Short communication: The essential role of *N*-glycosylation in the transport activity of bovine peptide transporter 2

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ABSTRACT

Bovine peptide transporter 2 (bPepT2), which mediates the absorption of di- and tripeptides in the bovine mammary gland, was predicted to contain multiple putative *N*-glycosylation sites of asparagine residues. *N*-Linked glycosylation is proven to be essential for the folding, stability, localization, and substrate binding of nutrient transporters and could therefore potentially have an essential role in the function of bPepT2. This study investigated the effect of mutagenesis of *N*-glycosylation sites on the transport function of bPepT2 in Chinese hamster ovary (CHO) cells. The bPepT2 cDNA was cloned and sequenced. BioXM (<http://202.195.246.60/BioXM/>) and TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>) software were used to predict the AA composition and transmembrane domain of bPepT2, respectively. The AA sequence of bPepT2 was predicted to have 12 transmembrane domains, with a large extracellular loop between the ninth and tenth transmembrane domains. All 5 putative *N*-glycosylation sites in this loop were altered by site-directed mutagenesis, and the mutant construct was transfected into CHO cells for transport activity assay. Compared with the wild type, the bPepT2 mutant had significantly lower uptake activity of β -alanine-L-lysine- ϵ -7-amino-4-methyl-coumarin-3-acetic acid (β -Ala-Lys-AMCA), a model dipeptide. Treatment with tunicamycin, an inhibitor of *N*-linked glycosylation, reduced the uptake of β -Ala-Lys-AMCA in CHO cells relative to the control group. Kinetic studies indicated that the Michaelis constant of bPepT2 was not affected by the mutation (98.03 ± 8.30 and 88.33 ± 4.23 μ M for the wild type and the mutant, respectively), but the maximum transport activity was significantly reduced (40.29 ± 8.30 and 13.02 ± 2.95 pmol/min per milligram of protein for the wild type and the mutant, respectively). In sum-

mary, this study demonstrated that *N*-glycosylation is critical for the function of bPepT2.

Key words: amino acid utilization, peptide transporter, post-transcriptional modification

Short Communication

Studies have shown that the supply of some EAA in the blood cannot meet the requirement of milk protein synthesis in the mammary gland (Metcalf et al., 1996; Bequette et al., 1999), but small peptides in the blood can make up the shortage (Pan et al., 1996; Yang et al., 2015). Tagari et al. (2008) revealed that peptide-bound AA represent a crucial portion of total AA flux across the portal-drained viscera and contribute to milk protein synthesis in the bovine mammary gland (Tagari et al., 2008). The arterial concentration of peptide-bound AA in cows fed a steam-flaked sorghum grain diet is 58.6 mg/L of plasma, whereas the mammary vein concentration of peptide-bound AA in these cows is 53.8 mg/L of plasma (Tagari et al., 2008). Previous research revealed that there was no arteriovenous difference for total peptide-bound AA, whereas the difference for the peptide containing Glu, Gly, and His was significant in the bovine mammary gland (Tagari et al., 2008). Although Tagari et al. (2008) indicated that there were significant mammary arteriovenous differences for some AA, later work found that the use of molecular weight cutoff membranes to prepare peptide samples as done by Tagari et al. (2008) was not a reliable method (Yoshida, 2004). Thus, it is unclear to what extent peptides contribute to the tissue AA supply. Our previous studies revealed that methionine-containing dipeptides have a higher efficiency in enhancing milk protein synthesis than free methionine in bovine mammary epithelial cells (BMEC; Yang et al., 2015; Wang et al., 2018). Proton-coupled oligopeptide transporter plays a great role in absorbing dipeptides, tripeptides, and peptidomimetic drugs in different epithelial cells (Kottra et al., 2013). Four proton-coupled oligopeptide transporters, including peptide transporter 1 (PepT1), peptide transporter 2 (PepT2; gene symbol: *SLC15A2*), peptide histidine

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transporter 1, and peptide histidine transporter 2, have been identified (Tai et al., 2013). Our previous works have shown that PepT2 and peptide histidine transporter 1 are abundantly expressed in the bovine mammary gland but that only PepT2 plays a major role in the uptake of a wide range of dipeptides in BMEC (Yang et al., 2015; Wang et al., 2018, 2019).

N-Glycosylation acts as a mechanism for post-translational modifications of protein synthesis (Suzuki et al., 1995). Many studies have revealed that *N*-glycosylation plays a crucial role in the folding, subunit assembly, stability, localization, and substrate binding of nutrient transporters (Jenkins et al., 1996). However, little is known about the regulatory role of *N*-glycosylation in the function of bovine PepT2 (**bPepT2**). Thus, the objective of this study was to explore the effect of *N*-glycosylation on the function of bPepT2.

Total RNA was isolated from bovine mammary gland tissue with an RNA isolation kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. The concentration of RNA was measured, and then the cDNA was reverse transcribed from 1 µg of RNA using a reverse transcription kit (Takara, Shiga, Japan). Polymerase chain reaction was performed to amplify bPepT2 cDNA with a SYBR PrimeScript reagent kit (Takara) and primers (5'-ATGCTTGAAGAGAGAGAGAGTGAG-3'; 5'-TTAGAGCCTTGTCTTCTTGGTTTCC-3'). Amplified cDNA was electrophoresed on a 1% agarose gel and purified and cloned into the pMD19-T vector (Tsingke, Beijing, China). The recombinant plasmid was transformed into *Escherichia coli* DH5α (Sangon Biotech), followed by DNA extraction and verification by restriction enzyme digestion. The positive colonies were sequenced by Genescript Biotech Company (Nanjing, China). The AA sequence of bPepT2 was deduced from the cDNA sequence by BioXM software (<http://202.195.246.60/BioXM/>). The topology structure of bPepT2 was analyzed by TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>). The NetGlyc 1.0 platform was used to predict the *N*-glycosylation sites, with N-X-S/T [asparagine (N), a variable AA except proline (X), and serine (S) or threonine (T)] being considered putative *N*-glycosylation sites (Stelzl et al., 2016). Site-directed mutagenesis of bPepT2 was done following the procedures described previously (Chan et al., 2016). Asparagines in positions 435, 472, 508, 528, and 587 were replaced by glutamine (N435Q, N472Q, N508Q, N528Q, N515Q, and N587Q, respectively). Mutations were confirmed by DNA sequencing.

The Chinese hamster ovary (**CHO**) cells were cultured at 37°C and 5% CO₂ in Ham's F12 medium supplemented with 10% fetal bovine serum (Gibco, Scoresby, VIC, Australia) and transfected with plasmid

DNA using lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, plasmid DNA (5 µg), lipofectamine 3000 (3.75 µL), and P3000 (10 µL) were diluted into 125 µL with opti-MEM (Invitrogen), mixed, incubated for 5 min, and then added to the cells. For the deglycosylation experiment, the CHO cells transfected with bPepT2 wild type or mutant constructs for 48 h were treated with tunicamycin (50 µg/mL) for 8 h.

The dipeptide uptake assay was carried out in CHO cells as previously described using β-alanyl-L-lysyl-Nε-7-amino-4-methyl-coumarin-3-acetic acid (**β-Ala-Lys-AMCA**), which is a model dipeptide from a dipeptide derivative containing a fluorescent reporter molecule (Yang et al., 2002; Wang et al., 2018); β-Ala-Lys-AMCA is resistant to the hydrolysis of peptidase and often is used to detect the uptake of dipeptide in cells (Agu et al., 2011; Wang et al., 2018). Fluorescence-based methods have high sensitivity and are easy to measure with multiwell plate readers (Landowski et al., 2003). The CHO cells overexpressed with bPepT2 were preincubated with Krebs-Ringer modified buffer (**KRB**) for 30 min and then incubated with KRB containing 25 µM β-Ala-Lys-AMCA for 15 min at 37°C. For the transport kinetic study, the cells were incubated with 25, 50, 100, 150, and 200 µM β-Ala-Lys-AMCA. The cells were washed with ice-cold KRB 3 times to stop the peptide uptake followed by lysis with 1% triton-100. The uptake of β-Ala-Lys-AMCA in cells was measured with a microplate reader (Spectra MAX M5, Molecular Devices, Sunnyvale, CA; excitation at 350 nm and emission at 455 nm). Protein concentration was determined with the bicinchoninic acid assay method. Uptake of β-Ala-Lys-AMCA in each well was standardized to the amount of protein.

All data were analyzed with SPSS 19.0 (IBM Corp., Armonk, NY). Shapiro-Wilk and Levene tests were used to test normality and homogeneity of variance of all the data, respectively. For data conforming to Gaussian distribution, parametric statistical analysis was conducted using the independent-samples *t*-test, whereas for the data with skewed distribution, non-parametric statistical analysis was performed with the Wilcoxon test. Values were shown as means ± standard deviation, and *P* < 0.05 was considered a significant difference. All experiments were performed with 3 replicates.

Sequence analysis showed that bPepT2 contains 2,190 nucleic acids (Supplemental Figure S1; <https://doi.org/10.3168/jds.2019-16858>), which encode 729 AA, consistent with human and rat PepT2 (Liu et al., 1995; Saito et al., 1996). The large extracellular loop between the ninth and tenth transmembrane domains (**TMD**) of bPepT2 contains 5 potential *N*-glycosylation

sites (Table 1). The *N*-glycosylation is one of the most frequent post-translational modifications of proteins in eukaryotes and plays a great role in protein folding, stability, and trafficking (Krishna and Wold, 1993; Moharir et al., 2013). To study the role of *N*-glycosylation in bPepT2 transport activity, we transfected bPepT2 expression vector into the CHO cells and measured the uptake activity of β -Ala-Lys-AMCA in CHO cells treated with or without tunicamycin, which causes the deglycosylation of proteins (Chan et al., 2016). We did not carry out the experiment in BMEC because PepT2 is abundantly expressed in BMEC (Wang et al., 2019). In contrast, CHO cells do not express PepT2 and are equipped with glycosylation machinery, making it an ideal host for exogenous transporter expression and kinetics study (Jenkins et al., 1996). For example, the functional characteristics of a bovine peptide transporter, bovine PepT1, has been studied in CHO cells (Xu et al., 2018). As shown in Figure 1A, treatment with tunicamycin reduced the uptake of β -Ala-Lys-AMCA in CHO cells transfected with bPepT2 cDNA, suggesting that bPepT2 may be glycosylated and that the glycosylation may play an important role in the transport activity of bPepT2.

The functional importance of asparagine residues in putative *N*-glycosylation sites of bPepT2 was further evaluated by site-directed mutagenesis. Replacement of all 5 potential *N*-glycosylation sites (asparagine residues) with glutamine (Supplemental Figures S2 and S3; <https://doi.org/10.3168/jds.2019-16858>) in the large extracellular loop of bPepT2 significantly decreased its uptake activity of β -Ala-Lys-AMCA in CHO cells (Figure 1B). This was consistent with previous research showing that the impairment of substrate transport of human PepT1 and system ASC AA transporter 2 (ASCT2) was observed after disruption of their *N*-glycosylation sites (Lara et al., 2015; Chan et al., 2016). Our result revealed that the function of bPepT2 requires *N*-glycosylation and the asparagine residues between the ninth and tenth TMD.

Table 1. Prediction results of *N*-glycosylation sites in bovine peptide transporter 2 (bPepT2) with the NetNGlyc 1.0 platform

Position	Potential	<i>N</i> -glycosylation result ¹
435 NNLT	0.5271	+
472 NVSV	0.7802	+++
508 NKTT	0.5002	+
528 NVSL	0.5608	+
587 NRTN	0.6416	+

¹Describes a potential score for predicted *N*-glycosylation sites of bPepT2 predicted by the NetNGlyc platform (<http://www.cbs.dtu.dk/services/NetNGlyc/>). Predictions indicating positive sequon occupancy are marked with + (+ = potential > default threshold of 0.5; +++ = potential > 0.75).

Figure 1. The effect of tunicamycin and *N*-glycosylation of bovine peptide transporter 2 (bPepT2) on the cellular uptake of β -alanyl-L-lysyl-N ϵ -7-amino-4-methyl-coumarin-3-acetic acid (β -Ala-Lys-AMCA). (A) Tunicamycin reduced uptake of β -Ala-Lys-AMCA in Chinese hamster ovary (CHO) cells transfected with bPepT2. The uptake of β -Ala-Lys-AMCA was measured at 25 μ M for 15 min at pH 6.5 with or without tunicamycin in CHO cells. (B) The bPepT2 mutated at all 5 predicted *N*-glycosylation sites (5NQ) showed significantly lower uptake activity of β -Ala-Lys-AMCA compared with nonmutated wild type in CHO cells. Uptake of β -Ala-Lys-AMCA was measured at 25 μ M for 15 min at pH 6.5. Values are means $\pm$ SD ($n = 3$). * $P < 0.05$.

Furthermore, the transport kinetics of bPepT2 mutant for β -Ala-Lys-AMCA was studied in CHO cells. The Michaelis constant (98.03 ± 8.30 and 88.33 ± 4.23 μ M for the wild type and the mutant, respectively) was not altered by asparagine mutations compared with the wild type, but the maximal transport velocity (40.29 ± 8.30 and 13.02 ± 2.95 pmol/min per milligram of protein for the wild type and the mutant, respectively) was reduced (Figure 2). The reduced maximal transport velocity of bPepT2 mutant may result from impaired translocation of bPepT2 to plasma membrane. Previ-

Figure 2. The effect of *N*-glycosylation on the transport kinetics of bovine peptide transporter 2 (bPepT2). (A) Uptake of β -alanyl-L-lysyl-N ϵ -7-amino-4-methyl-coumarin-3-acetic acid (β -Ala-Lys-AMCA) was measured at 25, 50, 100, 150, and 200 μ M for 15 min at pH 6.5 in Chinese hamster ovary cells transfected with the expression vector of wild type bPepT2 or bPepT2 mutated at all 5 putative *N*-glycosylation sites in transmembrane domains 9 and 10 (5NQ). The mutation (5NQ) had no effect on the Michaelis constant (K_m) (B) but significantly reduced the maximal transport velocity (V_{max}) of β -Ala-Lys-AMCA (C). Values are means $\pm$ SD ($n = 3$). * $P < 0.05$.

ous studies also showed that *N*-glycosylation between TMD 9 and 10 is vital for the membrane expression of human PepT1 (Jappar et al., 2010). *N*-Glycosylation can regulate membrane expression of many other nutrient transporters (e.g., organic anion transporting polypeptide 1B1 and glucose transporter 4) by affecting membrane protein targeting and membrane protein stability (Haga et al., 2011; Yao et al., 2012). Whether *N*-glycosylation can affect the membrane targeting or protein stability of bPepT2 remains to be investigated. In summary, bPepT2 is likely *N*-glycosylated in the large extracellular loop between the ninth and tenth TMD, and this post-translational modification is critical for the function of bPepT2.

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