constituting around 70% of the sample, although higher oligomeric states were also visible in the remaining 30% of the particles (Fig. 6A). Each monomer contains one ellipsoidal disc like structure with a domain protruding from it (Fig. 6B). All in all, our EM results are in accordance with the SEC results although the smaller species are more predominant. This may be since the EM sample was significantly diluted compared to the SEC fractions.

In conclusion, we have performed successful expression, purification, functional characterization, of a folded and active detergent solubilized hPEPT1, which can be used for structural and biochemical studies.

CRediT authorship contribution statement

Maria Rafiq: Writing – original draft, Investigation. **Heidi A. Ernst:** Investigation. **Nanda G. Aduri:** Investigation. **Bala K. Prabhala:** Investigation. **Soban Tufail:** Investigation. **Moazur Rahman:**

Investigation. **Magnus Borup Bloch:** Investigation. **Nadia Mirza:** Investigation. **Nicholas M.I. Taylor:** Investigation. **Thomas Boesen:** Investigation. **Michael Gajhede:** Writing – original draft. **Osman Mirza:** Writing – review & editing.

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