Journal of Animal and Veterinary Advances 10 (1): 100-105, 2011
ISSN: 1680-5593

Metallothionein in Yak Characterization of Metallothionein-III in Yak (*Bos grunniens*)

[1]L. Yang, [1]L.P. Zhang, [1]J.P. Wu, [2]M.A. Brown, [1]B. Liu, [1]B.Y. Ma and [1]L. Wang
[1]College of Animal Science and Technology,
Gansu Agricultural University, Lanzhou 730070, China
[2]Grazinglands Research Laboratory, USDA-ARS, El Reno, OK 73036

Abstract: Metallothionein-III (MT-III) as a new member of the Metallothionein (MT) family has specific physiological effects different from known MT-I and MT-II. In this study, the yak MT-III gene coding region was amplified and cloned by RT-PCR from brain tissue of yak using YMT-III$_{SP1}$ and YMT-III$_{SP2}$ as specific primers. The isolated cDNA sequence of MT-III was 207 bp in length (Genbank Accession, NO, DQ323545) and was subjected to BLASTn searching in NCBI. Results of the search indicate that nucleotide sequences of yak share 98, 97, 96, 92, 91, 90, 89, 88 and 86% sequence similarity with cattle, milk goat, hair goat, pig, sheep, chimpanzee, human, dog and house mouse, respectively. Comparing homologies of MT-III sequences with MT-I and MT-II in yak, we found 69 and 67% homologies, respectively. The MT-III protein was composed of 68 amino acids, including 19 cysteines, similar to the number of cysteines of sheep but not human and mouse which lack the conserved ninth cysteine and have no aromatic amino acids. There were conserved motifs of MTs, such as C-X-C, C-C-X-C-C, C-X-X-C and KKS and specific motifs including MDPE, CPCP in MT-III. This conservation of motifs suggests a conservation of MT-III in molecular evolution. The MT-III in yak had no signal peptide and represented a form of cytoplasmic protein similar to MT-I/II. There were few sheets in secondary protein structures, obvious helices in 39-46th AA and mainly irregular curling in the 2D-structure of MT-III protein. The lack of the conserved ninth cysteine in yak MT-III merits further research.

Key words: Yak, metallothionein-III, cDNA, protein structure, nucleotide sequence, gene coding

INTRODUCTION

Metallothionein-III (MT-III) is an isoform of Metallothioneines (MT) expressed mainly in the central nervous system, cerebra and reproductive system of animals (Valls *et al*., 2001; Nuria *et al*., 2002; Richard *et al*., 1992). Like other members of the MT family, MT-III is a low molecular weight, cysteine-rich, metal-binding protein with 20 cysteine residues at conserved positions. The functionally unique properties of MT-III are not found for MT-I/-II. It has neuroinhibitory functions, associated with neuronal Growth Inhibitory Factor (GIF) and seems to play a role in neuronal regeneration and degeneration. It protects cultured cortical neurons from toxic effects of amyloid β peptides and is not induced by typical inducers of MT-I/-II biosynthesis such as metal ions (zinc, cadmium and copper), hormones, inflammatory stimuli and stress agents (Nuria *et al*., 2002; Palmiter, 1998). Neuronal inhibitory activity has been mapped to the N-terminal domain of the MT-III protein and has been established for Cu_4, Zn_3-MT-III isolated from human and bovine brains and for recombinant human and mouse Zn_7-MT-III. In addition, changes in MT-III mRNA levels in response to central nervous system injury seem to indicate an important role of MT-III brain repair. Under conditions of γ-ray oxidative stress, MT-III prevents γ radiation-induced 8-oxoG accumulation and mutation in normal and hOGG1-depleted cells which at least in part may contribute to the anticarcinogenic and neuroprotective role of MT-III (Hye *et al*., 2004). The yak species (*Bos grunniens*) represents a unique bovine species adapted to the Tibetan plateau of China at altitudes of 3,000 m above sea level where oxygen content is only 33% of that at sea level and intensity of ultraviolet radiation is 3-4 times that in lowland areas (Zhang *et al*., 1994). Consequently, yak adapted to this environment likely have special physiological mechanisms to protect their central nervous systems against hypoxic and oxidative injury. Based on the function of MT-III, it is predicted that MTs may play an important role for yak adaptation to the Tibetan plateau environment. The objectives of this research were to characterize the nucleotide sequence and protein structure of MT-III in yak.

Corresponding Author: J.P. Wu, College of Animal Science and Technology, Gansu Agricultural University, Lanzhou 730070, China

MATERIALS AND METHODS

Animals and RNA isolation: Preharvest animal care of the yak individuals used in this study were under the control of local farmers in Gannan, Gansu production area and their treatment was consistent with Gansu Agricultural University animal care and use requirements. Animals were harvested at a commercial facility that must comply with state regulations governing processing of meat animals. To clone the MT-III, cerebra samples were aseptically obtained from 5 domestic yaks (*Bos. grunniens*) from Gannan, Small Tail Han Sheep (STHS, *Ovis aries*) and milk goats (*Capra aegagrus hircus*) from Lintao County and cashmere goats from Jingtai County, Gansu, China within 10 min after slaughter. Samples were flash-frozen in liquid nitrogen and stored at -80°C until thawed for RNA extraction. Total RNA was extracted with Trizol reagent (Invitrogen, Aukland, New Zealand). Yak cerebra tissue samples were homogenized in 1 mL of Trizol reagent per 50-100 mg of tissue using a glass homogenizer. Homogenized samples were incubated for 5 min at 15-30°C to permit complete dissociation of nucleoprotein complexes. Chloroform (0.2 mL mL^{-1} of Trizol) was added and samples were shaken and incubated at 15-30°C for an additional 2-3 min. Samples were then centrifuged at 12,000×g for 15 min at 2-8°C. After centrifugation, dissolved RNA was pipetted to another fresh tube and RNA was precipitated with isopropyl alcohol. Samples were then incubated at 15-30°C for 10 min and centrifuged at 12,000× g for 10 min at 2-8°C. The supernatant was removed and RNA precipitate was washed with 75% ethanol. The RNA and ethanol were vortexed and centrifuged at 7,500×g for 5 min at 2-8°C. The RNA was then redissolved in 100% formamide (deionized) and stored at -70°C.

Reverse-transcription PCR primers: Oligonucleotide Primers for Reverse-Transcription PCR (RT-PCR) were designed based on coding region sequences of MT-III in sheep (*Ovis aries*), pig (*Sus scrofa*), human, mouse (*Mus musculus*) published at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Forward and reverse primers, respectively, of the yak MT-III were YMT-$_{SP1}$ (23 bp): 5'-ATGGACCCTGA GRCC TGC CCCTG-3'and YMT-III$_{SP2}$ (23 bp): 5'-GGCRCAGC AGCTGCACTTGTCCG-3'. These primers were also used to clone MT-III of Small-tail Han sheep and milk and hair goats.

RT-PCR: The RT-PCR was carried out using a commercial RNA PCR kit (AMV Ver. 3.0 TaKaRa, Da Lian, China), for which Avian Myeloblastosis Virus (AMV) reverse transcription was used for first-strand DNA synthesis and Taq DNA polymerase was used for PCR in a single optimized RT-PCR buffer. First-strand cDNA synthesis was accomplished by RT-PCR at 30°C for 10 min, 50°C for 30 min, 99°C for 5 min and 4°C for 5 min. Subsequently, PCR was conducted using primers of YMT-III$_{SP1}$ and YMT-III$_{SP2}$ and DNA Taq polymerase. Reaction conditions and parameters for cycles were: predenatured 94°C for 2 min, 94°C for 30 sec, 58°C for 1 min, 72°C for 1.5 min, 72°C for 10 min for a total of 35 cycles. After completion of the reaction, products were separated by electrophoresis on a 1.5% agarose gel. Fragments of expected size were cut from the gel and purified using TIANgel Midi Purification Kit (Tiangen, Beijing, PRC).

Cloning and sequencing of cDNA encoding the MT-III gene: The plasmid library was constructed with T4 polymerase by ligating amplified DNA fragments into sphI (TaKaRa, Da Lian, China) and SalI (TaKaRa, Da Lian, China) sites of the cloning plasmid pGEM-T Vector System 1 (Promega, Madison, Wisconsin, USA), following the manufacturer's described procedures. Isolation of library DNA, transfection of competent *E. coli* JM109 and extraction of DNA from transfected cells were performed according to published methods (Sambrook and Russell, 2001). Recombinant plasmids containing relevant yak DNA fragments were sequenced on an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA).

Sequence analysis of the nucleotides and amino acid: The nucleotides and AA sequences of MT-III in yak were subjected to BLAST searching at the National Center for Biotechnology Information. Multiple comparisons of nucleotide sequences were then performed using Clustral W (www.ebi.ac.uk). Characterizations of MT-III included determination of their molecular weight, AA composition, hydrophobicity, transmembrane region characteristics and signal peptide analysis.

Hydrophobicity was analyzed using ProtScale and ExPASy (www.expasy.ch; Gasteiger *et al*., 2005) and evidence for transmembrane regions was analyzed using ExPASy (prediction parameters: TM-helix length between 17 and 33). Parameters used in ProtScale analyses included a window size of 9, window weight on the edges of 100%, the linear weight variation model and no normalization of scale. Protein signal peptides were analyzed using SignalP 3.0 software (http://www.cbs.dtu.dk/services/SignalP-3.0/) using Neural Networks (NN) and Hidden Markov Models (HMM) trained on eukaryotes. Molecular evolutionary genetics analysis used MEGA software (version 3.1, www.megasoftware.net. Tree inference: Method: Neighbor-Joining; Phylogeny Test and options: Interior Branch Test (1000 replicates; seed = 41699); Include Sites: Gaps/Missing Data: Complete Deletion; Substitution Model: Amino: JTT Model; Substitutions to Include: All; Pattern among

Lineages: Same (Homogeneous); Rates among sites: Uniform rates). Secondary structures of MT-III were predicted by APSSP2 (Raghava, 2000; http://www.expasy.ch/) and tertiary structures of MT-III were predicted by Swiss-Model (Schwede *et al*., 2003; http://www. expasy.ch/ swissmodel/).

RESULTS AND DISCUSSION

Nucleotide and AA sequences of MT-III: Results of nucleotide and AA sequences of MT-III of yak are shown in Fig. 1. Sequencing results among numerous clones were consistent and the consensus nucleotide length was 207 bp of Coding Sequence (CDS) (Genbank Accession, NO, DQ323545). Using the same method, the CDS of Small-tail Han sheep (Genbank Accession No., NM_001009755), milk goats (Genbank Accession No. EF471076) and hair goats (Genbank Accession No. EF195236) were obtained. A BLAST search of CDS sequences in yak MT-III found nucleotide sequences of yak shared 98, 97, 96, 92, 91, 90, 89, 88 and 86% sequence similarity with cattle, milk goat, hair goat, pig, sheep, chimpanzee, human, dog and house mouse, respectively. Consequently it is evident that MT-III is highly conserved among mammals. Comparing homologies of MT-III sequences with MT- I and MT-II in yak, About 69 and 67% homology is founded, respectively. The cDNA nucleotide sequences were translated into AA sequences encoding 68 amino acids, including 19 Cysteines (Cys) (27.94%), non-aromatic amino acids and no disulfide amino acids. Comparison of the AA sequences of MT-III with AA sequences of MT-I/II (Wu *et al*., 2007) in yak is shown in Fig. 2. We found that there were tripeptides conserved across MT-I/-II in yak: MDPN CXC ----- CXC --- CXC – CXC – - --- CCXCC --- CXXC --- CXC -----CXCC -where X designates AA excluding cysteines (Genbank Accession No.: Yak MT-I (AY513744), Yak MT-II (AY513745). However, there are 2 inserts in the MT-III sequence that show differences from MT-I/-II: a single Thr in the N-terminal region and an acidic hexapeptide (Glu-Ala- Ala-Glu- Ala- Glu) in the C-terminal region, similar to other reports in other species (Uchida *et al*., 1991; Tsuji *et al*., 1992; Richard *et al*., 1992).

However, the corresponding conservative Cys^{29} of MT-I/-II is replaced by Ser^{30} in MT-III, due to a mutation at the 88th nucleotide acid where T mutated into A. Analysis of AA sequences of MT-III in yak, cattle, sheep, human, house mouse, horse, hair goat, chimpanzee, dog and pig are shown in Fig. 3. It is founded that Cys^{29} was replaced by Ser in yak, sheep and goat, indicating the number of –SH groups in MT-III were decreased, compared to cattle, humans and horses. As such, it hypothesize that the metal binding capacity of MT-III may be lesser than that of MT-I and MT-II. Further research is needed to validate this hypothesis.

Yak MT-III protein sequence analysis: A homology search for MT-III protein at NCBI found that yak have 98, 97, 91, 91, 91, 92, 89 and 88% similarity with cattle, hair goat, sheep, human, horse, pig, house mouse and dog MT-III proteins. This suggests that MT-III has a high level of conservation in development and evolution. The

1	ATG	GAC	CCT	GAG	ACC	TGC	CCC	TGC	CCT	ACT	GGT	GGC	TCC	TGC	ACC	45
1	M	D	P	E	T	C	P	C	P	T	G	G	S	C	T	15
46	TGC	TCC	GAC	CCC	TGC	AAG	TGT	GAG	GGC	TGC	ACG	TGC	GCC	TCC	AGC	90
16	C	S	D	P	C	K	C	E	G	C	T	C	A	S	S	30
91	AAG	AAG	AGC	TGC	TGC	TCC	TGC	TGC	CCT	GCA	GAG	TGT	GAG	AAA	TGT	135
31	K	K	S	C	C	S	C	C	P	A	E	C	E	K	C	45
136	GCC	AAG	GAT	TGT	GTG	TGC	AAA	GGT	GGA	GAG	GGG	GCC	GAA	GCT	GAG	180
46	A	K	D	C	V	C	K	G	G	E	G	A	E	A	E	60
181	GAG	AAG	AAG	TGC	AGC	TGC	TGC	CAG	TGA	207						
61	E	K	K	C	S	C	C	Q	End	68						

Fig. 1: Open reading frames and AA sequences encoded by MT-III in the yak (note total open reading frame has length of 207 nucleotides, representing a structurally intact protein of 68 AA which includes 19 cysteines)

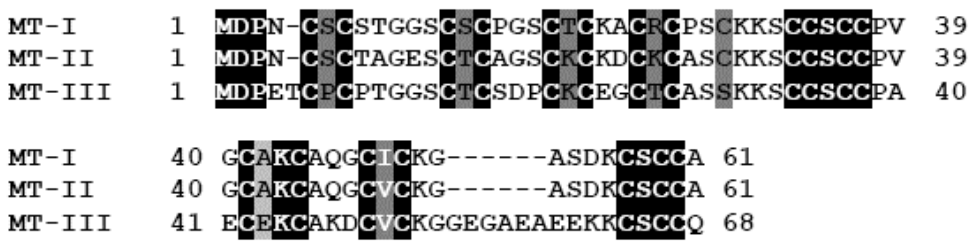

Fig. 2: Analysis of AA sequences of MT-I/-II/-III in yak (note that MT-III differs from MT-I/-II in a single Thr in the N-terminal region and an acidic hexapepitide (Glu-Ala- Ala-Glu- Ala- Glu) in C-terminal region)

```
Yak MT-III           1  MDPETCPCPTGGSCTCSDPCKCEGCTCASSKKSCCSCCPA  40
Cattle MT-III        1  MDPETCPCPTGGSCTCSDPCKCEGCTCASCKKSCCSCCPA  40
Goat MT-III          1  MDPETCPCPTGGSCTCSDSCKCEGCTCASSKKSCCSCCPA  40
Sheep MT-III         1  MDPEACPCPTGGSCTCSDSCKCEGCTCASSKK---SCCPA  40
Pig MT-III           1  MDPETCPCPTGGSCTCAGSCKCEGCKCTSCKKSCCSCCPA  40
Chimpanzee MT-III    1  MDPETCPCPSGGSCTCADSCKCEGCKCTSCKKSCCSCCPA  40
Human MT-III         1  MDPETCPCPSGGSCTCADSCKCEGCKCTSCKKSCCSCCPA  40
Dog MT-III           1  MDPETCPCPTGGSCTCDGSCKCEGCKCTSCKKSCCSCCPA  40
House mouse MT-III   1  MDPETCPCPTGGSCTCSDKCKCKGCKCTNCKKSCCSCCPA  40
Horse MT-III         1  MDPETCPCPTGGSCTCSGECKCEGCKCTSCKKSCCSCCPA  40

Yak MT-III          41 ECEKCAKDCVCKGGEGAEAEEKKCSCCQ  68
Cattle MT-III       41 ECEKCAKDCVCKGGEGAEAEEKKCSCCQ  68
Goat MT-III         41 ECEKCAKDCVCKGGEGAEAEEKKCGCCQ  68
Sheep MT-III        41 ECEKCAKDCVCKGGEGAEAEEKKCGCCQ  65
Pig MT-III          41 ECEKCAKDCVCKGGEGAEAEEEKCSCCQ  68
Chimpanzee MT-III   41 ECEKCAKDCVCKGGEAAEAEAEKCSCCQ  68
Human MT-III        41 ECEKCAKDCVCKGGEAAEAEAEKCSCCQ  68
Dog MT-III          41 ECEKCAKDCVCKGGEGTEAEAEKCSCCQ  68
House mouse MT-III  41 GCEKCAKDCVCKGEEGAKAEAEKCSCCQ  68
Horse MT-III        41 ECEKCAKDCVCKGGEGAEAEAEKCSCCQ  68
```

Fig. 3: Analysis of sequences of MT-III in yak, cattle, sheep, horse, pig , goat, human, chimpanzee, house mouse and dog [note tripeptides conserved across species are: MDPET CXC ----- CXC --- CXC – CXC------ CCXCC --- CXXC --- CXC ---EXAEAE---CXCC -. GenBank Accession No.: Yak (DQ492300), Cattle (NP_01106775), Sheep (NP_001009755), Horse (P37360), Pig (NP_999221), Goat (ABM 69167), Human (NP_005945), Chimpanzee (XP_001139417), House mouse (NP_038631), Dog (NM_013603)

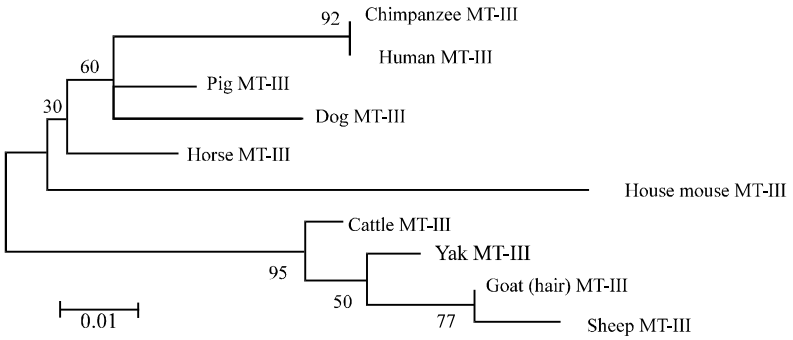

Fig. 4: Molecular phylogenetic evolution tree from MT-III amino acid sequences among yak, cattle, goat, sheep, human, pig, horse, chimpanzee, dog and house mouse

constructed phylogenetic tree of MT-III including yak, cattle, hair goat, sheep, pig, human, horse, chimpanzee, dog and house mouse by MEGA (http://www.megasoftware.net) further suggests that yak MT-III was very conservative in development and evolution (Fig. 4). Conserved Cys^{29} in MT-III in horse, rat, human, bovine, pig was replaced by Ser which may imply some structural/or dynamic and physiological function differences.

Characterization of MT-III proteins: The molecular weight of MT-III analyzed by BioEdit (www.psc.edu/biomed/genedoc/) gave a molecular weight of yak MT-III of 6936.70 Da. Hydrophobicity and transmembrane region analysis of MT-III in yak are shown in Fig. 5 and 6. Yak MT-III is similar to other mammals having no apparent hydrophobic or transmembrane regions. Results from protein signal peptide analysis suggested that MT-III proteins are nonsecretory cytoplasmic proteins (Table 1).

Yak MT-III protein high structure analysis: The secondary structure prediction of MT-III by APSSP2 indicates that there are obvious helices between the 39-46th AA and strands between the 21st and 22nd AA and the 26th and 27th AA. Others sequence regions suggest obvious coils.

Table 1: Analysis of protein signal peptides with signal P3.0 server

				SignalP-NN[2]		Signal P-HMM		
Measure[3]	Position	Value	Cutoff	Signal peptide	Signal peptide probability	Signal anchor probability	Max cleavage site probability	Prediction
Max. C	23	0.110	0.32	NO	0.001	0.000	0.000	Non-secretory protein
Max. Y	8	0.033	0.33	NO	-	-	-	-
Max. S	3	0.196	0.87	NO	-	-	-	-
Mean S	1-7	0.140	0.48	NO	-	-	-	-
D	1-7	0.086	0.43	NO	-	-	-	-

http://www.cbs.dtu.dk/services/SignalP-3.0/; last accessed Dec. 6, 2006; 2NN = Neural Networks; HMM = Hidden Markov Models; 3C = Raw cleavage site score; S = Signal peptide score; Y = Combined cleavage site score

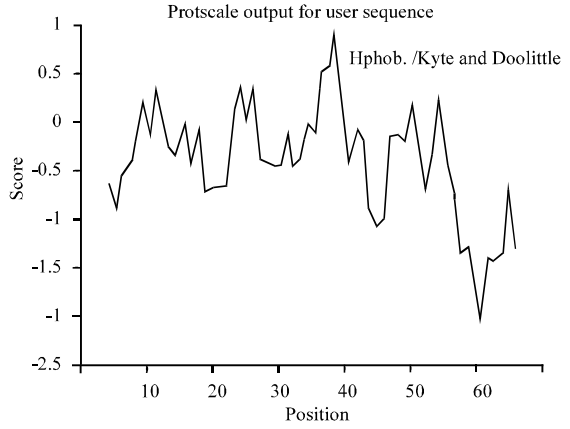

Fig. 5: Hydrophobicity analysis of MT-III in Yak

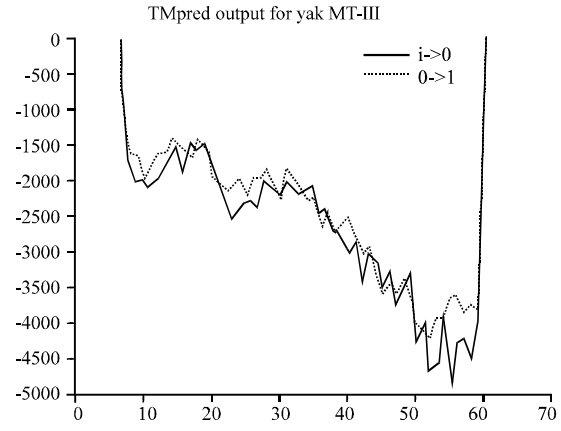

Fig. 6: Transmembrane helix of MT-III in yak

In this study, the coding sequence of MT-III in yak was cloned by RT-PCR using specific primers. Results of MT-III sequencing suggest that the full-length coding sequence was 207 bp, encoding 68 AAs in which specific initiation and terminal codons reside. The characterization and structure of yak MT-III suggest highly conservative, low-molecular-weight (approximately 7000 Da.), cysteine-rich metal-binding nonsecretory cytoplasmic proteins in which there are no obvious hydrophobic domains, transmembrane regions or signal peptides. Compared with MT-I/-II, there are 2 inserts: a single Thr in the N-terminal region and an acidic hexapeptide (Glu-Ala- Ala-Glu- Ala-Glu) in the MT-III C-terminal region. This difference is similar to other reports in other species (Uchida *et al*., 1991; Tsuji *et al*., 1992). One cysteine of MT-III had mutated to a Ser in yak, so that there is 1 less –SH region in MT-III of yak than MT-I/-II and MT-III in cattle, goat and human.

Structural data of MT-I/-II and MT-III were similar. Both included 2 domains, the N-terminal β-domain (residues 1-30) and a C-terminal α-domain (residues 31-68) (Nuria *et al*., 2002). The cysteine mutation was located in the β-domain which is the domain of the growth inhibitory activity (Ghazi *et al*., 2006). Consequently, there are some implied structural differences from MT-I/II and physiological function differences among species. Results of MT-III protein secondary structure prediction indicate that there are obvious helices in the 39-46th AA and no obvious sheets. Generally, the secondary structure of MT-III in yak was similar to other species, except for the helix in MT-III of human and mouse located in between Lys44 and Lys47 (Oz *et al*., 2001; Wang *et al*., 2006).

CONCLUSION

Analysis of nucleotides and amino acid sequence suggest MT-III in yak is highly conserved, except where Ser is substituted for Cys^{30} as compared with human and mouse MT-III. The yak species (*Bos grunniens*) represents a unique bovine species adapted to the Tibetan plateau of China at altitudes of 3,000 m above sea level with intense ultraviolet radiation. Consequently, yak adapted to this environment likely possess unique physiological adaptive mechanisms to protect their central nervous systems against hypoxic and oxidative injury. However, there is a need for further study of MT-III tertiary structure, physiological function and MT-IV to better understand these potentially adaptive characteristics in yak.

REFERENCES

Gasteiger, E., C. Hoogland, A. Gattiker, S. Duvaud and M.R. Wilkins, 2005. Protein Identification and Analysis Tools on the ExPASy Server. In: The Proteomics Protocols Handbook, Walker, J.M. (Ed.). Humana Press, New Jersey, USA., pp: 571-607.

Ghazi, I. El, B.L. Martinand and I.M. Armitage, 2006. Metallothionein-3 is a component of a multiprotein complex in the mouse brain. Exp. Biol. Med., 231: 1500-1506.

Hye, J.G., C.K. Youn, H.J. Cho, S.H. Kim and M.H. Kim *et al*., 2004. Metallothionein-III prevents γ-ray-induced 8-oxoguanine accumulation in normal and hOGG1-depleted cells. J. Biol. Chem., 279: 34138-34149.

Nuria, R.I., L.T. Jensen, O. Zerbe, D.R. Winge and M. Vasak, 2002. Engineering of metallothionein-3 neuroinhibitory activity into the inactive isoform metallothionein-1. J. Biol. Chem., 277: 37023-37028.

Oz, G., K. Zangger and I.M. Armitage, 2001. Three-dimensional structure and dynamics of a brain specific growth inhibitory factor: Metallothionein-3. Biochemistry, 40: 11433-11441.

Palmiter, R.D., 1998. The elusive function of metallothioneins. Proc. Natl. Acad. Sci. USA., 95: 8428-8430.

Raghava, G.P.S., 2000. Protein secondary structure prediction using nearest neighbor and neural network approach. CASP, 4: 75-78.

Richard, D.P., S.D. Findley, T.E. Whitmore and D.M. Durnam, 1992. MT-III, a brain-specific member of the metallothionein gene family. Proc. Natl. Acad. Sci., 89: 6333-6337.

Sambrook, J. and D.W. Russell, 2001. Molecular Cloning: A Laboratory Manual. 3rd Edn., Cold Spring Harbor Laboratory Press, New York, ISBN: 9780879695774, pp: 1-99.

Schwede, T., J. Kopp, N. Guex and M.C. Peitsch, 2003. SWISS-MODEL: An automated protein homology-modeling server. Nucleic Acids Res., 31: 3381-3385.

Tsuji, S., H. Kobayashi, Y. Uchida and T. Miyatake, 1992. Molecular cloning of human growth inhibitory factor cDNA and its down-regulation in Alzheimer's disease. EMBO J., 11: 4843-4850.

Uchida, Y., K. Takio, K. Titani, Y. Ihara and M. Tomonaga, 1991. The growth inhibitory factor that is deficient in the Alzheimer's diseased brain is a 68 amino acid metallothionein-like protein. J. Neuron, 7: 337-347.

Valls, M., R. Bofill, R.G. Duarte, P.G. Duarte, M. Capdevila and S. Atrian, 2001. A new insight into metallothionein (MT) classification and evolution: the *in vivo* and *in vitro* metal binding features of homarus americanus recombinant MT. J. Biol. Chem., 276: 32835-32843.

Wang, H., Q. Zhang, B. Cai, H. Li and K. Sze *et al*., 2006. Solution structure and dynamics of human metallothionein-3 (MT-3). FEBS Lett., 580: 795-800.

Wu, J.P., B.Y. Ma, H.W. Ren, L.P. Zhang, Y. Xiang and M.A. Brown, 2007. Characterization of metallothioneins MT-I and MT-II in yak. J. Anim. Sci., 85: 1357-1362.

Zhang, R.C., J.P. Wu and J.L. Han, 1994. Yak production in China. J. Asian Livestock: Yak, 615: 115-118.