Journal of Animal and Veterinary Advances 11 (16): 2898-2904, 2012
ISSN: 1680-5593

Molecular Structure, Expression Analysis and Functional Characterization of Selenoprotein P (SEPP1) in Goat (*Capra hircus*)

[1]Qian Wang, [2]Chunxiang Zhang, [2]Youshe Ren, [2]Wenbin Yue, [2]Liguang Shi and [3]Fulin Lei
[1]Lab of Animal Reproduction, [2]College of Animal Science and Technology,
Shanxi Agricultural University, 030801 Taigu, P.R. China
[3]Licheng Breeding Goat Center, 047600 Lichen, P.R. China

Abstract: Selenoprotein P (Sepp1) is a protein of considerable intrigue due to its unusual composition and requirements for its biosynthesis. Most selenoproteins contained a single selenocysteine residue, the human, bovine and rodent selenoprotein *P* genes encode proteins contained 10±12 selenocysteines. In this study, a full-length cDNA was cloned and characterized from Taihang black goat testes. The full-length of Sepp1 (1511 bp) cDNA contained an open reading frame encoding 347 amino acids. Sequence analysis showed that the predicted Sepp1 amino acids shared high identities with that of other species. The order of Sepp1 mRNA expression levels was: liver>lung>spleen>kidney, heart, testes, caput epididymidis and corpus epididymidis.

Key words: Goat, selenoprotein P, real-time PCR, expression, CDNA

INTRODUCTION

Selenium is a dietary micronutrient required by mam malian tissues and is essential for the maintenance of spermatogenesis and male fertility (Maiorino *et al*., 1999; Kohrle *et al*., 2000; Beckett and Arthur, 2005). Selenium exerts its biological roles as selenocysteine which is incorporated into the primary structure of selenoproteins. To date, 25 selenoproteins have been identified in humans such as Glutathione Peroxidase (GSH-Px), thioredoxin reductase, Selenoprotein P (Sepp1) and selenoprotein W (Hoffmann *et al*., 2007). Except for selenoprotein P, the selenoenzymes typically contain a single selenocysteine residue that functions at the active site.

Sepp1 is an extracellular glycoprotein and a unique member of the selenoprotein family in that it contains many selenocysteine residues, encoded by UGA stop codons in the open reading frame of its mRNA, 10 in mice and humans, 12 in bovine and 17 in zebrafish in its primary structure (Hill *et al*., 1991; Akesson *et al*., 1994; Burk and Hill, 1994; Steinert *et al*., 1998; Kryukov and Gladyshev, 2000). It is proposed to function in oxidant defense, selenium transport and homeostasis. Most Sepp1 is produced and secreted by the liver although, other tissues produce it as well (Burk and Hill, 2005). Sepp1 contains 50% of the total Se in plasma (Burk and Hill, 1999) and plasma SEPP1 concentration is regarded as a functional biomarker of human selenium status (Nakayama *et al*., 2007). Sepp1 is composed of two domains, the larger N-terminal domain, approximately two-thirds of the amino acid sequenc, contains one Sec residue and the smaller C-terminal domain containing multiple Sec residues which is connected with a bridge containing two His-rich regions.

The N-terminal domain displays enzymatic activity, reducing phospholipid hydroperoxide in the presence of thiol while the C-terminal domain may demonstrate Se carrier activity, delivering Se to cells. Thus, Sepp1 has a unique protein structure and is considered to be a multi-functional protein (Saito and Takahashi, 2000). So far, the cDNA of Sepp1 and its analog have been cloned from rat, human, bovine and murine cDNA libraries, etc. (Hill *et al*., 1991, 1993; Saijoh *et al*., 1995; Steinert *et al*., 1997). However, little is known about Sepp1 in goats and tissue specific expression patterns of Sepp1 in goat have not been examined.

It's also interesting to study thow the Sepp1 activity changes during the life-span in testes in goat. Thus, the current study was performed to identify the *Sepp1* gene of the goat and to determine relative expression levels of Sepp1 mRNA in various tissues and in different developmental stages of the testis in goat. And then provide useful molecular information and determine the expression patterns of this widely-distributed seleno-enzyme.

Corresponding Author: Chunxiang Zhang, College of Animal Science and Technology, Shanxi Agricultural University, 030801 Taigu, P.R. China

MATERIALS AND METHODS

Animals and tissue collection: The male Taihang black goats used in this study were provided by Licheng Sheep Breeding Center in Shanxi province, China. For the cloning and tissue expression profile of goat Sepp1, 5 adult bucks were killed by exsanguinations and the heart, liver, spleen, lung, kidneys, testis, epididymis and were collected, frozen in liquid nitrogen and stored at -80°C until use. For age-dependent expression analysis of Sepp1, 1, 2, 4, 6, 8, 12 and 20 weeks old male bucks were castrated. The left testes was frozen in liquid nitrogen for total RNA extraction.

RNA extraction: The total RNA was extracted from adult goat testes, different age testes and various tissues using TRIzol^{R} reagent (Invitrogen) according to the manufacturer's protocol. The extracted RNA was dissolved in DEPC-treated water and the concentration, purity and integrity were assessed using an Eppendorf Biophotometer (Germany) at 260/280 nm (OD260/OD280 = 1.8-2.0) and by electrophoresis in a 1% agarose gel to verify its integrity.

Cloning the full-length cDNA of goat testes Sepp1: Total RNA (2 μg) was used as a template for cDNA synthesis using PrimeScript™ RT Reagent kit (Takara, Japan) according to the instructions of the manufacturer. The two PCR-amplification primers (Table 1) were designed based on highly conserved region by comparing all known Sepp1 sequences using the Blastn program and used to amplify the full-length cDNA of goat Sepp1. The PCR reaction was performed at 94°C for 5 min, 30 cycles of 94°C for 30 sec, 54.3 and 52.4°C for 30 sec, 72°C for 30 sec followed by 72°C for 10 min. The amplified products were gel-extracted, purified using a TaKaRa agarose gel purification kit, ligated into pMD18-T (TaKaRa) and sequenced. The two obtained Sepp1 sequences were assembled using DNAStar Lasergene 7.1 Software to obtain full-length cDNA.

Sequence analysis: Sequence alignments, Open Reading Frame (ORF) translation and molecular mass calculation of predicted protein were carried out with Vector NTI Suite 8.0. Hydrophobic nature of protein Sepp1 was predicted by the ExPASy servers (http://web.expasy.org/protscale/), whereas secondary structural analysis of predicted LsTMT was carried out on the website http://www.expasy.org. The prediction of the signal peptide was predicted by the SignalP program (http://www.cbs.dtu.dk/services/SignalP/). Potential N-glycosylation sites were predicted by NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/). O-glycosylation sites were predicted by NetOGlyc 3.1 (http://www.cbs.dtu.dk/services/NetOGlyc/).

Analysis of mRNA expression profile in various tissues and different age of testes: Mx3000P real-time PCR system (Stratagene, USA) and SYBR® Permix Ex Taq™ kit (Takara, Japan) were used to study the mRNA expression of Sepp1 in different samples according to the manufacturer's instructions. Primer was designed by using Primer Premier 5.0 Software (Table 1), based on the sequence of goat Sepp1 cDNA (JQ316673), GAPDH as a housekeeping gene based on goat GAPDH (NM001034034) The PCR reaction was performed at 95°C for 10 sec followed by 40 cycles of 95°C for 10 sec and 61°C for 30 sec and a following cycle of 61°C for 30 sec and 95°C for 10 sec to obtain the dissociation curves.

And the reaction specificity was determined when there was only one specific peak in the dissociation curve. PCR efficiencies were detected using relative standard curve derived from diluted cDNA reaction mixture (a 2 fold dilution series with 5 measuring points). The R^2 values for all standard curves generated ranged between 0.997 and 0.999 and PCR efficiencies were between 90 and 110%. The CT values were used to quantify the PCR product, i.e., the relative expression level of the target gene was expressed as 2^{-CT} and CT was calculated by subtracting CT (housekeeping gene: *GAPDH*) from CT (target gene).

Statistical analysis: The data of different groups were analyzed by ANOVA. All values were expressed as the Mean±Standard Error of Mean (SEM). Differences were considered significant at $p<0.05$ using Tukey's test in SPSS Software Program (Version 13.0 for Windows).

Table 1: Sequences of primers used in the study

Names	Primer sequence	Size (bp)	Useage
P1	F: 5' TAAACTTCTGACTTTCGCTC 3' R: 5' CTGTTTTCTCCTTGGTAGCC 3'	736	Gene cloning
P2	F: 5' GTCTGGACTCTCTTAAATGG 3' R: 5' CTTCCTCCATTCTTAGTTGC 3'	1162	Gene cloning
P3	F: GATTGGAGGACCTGCGAGT R: GGTTGGGCGTCTTCTTGT	160	expression
GAPDH	F: 5' GGTGATGCTGGTGCTGAG 3' R: 5' TGACAATCTTGAGGGTGTTG 3'	181	expression

RESULTS AND DISCUSSION

Cloning and analysis of Sepp1 cDNA: The full-length cDNA was assembled by the two obtained Sepp1 sequences using DNA Star Lasergene 7.1 Software. The full-length cDNA of Sepp1 was 1511 bp long including 5 and 3-untranslated regions and a 1041 bp ORF encoding a 347 amino acid protein (Fig. 1). The complete cDNA sequence of the *Sepp1* gene was submitted to NCBI GeneBank (Accession No.: JQ316673). A conserved stem-loop structure in 3'UTR, SECIS has been reported in selenoprotein mRNA (18, 19) and the recoding of UGA to translate as Sec instead of a stop codon requires this specific structure. In the present study, the predicted Seleno-cysteine Insertion Sequence (SECIS) in 3'-UTR was also observed with the same structure of SECIS of other selenoproteins (Cossio-Bayugar *et al*., 2005; Cai *et al*., 2008; Yeh *et al*., 2009). The deduced Sepp1 amino acid sequences showed that the predicted protein has an estimated 37.5 kDa molecular weight and with

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1    TTAAACTTCTGACTTTCGCTCAGAGGGTGAGGTAAACAACAGGACTATAAATAGCCAAGT
61   TTCTCACGGTGGCTTTGTGGAGCCGCCCAGCTGAGCAAAGAGAAAGGAAGCAAGCTGGTC
121  GGAAGGGGTTCTGACAACCCCAGCC ATG TGG AGA GGC CTG GGG CTG GCT CTG GCT CTC TGC

                          M  W  R  G  L  G  L  A  L  A  L  C
182  CTC CTC CTA ACC GGA GGA ACA GAG AGC CAG GGC CAA AGC TCC TAT TGT AAG CAA CCT CCA
13   L  L  L  T  G  G  T  E  S  Q  G  Q  S  S  Y  C  K  Q  P  P
242  GCC TGG AGC ATA AAG GAT CAA GAC CCT ATG CTG AAC TCC TAT GGT TCA GTG ACC GTG GTT
33   A  W  S  I  K  D  Q  D  P  M  L  N  S  Y  G  S  V  T  V  V
302  GCT CTT CTT CAA GCC AGC TGA TAC CTG TGC ATT CTG CAG GCA TCT AGA TTG GAG GAC CTG
53   A  L  L  Q  A  S  *  Y  L  C  I  L  Q  A  S  R  L  E  D  L
362  CGA GTA AAA CTG GAG AAA GAA GGA TAT TCT AAC ATC TCT TAT GTT GTT GTT AAT CAT CAA
73   R  V  K  L  E  K  E  G  Y  S  N  I  S  Y  V  V  V  N  H  Q
422  GAA ATC TCT TCT CGA TTA AAA TAT GTG CAT CCT AAG AAT AAG GTT TCA GAA CAT ATT CCT
93   E  I  S  S  R  L  K  Y  V  H  P  K  N  K  V  S  E  H  I  P
482  GTT TAC CAA CAA GAA GAC GAC CAA CCA GAT GTC TGG ACT CTC TTA AAT GGA AAT AAA GAT
113  V  Y  Q  Q  E  D  D  Q  P  D  V  W  T  L  L  N  G  N  K  D
542  GAC TTC CTC ATA TAT GAC AGA TGT GGC CGC CTT GTA TAT CAT CTT GGT TTG CCT TAT TCC
133  D  F  L  I  Y  D  R  C  G  R  L  V  Y  H  L  G  L  P  Y  S
602  TTC CTA ACT TTC ACA TAT GTA GAA GAT TCC ATT AAG ACT GTT TAC TGT GAA GAT AAA TGT
151  F  L  T  F  T  Y  V  E  D  S  I  K  T  V  Y  C  E  D  K  C
662  GGA AAC TGC TCT CTC AAG ACA CAG GAG GAT GAA GAC TTC TGT AAA AAT GTA TCT CTG GCT
173  G  N  C  S  L  K  T  Q  E  D  E  D  F  C  K  N  V  S  L  A
722  ACC AAG GAG AAA ACA GCT GAG GCT TCA CGG CGA CAT CAC CAC CAC CAT AGG CAC AAG GGT
193  T  K  E  K  T  A  E  A  S  R  R  H  H  H  H  H  R  H  K  G
782  CAC CAA AGA CAG GGT CAC TCA GAT AAC TGT GAT ACA CCA GTA GGA AGT GAA AAT TTA CAA
213  H  Q  R  Q  G  H  S  D  N  C  D  T  P  V  G  S  E  N  L  Q
842  CTT TCT CTT CCA CAA AAG AAG CTC TGA CGA AAG AGA TGC ATA AAT CAG TTA CTC TGA CAG
233  L  S  L  P  Q  K  K  L  *  R  K  R  C  I  N  Q  L  L  *  Q
902  TTT CCC AAA GAT TCA GAA TCT GCT TTG AGT AGC TGC TGT TGC CAC TGT CGA CAT CTG GTA
253  F  P  K  D  S  E  S  A  L  S  S  C  C  C  H  C  R  H  L  V
962  TTT GAA AAA ACA GGG TCT GCA ATC ACC TGA CAG TGT ACA GAA AAC CTC CCC TCT TTA TGT
273  F  E  K  T  G  S  A  I  T  *  Q  C  T  E  N  L  P  S  L  C
1022 AGC TGA CAG GGC CTT TTG GCA GAG GAG AAC GTC ATT GAA TCT TGA CAG TGA CGT TTG CCT
293  S  *  Q  G  L  L  A  E  E  N  V  I  E  S  *  Q  *  R  L  P
1082 CCA GCT GCC TGA CAG GCA GCA GGT CAG CAG CTC AAT CCC ACA GAA GCC AGC ACC AAG TGA
313  P  A  A  *  Q  A  A  G  Q  Q  L  N  P  T  E  A  S  T  K  *
1142 AGC TGA AAA AAT AAG GCC AAA ATG TGA AAA TGA CCT TCA AAT TAA ATATATGAAATTGG
333  S  *  K  N  K  A  K  M  *  K  *  P  S  N  *
1201 ATATATTCCCCAAGTCAATCTACACATTACATTCCCAGCATTTGTATAAGCTACCTAATT
1261 AATAGTGATTCAAAAATAGGAACTGGATTTGTGCAAACATGGAGAAATTGACTTCCACAT
1321 TTAAAAATTTAAGTCAAAGAAATTTTGACCCAAACCATATTTTTATTCAGCTGAAAGGTG
1381 GTTGCAGCATTTGGTTAATATGTTTTTCTTTTTCTTTTTCCAGTATTCTACTTGCATTAA
1441 TGAGAACAGAAACGTAAACTATAACCTAGGGGTTTCTGTTGGATAGTTGGCAGCTAAGAA
1501 TGGAGGAAGAA
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Fig. 1: The full-length cDNA and deduced amino acid sequence of Sepp1 from goat. The start codon (ATG) and the stop codon (TAA) are in green. Selenocysteine (Sec) are shown in the boxes. The signal peptide is shaded in gray. The predicted Selenocysteine Insertion Sequence (SECIS) in 3'-UTR is underlined and conserved ATGA, AA and TGA motif are in red

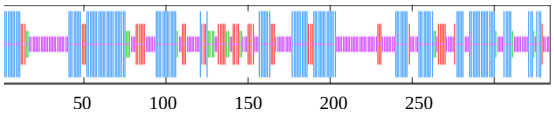

Fig. 2: The secondary structure of Sepp1. Alpha helix, extended strand, beta turn and random coil are indicated, respectively with the longest, the second longest, the second shortest and the shortest vertical lines

Fig. 3: 3D Protein Crystal Structural Model of goat Sepp1

an Isoelectric point (pI) of 7.85; the secondary structure of Sepp1 was analyzed by SOPMA. The result showed that the putative Sepp1 peptide contained 41.62% of α helix, 14.37% of extended strand, 5.69% of β turn and 38.22% of random coil (Fig. 2); the α-helix constituted the majority of the secondary structure.

The 3D Protein Crystal Structural Model of goat Sepp1 was predicted from amino acid sequences using SWISS Model server on-line (Fig. 3), the result showed that the protein contains one nucleus helix, three short-helix and some coiling. The 4 N-glycosylation sites were predicted in the goat Sepp1 sequence in contrast with a single in zebrafish, five in the rat and six in the human sequence (Fig. 4). Hwever, no O-glycosylation sites were observed in the sequence (Fig. 5).

The phylogenetic tree inferred from amino acid sequences of Sepp1 from all six mammalian species was based on maximum likelihood using DNAMAN (Fig. 6). The BLAST analysis showed that the sequence of *Sepp1* gene had 97.5% homology with bovine; 90% with *Sus scrofa* and 77.1, 79.7 and 86.7% homology with *Mus musculus*, *Rattus norvegicus* and human, respectively which indicated a close evolutionary relationship.

Expression analysis of goat Sepp1 mRNA in different tissue: Tissue distribution of a protein may be the

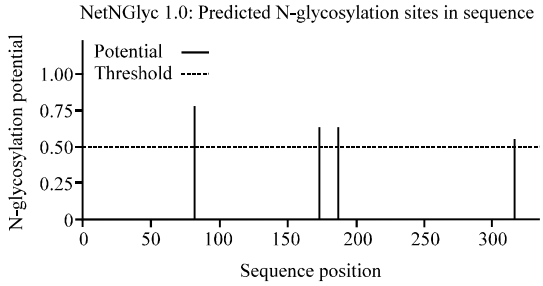

Fig. 4: Predicted N-glycosylation sites in goat Sepp1 sequence

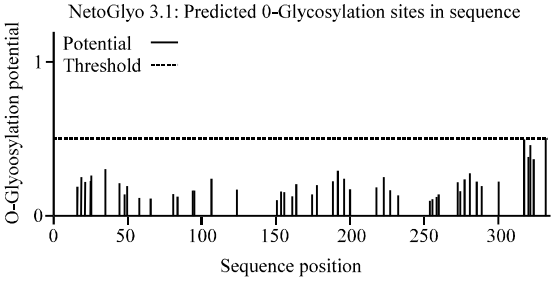

Fig. 5: Predicted O-glycosylation sites in goat Sepp1 sequence

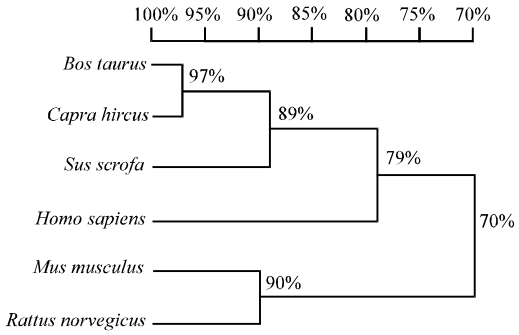

Fig. 6: Phylogenetic tree among Sepp1 protein of goat and other mammal

indication of its physiological function. The constitutive mRNA expression of Sepp1 in different tissues was analyzed by real-time PCR normalized against GAPDH levels. The order of Sepp1 mRNA expression in different tissues was: liver>lung>spleen>kidney, heart, testes, caput epididymidis and corpus epididymidis>cauda epididymidis>muscle (Fig. 7). The highest rate of transcription was observed in liver ($p<0.001$) very low expression was detected in the muscle ($p<0.001$).

Expression profile of Sepp1 in testes of different age: Figure 8 showed the relative mRNA expression levels of Sepp1 in different ages of goat testes and normalized with

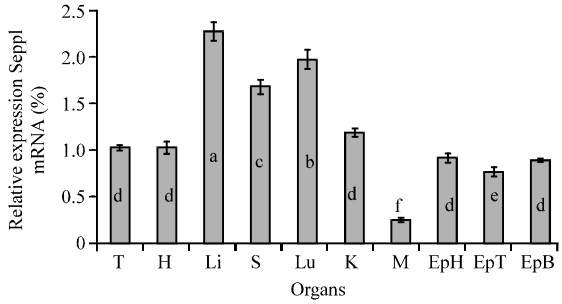

Fig. 7: Relative Sepp1 mRNA expression levels in various tissues which normalized with GAPDH mRNA in each tissue. Testes (T), Heart (H), Liver (Li), Spleen (S), Lung (Lu), Kidney (K), Muscle (M), caput Epididymidis (EpH), corpus Epididymidis (EpT), cauda Epididymidis (EpB). Different letters (a-f) show significantly different ($p<0.05$)

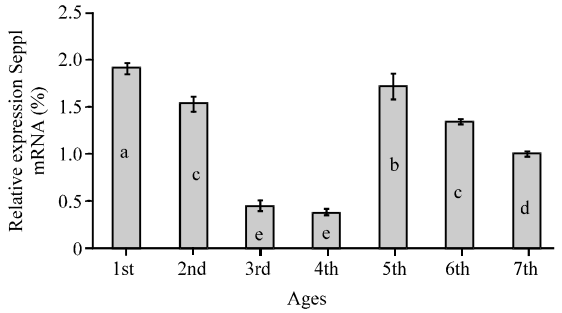

Fig. 8: Relative Sepp1 mRNA expression levels of goat testes in different ages and normalized with GAPDH in each age. Different letters (a-e) show significantly different ($p<0.05$). 1st, 2nd, 3rd, 4th, 5th, 6th and 7th represent 1, 2, 4, 6, 8, 12 and 20 weeks old male bucks, respectively

GAPDH in each age. The Sepp1 mRNA expression was observed in each developmental stage of testis. The first (1 week) had the highest Sepp1 mRNA expression while the third (4 weeks) and fourth (6 weeks) had the lowest Sepp1 mRNA expression. In this study, researchers described the identification, expression and characterization of Sepp1. By now, little was known about Sepp1 in goats and tissue specific expression patterns of Sepp1 in goat. Selenoprotein P (Sepp1) is a unique member of the selenoprotein family in that it contains multiple Sec residues per protein molecule, human and mouse Sepp1 both contain 10 Sec residues. In this study, researchers found that goat had 12 Sec residues in its protein molecule which is the same to the cow. Met1-Glu20 is high hydrophobic region which is also considered to be the signal peptide of Sepp1 which is consistent with the characteristics that the signal peptide is highly hydrophobic (Fig. 9). Se is an essential micronutrient and is incorporated into proteins in the form

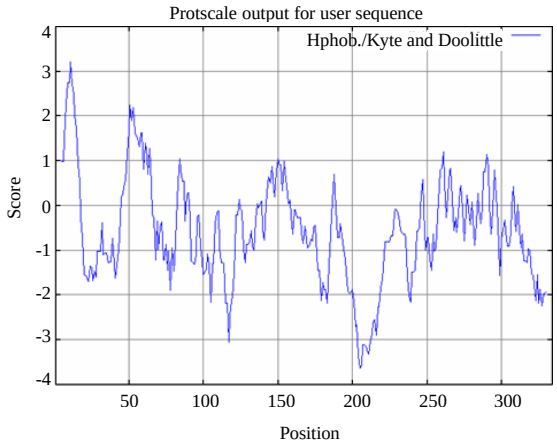

Fig. 9: Analysis of hydrophobility region of Sepp1 protein from goat testis

of selenocysteine. A conserved stem-loop structure in 3'UTR, SECIS has been reported in selenoprotein mRNA (Shen *et al*., 1993; Grundner-Culemann *et al*., 1999), the presence of SECIS is necessary for the recognition of UGA as a signal for Sec insertion (Mizutani *et al*., 2000; Lescure *et al*., 2002). In this study, the predicted Selenocysteine Insertion Sequence (SECIS) was also observed in the goat Sepp1 mRNA.

The high level of conservation of deduced amino acid sequence among the *Sepp1* genes from other species provides evidence that these sequence features are critical for function. The presence of Sepp1 in plasma was suspected in the mid 1970s, purification of the protein described in 1987 (Yang *et al*., 1987) and the first cDNA reported in 1991 (Hill *et al*., 1991). The highest expression rate of goat Sepp1 was detected in the liver which is conform to the fact that approximately 75% of the selenium in mouse plasma is in Sepp1 (Hill *et al*., 2007)and this protein has been postulated to transport the element from the liver to other tissues.

Sepp1 can be divided into two domains containing the N-terminal domain and the C-terminal domain. Each of the two domains has its own function. Thisse C (Thisse *et al*., 2003) showed that the shorter isoform was expressed primarily in the liver and the longer one primarily in the brain, kidney and intestine. The different Sepp1 mRNA expression levels are considered to be associated with the different functions. The expression profile of goat Sepp1 mRNA in other tissues is similar with the results reported by Burk and Hill (2009).

The second higher level of expression of Sepp1 is found in testis, suggesting that Sepp1 in testes have more than just an antioxidant function they also play an important role in male fertility. Previous studies indicated that Sepp1$^{-/-}$ mice have reduced fertility and produce

spermatozoa that have the same appearance as selenium-deficient ones (Andersen *et al*., 2003). Sepp1 plays an important role in transporting selenium and removing Reactive Oxygen Species (ROS). In this study, the relative Sepp1 mRNA expression had the highest level in the 1 week old goat.

This observation may attribute to the fact that a large number of metabolites required removal in the stage of the early stages of development. With the development of the testis, the requirement of selenium become steadily falling which is consistent with the results in the recent report. While the Sepp1 mRNA expression level increased in the 8 weeks old. That may be associated with the important role of Sepp1 in male reproduction and the testicular development is relatively mature in 8 weeks old when begin to produce sperm. Therefore, Sepp1 is in great need at the stage of sexually mature.

CONCLUSION

Sepp1 cDNA was cloned and characterized from the goat (*Capra hircus*). Sepp1mRNA was expressed in all goat tissues studied. That suggested that Sepp1 had multiple functions in goat. This study also provides the basis for investigations on the role of Sepp1 in any other animals. The mechanisms need further investigations. The function of Sepp1 is still unknown, the cloning and characterization of Sepp1 will be helpful to further understanding its role in the some aspects.

ACKNOWLEDGEMENTS

This project was supported by research fund for the Doctoral Program of Higher Education of China (No.: 20091403120001). The researchers also thank Fulin Lei, Xiaofeng Lei and other staff at the Taihang Black Goat Breeding Institute for their help in collection of testes tissue. The researchers thank staffs in the Lab of Animal Reproduction, Shanxi Agricultural University, P.R. China for assistance in the experiment. The researchers also thank Liguang Shi and Lei Shi for their contribution in the manuscript revision.

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