SUPPLEMENTARY MATERIAL AVAILABLE ONLINE

Indian Journal of Animal Sciences **91** (5): 360–367, May 2021/Article
https://doi.org/10.56093/ijans.v91i5.115388

Cloning of oligopeptide transport carrier PepT2 and comparative analysis of PepT2 expression in response to dietary nitrogen levels in yak and cattle

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Received: 17 November 2020; Accepted: 21 July 2021

ABSTRACT

The objectives of this study were clone oligopeptide transport carrier PepT2 and compare its abundance in kidney tissues of yak and cattle in response to different dietary nitrogen levels. Four adult female yaks were enrolled as donor animals for profiling the molecular characteristics and expression specificity of yak PepT2 (yPepT2), and twenty-four castrated males of each of two genotypes, yak (*Bos grunniens*) and indigenous cattle (*Bos taurus*) were used to explore PepT2 mRNA expression in kidney tissue in different nitrogen (N) levels (10.3, 19.5, 28.5 or 37.6 g N/kg dry matter; DM). Our results showed that the yPepT2 coding sequence region contains 2190 bp, and encodes a putative protein of 729 amino acids (AA) residues. The yPepT2 AA sequence identified eight putative extracellular N-glycosylation sites (Asn^{7}, Asn^{80}, Asn^{373}, Asn^{435}, Asn^{472}, Asn^{508}, Asn^{528}, Asn^{587}) and eight intracellular putative protein kinase C sites (Ser^{34}, Ser^{264}, Ser^{274}, Ser^{376}, Ser^{442}, Ser^{586}, Ser^{640}, Ser^{724}). The yPepT2 AA sequence was 98 and 94% identical to PepT2 from zebu cattle (*Bos indicus*) and sheep (*Ovis aries*), respectively. The relative PepT2 expression in kidney tissue for yak was greater than of indigenous cattle in the10.3 and 28.5 N/kg DM diet, but it was lower for yaks in 37.6 N/kg DM diet. These implied that relative PepT2 mRNA expression was higher in yak kidney than that in indigenous cattle at lower dietary N supplies, but more research on PepT2 will be required to determine the renal regulatory mechanisms.

Keywords: Cattle, Cloning, Dietary nitrogen level, Expression, PepT2, Yak

Yak (*Bos grunniens*) is a special livestock whose physiological and digestive characteristics are different from other species, and can survive a long period of forage shortage each year traditional grazing management under extreme harsh environment on the Qinghai-Tibetan Plateau (Fan *et al*. 2020). Previous studies revealed that yak have evolved some genetic mechanisms (Qiu *et al*. 2012) and tissue structures (Yang *et al*. 2017) which adapts to cold, anoxia and lack of forage grass. It has been reported that yak had a lower endogenous urine nitrogen (N) excretion and lower glomerular filtration rate than indigenous cattle (Wang *et al*. 2011), which indicated that yak developed special reabsorption mechanisms in the kidney. Moreover, previous studies showed that the kidney can reabsorb small peptide by the peptide transport carrier PepT2, which is located in the brush marginal membrane of renal medulla cells and epithelial cells (Oppermann *et al*. 2019). The yak PepT1 profile and its expression were varied from those of cattle in gastrointestinal epithelial cells in different dietary nitrogen levels (Wang *et al*. 2016), and study indicates that PepT2 was functional complementary to PepT1 (Con *et al*. 2017), which implies that yak may have a specific mechanism of nonprotein utilization including a peptide absorption mechanism in kidneys. N recycling is a vital function for the survival of ruminants under conditions of insufficient nutrient intake. However, the mechanism of peptides utilization in yak kidney has not been clarified. Hence, we hypothesized that, in response to harsh conditions, yak kidneys possess better oligopeptide transport carrier PepT2 expression than cattle. To test this hypothesis, two experiments were conducted: profiling molecular characteristics of yak PepT2 (yPepT2) cDNA and mRNA expression specificity in gastrointestinal tract, breast, kidney and liver tissues; determining the effects of PepT2 mRNA expression between yak and indigenous cattle fed with different N level diets.

MATERIALS AND METHODS

Animals, experimental design, management and sample collection: Four adult female yaks were selected as donor animals to sample gastrointestinal tract, breast, kidney and liver tissues to profile molecular characteristics of yPepT2 and expression specificity from Wushaoling pasture farm located in the alpine meadow grassland of the Tianzhu Autonomous County, Gansu province.

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Table 1 Composition ratio of amino acid (AA) in yPepT2 coding sequence region

AA	Quantity	Proportion (%)
Alanine	49	6.7
Arginine	19	2.6
Asparagine	31	4.3
Asparticacid	25	3.4
Cystine	12	1.6
Glutarnine	31	4.3
Glutamicacid	35	4.8
Glycine	44	6.0
Histidine	14	1.9
Ilesoleucine	42	5.8
Leucine	83	11.4
Lysine	41	5.6
Methionine	27	3.7
Phenylalanine	46	6.3
Proline	36	4.9
Serine	55	7.5
Threonine	37	5.1
Tryptophan	12	1.6
Tyrosine	30	4.1
Valine	60	8.2

Twenty-four 3-years old castrated male of each of two genotypes, yak (198±15 kg average body weight; BW) and indigenous cattle (203±5 kg average BW) were also used as feeding experimental animals selected from above-mentioned site. Before the feeding experiment, the castrated male animals had free access to oat hay for a 15-days period followed by a feeding trial, therefore, the trial used a 2 × 4 factorial arrangement of treatments. Each genotype was first divided into 4 groups, and six animals of each group were fed 1 of 4 experimental diets with different N levels: 10.3 g N/kg dry matter (DM), 19.5 g N/kg DM, 28.5 g N/kg DM or 37.6 g N/kg DM (Ingredient and chemical composition of experimental diets as described by Wang *et al.* 2016 (Table 1). During the feeding trial period, the animals were fed 3 kg of the experimental diet daily, half at 08:00 h and half at 18:00 h. All animals were individually housed in metabolism crates with *ad lib.* access to water. Following the feeding trial, the animals were slaughtered and tissues were evaluated. Animals were slaughtered in our meat lab on the morning of day 31 of the feeding experiment, and all animals were fasted for 1 day prior to slaughter.

All animals were stunned by a captive bolt pistol and killed by exsanguination. The samples were collected according to the procedure by Wang *et al.* (2016). Part of the yak kidney sample was used for cloning the PepT2 cDNA. All tissue samples were used for the relative PepT2 mRNA expression analysis.

RNA isolation and first-strand cDNA synthesis: Total RNA was isolated from kidney tissues using a RNA extraction kit (Takara, Shiga, Japan). The quantity of RNA was detected by a NanoDrop2000 spectrophotometer (Thermo Scientific, Wilmington, DE) and the purity assessed by the A260/A280 ratio. The RNA integrity was assayed by 1.5% agarose gel containing 1 pg/mL ethidium bromide. The first-strand cDNA was synthesized using 3 μg total RNA, 1 μL oligo dT, 1 μL deoxyribonucleotide triphosphate mixture, and water to 10 μL; incubated at 65°C for 5 min; and cooled on ice. Then, 4 μL 5× PrimeScript 2 buffer (Takara), 0.5 μL ribonuclease inhibitor, 1 μL PrimeScript 2 Rtase (Takara), and water were added to 20 μL and reaction was extended for 50 min at 42°C, inactivated at 95°C for 5 min, and stored at –80°C.

Cloning of PepT2 cDNA: The primers for yPepT2 cDNA sequence were designed using Primer 5.0 (Premier Biosoft International, Palo Alto, CA) based on zebu cattle (XP_019809864.1) and sheep (XP_005675070.2) PepT2 cDNA sequences. The PCR primers were forward 5′-CATGGACTGCACCCTACCA-3′ and reverse 5′-AGCCCAAAGTGAGATAGAAACCA-3′. The yPepT2 cDNA from kidney tissue was amplified by conventional PCR. The PCR conditions were 94°C for 5 min, 30 cycles of 98°C for 10 sec, 54.5°C for 30 sec, and 72°C for 1 min. The PCR products were separated by electrophoresis on a 1.5% agarose gel. The DNA products were cloned into pMD18-T (Takara) and then sequenced to obtain the central core sequence of yPepT2.

Quantification of mRNA by real-time PCR analysis: Real-time PCR was determined by Applied Biosystems 7500 quantitative PCR (Applied Biosystems, Foster City, CA). 18S RNA was used as an endogenous control to normalize target gene transcript levels. The cDNA was diluted 50 ug/μL before all Real-time PCR analyses. The real-time quantitative PCR primers for 18S RNA were forward 5′-CAACACGGGAAACCTCACC-3′ and reverse 5′-CCCAGACAAATCGCTCCAC-3′ . Real-time PCR was completed using SYBR Premix Ex Tap 2 (Takara) containing $MgCl_2$, deoxyribonucleo-tide triphosphates, and HotStarTaq polymerase, and 1 μL each of the forward and reverse primers, and 12.5 μL SYBR Premix Ex Taq 2 (Takara) and 2 μL of cDNA template were added to a 25-μL total volume, the final concentrations of cDNA and 18S RNA primer were 4 ng/μL and 0.4 μmol/L, respectively. A no-template (sterile distilled water) negative control was added on each plate run to screen for possible contamination and dimer formation and to set background fluorescence for plate normalization. Real-time PCR was initiated with 30 sec of denaturation at 95°C followed by 40 cycles of amplification at 95°C for 5 sec and 60°C for 30 sec. A melting curve was produced from 60°C to 95°C to confirm the specificity of the amplified product. Each amplification was duplicated (Cycle threshold [Ct] values ranged from 21.34 to 31.55) and the mean value was calculated using the ΔΔCt method (Livak and Schmittgen 2001), the relative PepT2 mRNA expression was calculated as $R = 2^{-\Delta\Delta Ct}$, and all the relative PepT2 mRNA expression in yak kidney tissue (10.3 g N/kg DM) was used as calibrator for all the other tissue of yak relative PepT2 mRNA expression.

Bioinformatics analysis: The tools or procedures used in bioinformatics analysis are listed in Supplementary Material S1.The amino acid (AA) sequences were analyzed using the open reading frame finder program from National Center for Biotechnology Information; TMHMM Server2.0 software was used to predict transmembrane domains of yPepT2; NetNGlyc software was used to predict the potential N-glycosylation sites; Protein kinase A (PKA) and protein kinase C (PKC) sites were predicted by PROSITE 20.40 computational tools. It was predicted that theoretical isoelectric point (pI), molecular weight, formula, instability index (2), aliphatic index, Grand average of hydropathicity (GRAVY) and estimated half-life by a tool ProtParam; Signal peptide was predicted using software SignalP; AA and homology of yPepT2 sequences were aligned with the multiple alignment program of the biological analysis software DNAman (Lynnon Corporation, Pointe-Claire, Canada); Secondary structure was predicted referring to the procedure PRABI Lyon Gerland.

Statistical analysis: Data were analyzed using the MIXED procedure of SAS 9.2 (SAS Inst. Inc., Cary, NC) according to the following model: $Y = \mu + N + G + (N \times G) + E$, in which Y is the dependent variable, μ is the overall mean, N is the effect of dietary N level, G is the effect of animal genotype, $N \times G$ is the interaction between dietary N level and genotype, and E is the residual error. Dietary N level and genotype were fixed effects. Comparisons between genotypes within each level of dietary N were made using t tests when at least one contrast assessing the genotype × dietary N level interaction was significant. These t tests were performed to ensure that interpretations of the contrasts were clear. Differences were considered significant at $P<0.05$.

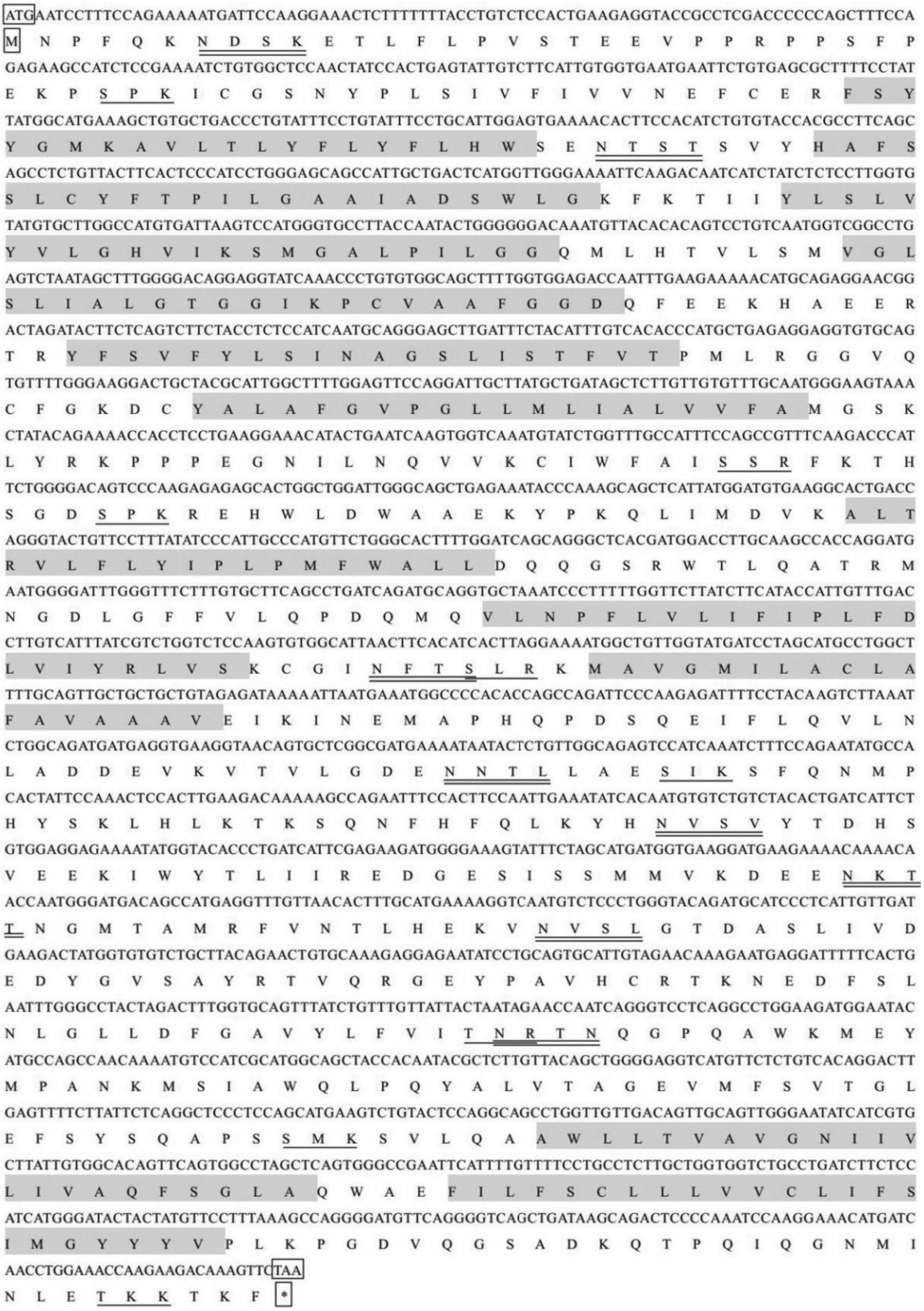
RESULTS AND DISCUSSION

Molecular characteristics of ypepT2 cDNA and AA sequence analysis: The yPepT2 cDNA was isolated from yak kidney tissues, cloned by reverse transcription PCR and sequenced. The full length of PepT2 cDNA coding sequence region is 2190 bp (GenBank accession code MH932071) encoding 729 AA. The yPepT2 (*Bos grunniens*) is predicted to adopt a putative 11-transmembrane-domain structure, with intracellular amino and carboxyl termini, an elongated intracellular loop of 61residues between the sixth and seventh transmembrane domains, and a large extracellular loop of 250 residues between the ninth and tenth transmembrane domains. The large extracellular hydrophilic ring between the ninth and tenth transmembrane domains played a key role in the functional differences of PepT2 (Wang *et al.* 2010). There were modification sites of protein PKA and PKC on PepT2 carrier protein (Fei *et al.* 1994), and the activated PKC decreased the absorption of peptide (Wenzel *et al.* 1999). It was predicted to contain 8 putative extracellular N-glycosylation sites (Asn^{7}, Asn^{80}, Asn^{373}, Asn^{435}, Asn^{472}, Asn^{508}, Asn^{528}, Asn^{587}), 8 putative intracellular PKC sites in present study (Ser^{34}, Ser^{264}, Ser^{274}, Ser^{376}, Ser^{442}, Ser^{586}, Ser^{640}, Ser^{724}; Fig. 1). Therefore, it was speculated that yPepT2 may need phosphorylation before performing physiological and biochemical functions or interacting with other proteins.

The predicted theoretical pI of yPepT2 is 7.19, the molecular weight is 81.86 kDa; Formula is $C_{3763}H_{5809}N_{929}O_{1034}S_{39}$, and the instability index (2) is computed to be 32.49. The sequence has the greatest Leu content (11.4%), the lowest Cys and Try content (1.6%); The non-polar and polar (hydrophobic) AA account for 54.6% and 45.4%, respectively; And it accounted 26.9%, 10.1% and 8.2% for neutral, basic and acid AA, respectively; The hydrophobic AA is slightly greater than hydrophilic AA indicating yPepT2 is hydrophobic AA (Table 1). The sulfhydryl group of Cys residue in protein was oxidized to form a disulfide bond, and the hydrophobic AA residue encircled the disulfide bond forming a local hydrophobic center and denying water molecules to entry into the peptide and destroy the hydrogen bond (Hu *et al.* 2020).There are 12 Cys in yPepT2 protein sequence, therefore, it can be inferred that the yPepT2 may contain disulfide bonds, which may be of great significance of to maintain its structural stability and function. The C value of AA sequence was not close to one, indicating that yPepT2 is no signal peptide, and the shear site of yPepT2 can be estimated comprehensively from y-max and C value at the forty-fifth AA; The S-mean (The mean value of S of each AA from the n-terminal to the shear site) and D value (The weighted mean of s-mean and y-max) was respective 0.103 and 0.106 (Supplementary Table 1) indicating that yPepT2 was a non-secretory protein. The secondary structure prediction (Fig. 2) show that Alpha helix contains 207 AA (accounting for 28.40%), the Extended strand is 172 AA (accounting for 23.59%), Random coil contains 321 AA (accounting for 44.03%), and Beta turn contains 29 AA (accounting for 3.98%).

PepT2 homology analysis: Table 2 shown the homology of yPepT2 sequences compared with other species. Analysis using the biological software DNAman showed that there were differences between PepT2 AA sequence of yak (*Bos grunniens*) and zebu cattle (*Bos indicus*) at F^{46}(yak):I^{46}(zebu cattle), G^{159}(yak):X^{159}(zebu cattle), L^{226}(yak):R^{226}(zebu cattle), M^{228}(yak):L^{228}(zebu cattle), L^{229}(yak):I^{229}(zebu cattle), A^{231}(yak):R^{231}(zebu cattle), L^{232}(yak):A^{232}(zebu cattle), S^{245}(yak cattle):P^{245}(zebu cattle), T^{269}(yak):N^{269} (zebu cattle), S^{274}(yak):N^{274}(zebu cattle), P^{279}(yak):H^{279}(zebu cattle), A^{535}(yak):X^{535}(zebu cattle), N^{565}(yak):K^{565}(zebu cattle) (Fig. 3).

yPepT2 tissue expression specificity: In the present study, we found that yPepT2 mRNA was mainly expressed in kidney, breast and liver tissue (Fig. 4), while its expression not seen in rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum, cecum and colon tissues. PepT2 belonged to the proton-dependent oligopeptide transporter family (Wang *et al.* 2020) and had a higher affinity than PepT1 (Kudo *et al.* 2020). PepT2 possessed higher affinity than PepT1 not only for neutral peptides but also for peptides consisting of anionic and/or cationic AA (Ramamoorthy *et al.* 1995). Previous research had shown that PepT2mRNA was expressed in variety tissues, including lung (Groneberg *et al.* 2001), brain, spleen



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ATGAATCCTTTCCAGAAAAATGATTCCAAGGAAACTCTTTTTTTACCTGTCTCCACTGAAGAGGTACCGCCTCGACCCCCCAGCTTTCCA
M N P F Q K N D S K E T L F L P V S T E E V P P R P P S F P
GAGAAGCCATCTCCGAAAATCTGTGGCTCCAACTATCCACTGAGTATTGTCTTCATTGTGGTGAATGAATTCTGTGAGCGCTTTTCCTAT
E K P S P K I C G S N Y P L S I V F I V V N E F C E R F S Y
TATGGCATGAAAGCTGTGCTGACCCTGTATTTCCTGTATTTCCTGCATTGGAGTGAAAACACTTCCACATCTGTGTACCACGCCTTCAGC
Y G M K A V L T L Y F L Y F L H W S E N T S T S V Y H A F S
AGCCTCTGTTACTTCACTCCCATCCTGGGAGCAGCCATTGCTGACTCATGGTTGGGAAAATTCAAGACAATCATCTATCTCTCCTTGGTG
S L C Y F T P I L G A A I A D S W L G K F K T I I Y L S L V
TATGTGCTTGGCCATGTGATTAAGTCCATGGGTGCCTTACCAATACTGGGGGGACAAATGTTACACACAGTCCTGTCAATGGTCGGCCTG
Y V L G H V I K S M G A L P I L G G Q M L H T V L S M V G L
AGTCTAATAGCTTTGGGGACAGGAGGTATCAAACCCTGTGTGGCAGCTTTTGGTGGAGACCAATTTGAAGAAAAACATGCAGAGGAACGG
S L I A L G T G G I K P C V A A F G G D Q F E E K H A E E R
ACTAGATACTTCTCAGTCTTCTACCTCTCCATCAATGCAGGGAGCTTGATTTCTACATTTGTCACACCCATGCTGAGAGGAGGTGTGCAG
T R Y F S V F Y L S I N A G S L I S T F V T P M L R G G V Q
TGTTTTGGGAAGGACTGCTACGCATTGGCTTTTGGAGTTCCAGGATTGCTTATGCTGATAGCTCTTGTTGTGTTTGCAATGGGAAGTAAA
C F G K D C Y A L A F G V P G L L M L I A L V V F A M G S K
CTATACAGAAAACCACCTCCTGAAGGAAACATACTGAATCAAGTGGTCAAATGTATCTGGTTTGCCATTTCCAGCCGTTTCAAGACCCAT
L Y R K P P P E G N I L N Q V V K C I W F A I S S R F K T H
TCTGGGGACAGTCCCAAGAGAGAGCACTGGCTGGATTGGGCAGCTGAGAAATACCCAAAGCAGCTCATTATGGATGTGAAGGCACTGACC
S G D S P K R E H W L D W A A E K Y P K Q L I M D V K A L T
AGGGTACTGTTCCTTTATATCCCATTGCCCATGTTCTGGGCACTTTTGGATCAGCAGGGCTCACGATGGACCTTGCAAGCCACCAGGATG
R V L F L Y I P L P M F W A L L D Q Q G S R W T L Q A T R M
AATGGGGATTTGGGTTTCTTTGTGCTTCAGCCTGATCAGATGCAGGTGCTAAATCCCTTTTTGGTTCTTATCTTCATACCATTGTTTGAC
N G D L G F F V L Q P D Q M Q V L N P F L V L I F I P L F D
CTTGTCATTTATCGTCTGGTCTCCAAGTGTGGCATTAACTTCACATCACTTAGGAAAATGGCTGTTGGTATGATCCTAGCATGCCTGGCT
L V I Y R L V S K C G I N F T S L R K M A V G M I L A C L A
TTTGCAGTTGCTGCTGCTGTAGAGATAAAAATTAATGAAATGGCCCCACACCAGCCAGATTCCCAAGAGATTTTCCTACAAGTCTTAAAT
F A V A A A V E I K I N E M A P H Q P D S Q E I F L Q V L N
CTGGCAGATGATGAGGTGAAGGTAACAGTGCTCGGCGATGAAAATAATACTCTGTTGGCAGAGTCCATCAAATCTTTCCAGAATATGCCA
L A D D E V K V T V L G D E N N T L L A E S I K S F Q N M P
CACTATTCCAAACTCCACTTGAAGACAAAAAGCCAGAATTTCCACTTCCAATTGAAATATCACAATGTGTCTGTCTACACTGATCATTCT
H Y S K L H L K T K S Q N F H F Q L K Y H N V S V Y T D H S
GTGGAGGAGAAAATATGGTACACCCTGATCATTCGAGAAGATGGGGAAAGTATTTCTAGCATGATGGTGAAGGATGAAGAAAACAAAACA
V E E K I W Y T L I I R E D G E S I S S M M V K D E E N K T
ACCAATGGGATGACAGCCATGAGGTTTGTTAACACTTTGCATGAAAAGGTCAATGTCTCCCTGGGTACAGATGCATCCCTCATTGTTGAT
T N G M T A M R F V N T L H E K V N V S L G T D A S L I V D
GAAGACTATGGTGTGTCTGCTTACAGAACTGTGCAAAGAGGAGAATATCCTGCAGTGCATTGTAGAACAAAGAATGAGGATTTTTCACTG
E D Y G V S A Y R T V Q R G E Y P A V H C R T K N E D F S L
AATTTGGGCCTACTAGACTTTGGTGCAGTTTATCTGTTTGTTATTACTAATAGAACCAATCAGGGTCCTCAGGCCTGGAAGATGGAATAC
N L G L L D F G A V Y L F V I T N R T N Q G P Q A W K M E Y
ATGCCAGCCAACAAAATGTCCATCGCATGGCAGCTACCACAATACGCTCTTGTTACAGCTGGGGAGGTCATGTTCTCTGTCACAGGACTT
M P A N K M S I A W Q L P Q Y A L V T A G E V M F S V T G L
GAGTTTTCTTATTCTCAGGCTCCCTCCAGCATGAAGTCTGTACTCCAGGCAGCCTGGTTGTTGACAGTTGCAGTTGGGAATATCATCGTG
E F S Y S Q A P S S M K S V L Q A A W L L T V A V G N I I V
CTTATTGTGGCACAGTTCAGTGGCCTAGCTCAGTGGGCCGAATTCATTTTGTTTTCCTGCCTCTTGCTGGTGGTCTGCCTGATCTTCTCC
L I V A Q F S G L A Q W A E F I L F S C L L L V V C L I F S
ATCATGGGATACTACTATGTTCCTTTAAAGCCAGGGGATGTTCAGGGGTCAGCTGATAAGCAGACTCCCCAAATCCAAGGAAACATGATC
I M G Y Y Y V P L K P G D V Q G S A D K Q T P Q I Q G N M I
AACCTGGAAACCAAGAAGACAAAGTTCTAA
N L E T K K T K F *
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Fig. 1. The cDNA and deduced AA sequences for yPepT2. ☐, initiation and termination codons; ▨, putative transmembrane domain structure; -, intracellular putative protein kinase C sites; =, putative extracellular N-glycosylation sites. *, termination signal.

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Pred: CCCCCCCCCCCCEEEECCCCCCCCCCCCCCCCCCCCCCCCCCCCCEEEEEHHHHHHHHHH
A A : MNPFQKNDSKETLFLPVSTEEVPPRPPSFPEKPSPKICGSNYPLSFVFIVVNEFCERFSY
               10        20        30        40        50        60
Pred: HHHHHHHHHHHHHHHHCCCCCCHHHHHHHHHHHHHHECCCHHHHHHHHHHHHTCCCEEEEEEEEE
A A : YGMKAVLTLYFLYFLHWSENTSTSVYHAFSSLCYFTPILGAAIADSWLGKFKTIIYLSLV
               70        80        90       100       110       120
Pred: EHTTHEEEEECCCCCCCCCCCHHHHHHHHHEEEEECCCCCCCEEEEETCCCCCCCHHHHH
A A : YVLGHVIKSMGALPILGGQMLHTVLSMVGLSLIALGTGGIKPCVAAFGGDQFEEKHAEER
              130       140       150       160       170       180
Pred: HHHHHEEEEEECTTCHHHHECCHHHHTCCCCCCCCCCEEEECCCHHHHHHHHHHEEETTCC
A A : TRYFSVFYLSINAGSLISTFVTPMLRGDVQCFGKDCYALAFGVPGLLMLIALVVFAMGSK
              190       200       210       220       230       240
Pred: EEECCCTTCCEEEEHHHHHHHHHHHHCCCCCCCCCCCHHHHHHHCCCCCHHHHHHHHHHH
A A : LYRKSPPEGNILNQVVKCIWFAISSRFKTHSGDSPKREPWLDWAAEKYPKQLIMDVKALT
              250       260       270       280       290       300
Pred: HHHEEECCCCHHEEEECTTTCCEEEEHHHHTTCCCCEECCCTHHHHHHHHEEEEECCCCC
A A : RVLFLYIPLPMFWALLDQQGSRWTLQATRMNGNLGFFVLQPDQMQVLNPFLVLIFIPLFD
              310       320       330       340       350       360
Pred: HEECCCCCCCCCCCCCCHHHHHHHHHHHHHHHHHHHHHHHHHHHCCCCCCCCCCCCHEEEEEE
A A : LVIYRLVSKCGINFTSLRKMAVGMILACLAFAVAAAVEIKINEMAPHQPDSQEIFLQVLN
              370       380       390       400       410       420
Pred: CCCCCEEEEECCCCCCCCCCCCEECCCCCCCCCCCCEEECCCCCCCCCEEEEECCCCCCCCCC
A A : LADDEVKVTVLGDENNTLLAESIKSFQNMPHYSKLHLKTKSQNFHFQLKYHNVSVYTDHS
              430       440       450       460       470       480
Pred: CCCCCCCEEEEECCCCCCEEEEEECCCCCCCTTCCEEEEEEECCCCCCEEEECCCCEEEEC
A A : VEEKIWYTLIIREDGESISSMMVKDEENKTTNGMTAMRFVNTLHEKVNVSLGTDASLIVD
              490       500       510       520       530       540
Pred: CCCCCCCCCEECCCCCCCCEEECCCCCCCCCCCCEEECTTCCEEEEEECCCCCCCCCEEEEC
A A : EDYGVSAYRTVQRGEYPAVHCRTKNEDFSLNLGLLDFGAVYLFVITNRTNQGPQAWKMEY
              550       560       570       580       590       600
Pred: CCTTCCEEEECCCTEEEEETTHEEEEETCCEEEECCCCHHHHHHHHHHHHHEEEHTTCEEE
A A : MPANKMSIAWQLPQYALVTAGEVMFSVTGLEFSYSQAPSSMKSVLQAAWLLTVAVGNIIV
              610       620       630       640       650       660
Pred: EEEHHCCCCCHHHHHHHHHHHHHHHHHHHHHHHHHHHEEEECCCCCCCCCCCCCCCCCCCHHHE
A A : LIVAQFSGLAQWAEFILFSCLLLVVCLIFSIMGYYYVPLKPGDVQGSADKQTPQIQGNMI
              670       680       690       700       710       720
Pred: EHHHCCCCH
A A : NLETKKTKF
             729
```

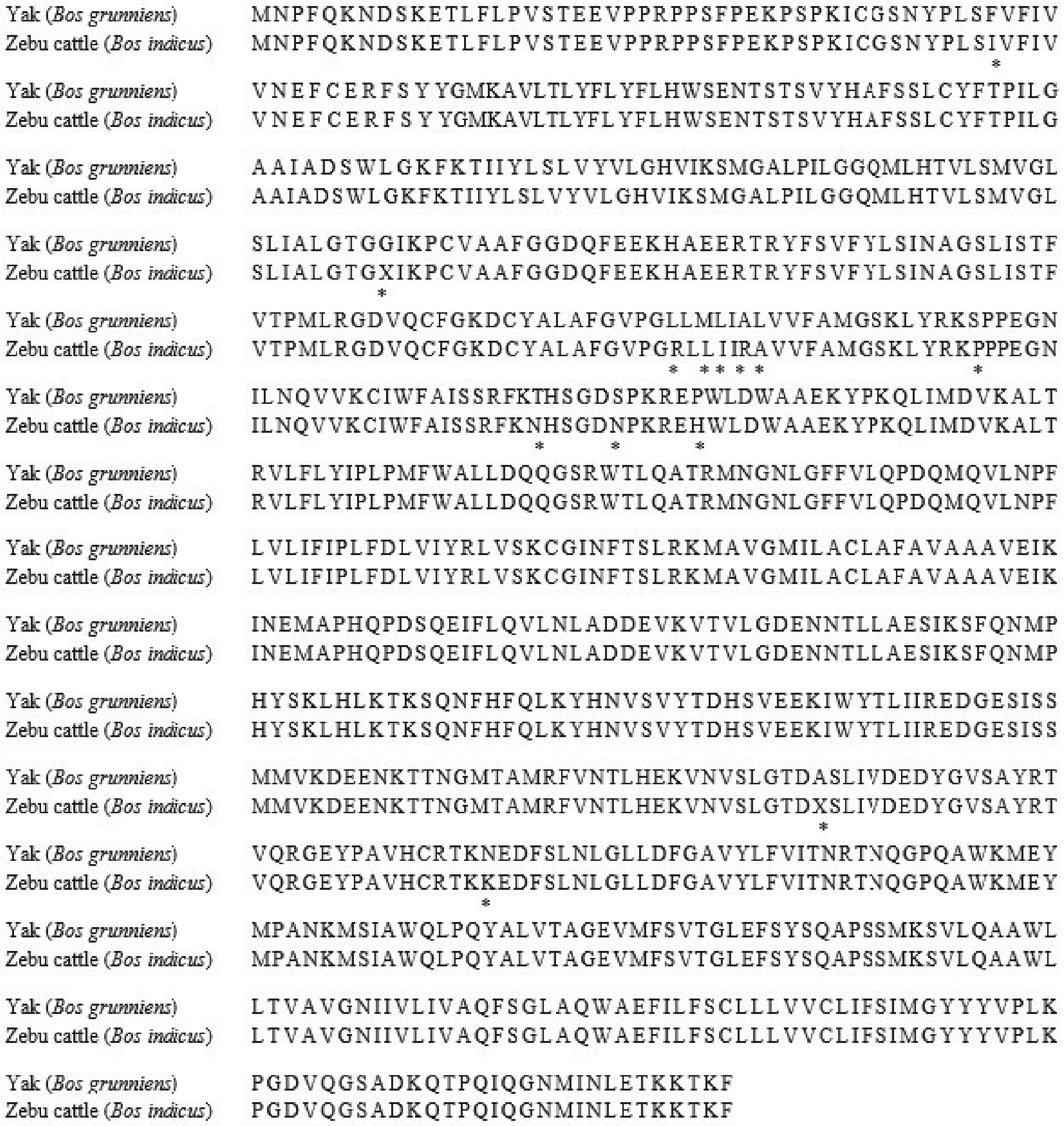
Fig. 2. Yak PepT2 secondary structure prediction. H, Alpha helix; E, Extended strand; C, Random coil; T, Beta turn.

(Doring *et al*. 1998), kidney (Minhas *et al*. 2020), and mammary gland (Alghamdi *et al*. 2020). It was also detected in the intestinal tract and inner ear in addition to the expression of PepT2 mRNA in the brain and kidney of zebrafish (Romano *et al*. 2006). PepT2 played a role in extracellular fluid of the brain and can transport peptide drugs (Chen *et al*. 2017). Study showed that PepT2 was expressed in human epidermal keratinocytes (Kudo *et al*. 2016). Moreover, PepT2 was expressed in cultured rat alveolar epithelium but not PepT1 (Gukasyan *et al*. 2017).

Effect of dietary nitrogen level on PepT2 mRNA expression in yak and indigenous cattle: As the feeding and slaughter trial described, yak and indigenous cattle were fed 10.3, 19.5, 28.5, and 37.6 g N/kg DM diets, and their

Table 2. The homology of yak PepT2 sequences compared with other species

Species	*Bos grunniens*	*Bos indicus*	*Capra hircus*	*Ovis aries*	*Sus scrofa*	*Homo sapiens*	*Mus musculus*	*Gallus gallus*	*Danio rerio*
Bos grunniens	100								
Bos indicus	98	100							
Capra hircus	94	93	100						
Ovis aries	94	93	99	100					
Sus scrofa	91	92	90	90	100				
Homo sapiens	89	90	88	88	91	100			
Mus musculus	83	82	83	83	82	84	100		
Gallus gallus	64	63	64	64	63	64	64	100	
Danio rerio	58	59	58	57	58	58	57	59	100



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Yak (Bos grunniens)         MNPFQKNDSKETLFLPVSTEEVPPRPPSFPEKPSPKICGSNYPLSFVFIV
Zebu cattle (Bos indicus)   MNPFQKNDSKETLFLPVSTEEVPPRPPSFPEKPSPKICGSNYPLSIVFIV
                                                                         *

Yak (Bos grunniens)         VNEFCERFSYYGMKAVLTLYFLYFLHWSENTSTSVYHAFSSLCYFTPILG
Zebu cattle (Bos indicus)   VNEFCERFSYYGMKAVLTLYFLYFLHWSENTSTSVYHAFSSLCYFTPILG

Yak (Bos grunniens)         AAIADSWLGKFKTIIYLSLVYVLGHVIKSMGALPILGGQMLHTVLSMVGL
Zebu cattle (Bos indicus)   AAIADSWLGKFKTIIYLSLVYVLGHVIKSMGALPILGGQMLHTVLSMVGL

Yak (Bos grunniens)         SLIALGTGGIKPCVAAFGGDQFEEKHAEERTRYFSVFYLSINAGSLISTF
Zebu cattle (Bos indicus)   SLIALGTGXIKPCVAAFGGDQFEEKHAEERTRYFSVFYLSINAGSLISTF
                                    *

Yak (Bos grunniens)         VTPMLRGDVQCFGKDCYALAFGVPGLLMLIALVVFAMGSKLYRKSPPEGN
Zebu cattle (Bos indicus)   VTPMLRGDVQCFGKDCYALAFGVPGRLLIIRAVVFAMGSKLYRKPPPEGN
                                                     * ****             *

Yak (Bos grunniens)         ILNQVVKCIWFAISSRFKTHSGDSPKREPWLDWAAEKYPKQLIMDVKALT
Zebu cattle (Bos indicus)   ILNQVVKCIWFAISSRFKNHSGDNPKREHWLDWAAEKYPKQLIMDVKALT
                                              *    *    *

Yak (Bos grunniens)         RVLFLYIPLPMFWALLDQQGSRWTLQATRMNGNLGFFVLQPDQMQVLNPF
Zebu cattle (Bos indicus)   RVLFLYIPLPMFWALLDQQGSRWTLQATRMNGNLGFFVLQPDQMQVLNPF

Yak (Bos grunniens)         LVLIFIPLFDLVIYRLVSKCGINFTSLRKMAVGMILACLAFAVAAAVEIK
Zebu cattle (Bos indicus)   LVLIFIPLFDLVIYRLVSKCGINFTSLRKMAVGMILACLAFAVAAAVEIK

Yak (Bos grunniens)         INEMAPHQPDSQEIFLQVLNLADDEVKVTVLGDENNTLLAESIKSFQNMP
Zebu cattle (Bos indicus)   INEMAPHQPDSQEIFLQVLNLADDEVKVTVLGDENNTLLAESIKSFQNMP

Yak (Bos grunniens)         HYSKLHLKTKSQNFHFQLKYHNVSVYTDHSVEEKIWYTLIIREDGESISS
Zebu cattle (Bos indicus)   HYSKLHLKTKSQNFHFQLKYHNVSVYTDHSVEEKIWYTLIIREDGESISS

Yak (Bos grunniens)         MMVKDEENKTTNGMTAMRFVNTLHEKVNVSLGTDASLIVDEDYGVSAYRT
Zebu cattle (Bos indicus)   MMVKDEENKTTNGMTAMRFVNTLHEKVNVSLGTDXSLIVDEDYGVSAYRT
                                                              *

Yak (Bos grunniens)         VQRGEYPAVHCRTKNEDFSLNLGLLDFGAVYLFVITNRTNQGPQAWKMEY
Zebu cattle (Bos indicus)   VQRGEYPAVHCRTKKEDFSLNLGLLDFGAVYLFVITNRTNQGPQAWKMEY
                                          *

Yak (Bos grunniens)         MPANKMSIAWQLPQYALVTAGEVMFSVTGLEFSYSQAPSSMKSVLQAAWL
Zebu cattle (Bos indicus)   MPANKMSIAWQLPQYALVTAGEVMFSVTGLEFSYSQAPSSMKSVLQAAWL

Yak (Bos grunniens)         LTVAVGNIIVLIVAQFSGLAQWAEFILFSCLLLVVCLIFSIMGYYYVPLK
Zebu cattle (Bos indicus)   LTVAVGNIIVLIVAQFSGLAQWAEFILFSCLLLVVCLIFSIMGYYYVPLK

Yak (Bos grunniens)         PGDVQGSADKQTPQIQGNMINLETKKTKF
Zebu cattle (Bos indicus)   PGDVQGSADKQTPQIQGNMINLETKKTKF
```

Fig. 3. Amino acid (AA) of yak PepT2 sequence comparison of zebu cattle. *, differences AA sequence.

kidney tissues were then analyzed for the relative PepT2 mRNA expression by PCR and real-time PCR. As shown in Table 3, there was a significant difference in the relative PepT2 mRNA expression in genotype, dietary N and interactions between genotype and dietary N ($P<0.001$). When the yak and indigenous cattle were fed 28.5 N/kg

Table 3. Effect of dietary nitrogen (N) level and genotype on relative PepT2 mRNA expression of kidney tissue in yak vs. indigenous cattle (IC)

Genotype[1]	Dietary N (g N/kg DM)				SEM	*P* value[2]		
	10.3	19.5	28.5	37.6		Genotype	N	Genotype × N
Yak	$1.00^{Ba}\pm0.19$	$0.75^{B}\pm0.43$	$3.83^{Aa}\pm0.68$	$0.76^{Bb}\pm0.17$	0.133	<0.001	<0.001	<0.001
IC	$0.65^{Bb}\pm0.12$	$0.60^{B}\pm0.17$	$2.01^{Ab}\pm0.28$	$1.42^{Aa}\pm0.49$				

^{a,b}Within a column, means differ between yak and indigenous cattle ($P<0.05$). ^{A,B}Within a row, means differ between diets differing in N concentrations ($P<0.05$). [1]Animal replicates were 6 for yak and 6 for indigenous cattle. [2]N means dietary nitrogen.

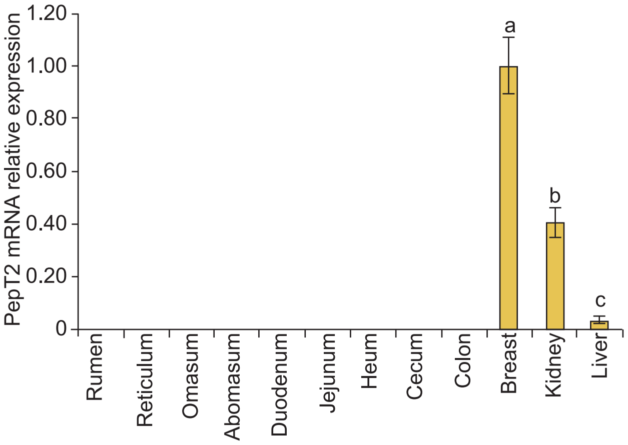

Fig. 4. Yak PepT2 mRNA tissue expression specificity.

DM diet, the relative PepT2 mRNA expression was greater than that in the 10.3, 19.5 and 37.6 g N/kg DM diet ($P<0.001$). Meanwhile, when the indigenous cattle was fed 37.6 N/kg DM diet, the relative PepT2 mRNA expression was greater than that in the 10.3 and 19.5 g N/kg DM diet ($P<0.05$). When 10.3 and 28.5 N/kg DM diet were fed, the relative PepT2 mRNA expression was greater in the yak kidney tissue than that in the indigenous cattle ($P<0.05$), however, when 37.6 N/kg DM diet was fed, the relative PepT2 mRNA expression was lower in the yak kidney tissue than that in the indigenous cattle ($P<0.05$). Yak has an strong ability to retrieve urea N when N intake is low relative to nutritional requirements for growth (Guo *et al*. 2012), a previous study proposed that glomerular filtration rate was lower in yaks than in cattle (Zhou *et al*. 2017), and yaks have special regulating mechanisms in kidneys in terms of glomerular filtration rate and purine derivatives excretion (Wang *et al*. 2011). Moreover, previous studies have suggested that the greater urea N recycled to gastrointestinal tract for yaks compared with that for cattle may be associated with urea transporters in the gut and/or kidney (Zhou *et al*. 2017). As small peptide transport carrier, PepT1 and PepT2 activity were regulated by animal genotypes (Wang *et al*. 2016) and diet nitrogen sources and level (Chen *et al*. 2005). These implied that relative PepT2 mRNA expression was higher in yak kidney than that in indigenous cattle at lower dietary nitrogen supplies, but more research on PepT2 will be required to determine the e renal regulatory mechanisms.

ACKNOWLEDGEMENTS

Financial support from the Second Tibetan Plateau Scientific Expedition and Research Program (2019QZKK0302), and National Youth Natural Science Foundation of China (31302001) are gratefully acknowledged. The authors would like to express their appreciations to Dr. Mahaboubil-haq Muhaiden for reviewing the manuscript.

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