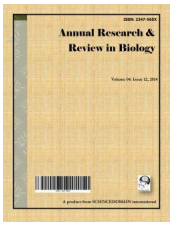

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Molecular Cloning and Expression Analysis of Bovine Alpha-tocopherol Transfer Protein (α-TTP)

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Authors' contributions

This work was carried out in collaboration between all authors. Authors YT and KS designed the study. Author YT wrote the manuscript. Authors NH and YK performed PCR analysis, northern analysis and sequencing. Authors YT and MKT prepared the polyclonal antibody and performed immunological analysis. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To understand the mechanisms by which cattle circulate and accumulate vitamin E, we cloned the cDNA for bovine α-tocopherol transfer protein (α-TTP) and examined its expression in different tissues.
Methodology: A full length α-TTP cDNA was amplified from bovine liver by RT-PCR. Poly $(A)^+$ RNA obtained from bovine tissues was subjected to RT-PCR analysis to examine α-TTP mRNA expression. Western blot analysis was performed using polyclonal antibody raised against an oligopeptide derived from bovine α-TTP to examine α-TTP protein expression in various bovine tissues. The localization of α-TTP in bovine lung tissue was examined by immunostaining with anti-bovine α-TTP polyclonal antibody.

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Results: The open reading frame consists of 846 nucleotides encoding 282 amino acids with 98% and 83% identities to sheep and rat orthologs, respectively. Bovine α-TTP mRNA and protein were expressed most strongly in liver and lung, whereas expression of α-TTP mRNA and protein are reported to be very weak or absent in human and rodent lungs. In the lung, immunostaining suggested that α-TTP is expressed specifically in alveolar walls, which consists of alveolar cells, epithelial cells of small bronchi, and endothelial cells of pulmonary blood vessels.
Conclusion: These results suggest that, in the lung, α-TTP is involved in supplying vitamin E to alveolar surfactant in order to protect the lung tissue from oxidative stress, and that this role may be more important in bovines than in other mammals.

Keywords: Vitamin E; α-TTP; α-tocopherol; bovine; lung alveolar cells.

ABBREVIATIONS

α-TTP: Alpha(α)-tocopherol transfer protein; AVED: Ataxia with vitamin E deficiency; RT-PCR: Reverse transcription polymerase chain reaction; RACE: Rapid amplification of cDNA ENDs.

1. INTRODUCTION

Vitamin E molecules are plant-derived lipophilic antioxidants that inhibit peroxidation of lipid molecules in biological membranes by scavenging reactive oxygen species [1,2]. By this activity, vitamin E molecules are thought to contribute to membrane stability, protect cells from oxidative stress, and thus prevent various oxidative stress-related pathologies, such as ataxia, cardiovascular disease, cancer, Alzheimer disease, and inflammation. At least eight kinds of vitamin E molecules occur naturally, and differ in the methylation pattern of their chromanol ring (α-, β-, γ-, and δ-), and the degree of saturation of the phytyl side-chain (tocopherol and tocotrienols). Plasma and tissues of animals are enriched in α-tocopherol, the most biologically active form of vitamin E, although foods of animals include many other tocols, such as γ-tocopherol [3].

The preferential retention is achieved by α-tocopherol transfer protein (α-TTP), a cytosolic protein that specifically binds α-tocopherol and is found mainly in the liver, where it regulates the amount of α-tocopherol secreted into the plasma [4,5]. In human, α-TTP mRNA and protein are strongly expressed in liver [6]. In mouse α-TTP protein is strongly expressed in liver, and weakly expressed in brain and uterus [7,8]. In rat, α-TTP protein is strongly expressed in liver [9], while its mRNA is expressed strongly in liver, and very weakly in brain, spleen, lung and testis [10]. α-TTP is defective in patients with ataxia with vitamin E deficiency (AVED) [11,12]. α-TTP knockout mice have much lower plasma concentrations of α-tocopherol and are unable to maintain pregnancy because of impaired placentas [7]. They also show severe neuronal degeneration unless fed a high dose of vitamin E [8]. Studies of AVED patients and α-TTP knockout mice suggest that hepatic α-TTP governs the plasma α-tocopherol level, and that the protein also works in tissues such as brain and uterus in which α-TTP is expressed.

Animals obtain vitamin E from plants, which can synthesize vitamin E on their own. Thus, plant-eating animals, such as bovines, might have quite different mechanisms for vitamin E circulation and accumulation than rodents and humans, and the roles of bovine α-TTP might be different from those of rat, mouse and human. To elucidate the role of bovine α-TTP, we isolated and sequenced a full-length cDNA of bovine α-TTP. We then investigated the mRNA and protein expressions of bovine α-TTP in different bovine tissues.

2. MATERIALS AND METHODS

2.1 Ethics

All experimental procedures involving animals were approved by the Animal Research Ethics Committee of Kindai University.

2.2 Animals

Bovine tissues were obtained from three Japanese Black calves (*Bos taurus*), purchased from Kurosawa Farm, Wakayama, Japan. For taking tissues, the cattle were anesthetized with an i.p. injection of pentobarbital and killed by exsanguination through the carotid arteries. Rabbits for preparation of the polyclonal antibody were purchased from Kiwa Laboratory Animals Science Co., Ltd.

2.3 RNA Extraction, RT-PCR and Northern Blot Analysis

Total RNA from bovine tissues was obtained by the acid guanidium-phenol-chloroform (AGPC) method [13], and poly $(A)^+$ RNA was selected from total RNA with the use of Oligotex-dT30 (Takara Shuzo Co., Ltd.). RT-PCR analysis for the expression of bovine α-TTP and GAPDH was performed by utilizing Qiagen One Step PCR kit (Qiagen) and gene specific primers (bovine α-TTP; sense primer (BaTTP-3GSP): CGAGACC-CCACCGGCAGCAAAGTTCT, antisense primer (BaTTP-5GSP): GAACAGCAGCAATCTTCTT-GGCTACAGA, GAPDH; sense primer: GTCTTC-CACTACCATGGAGAAGG, antisense primer: TCATGGATGACCTTGGCCAG). Gene specific primers for bovine α-TTP was designated by the partial sequence of bovine α-TTP cDNA (GenBank: AF185291). For Northern blot analysis, 2.3 μg of poly$(A)^+$ RNAs from the indicated bovine tissues were separated on a 1 % agarose gel containing formaldehyde, and blotted to nitrocellulose membrane. The blot was hybridized with ^{32}P-labeled probes for bovine α-TTP cDNA or β-actin at 65°C for 16 hours, washed in 0.5 x SSC at 65°C and then analyzed by autoradiography. The Northern blot probes were generated by a random priming and ^{32}P-labeling method using a fragment of bovine α-TTP cDNA, which was amplified by RT-PCR with gene specific primers, BaTTP-3GSP and BaTTP-5GSP, as described above, and a human β-actin cDNA control probe (Clontech).

2.4 PCR Amplification and Sequencing of Full-length cDNA for Bovine α-TTP

The upstream and downstream regions of bovine α-TTP cDNA were amplified by RACE PCR with a SMART RACE cDNA Amplification Kit (Clontech Laboratories, Inc.), as the sequences of these regions were not available at the start of this study.

For 5'-RACE, cDNA was synthesized by reverse transcription from 2 μg of poly(A) RNA isolated from bovine liver tissue, using a gene specific primer (BaTTP-5GSP), SMART II A oligonucleotide, and PowerScript reverse transcriptase from the RACE Kit. The upstream region of bovine α-TTP cDNA was amplified by PCR using a forward degenerate primer (GGCGGCGGGCATGGCNGAN) and the reverse gene specific primer BaTTP-5GSP. The forward degenerate primer was designed by comparison of the sequences near the translation start sites of human, mouse, and rat α-TTP cDNAs. The amplified DNA containing the translation start sites of bovine α-TTP was cloned using pGEM-T easy vector system (Promega), and sequenced. For the region farther upstream containing the 5' untranslated region, 5' RACE PCR was performed using a newly synthesized primer based on the sequence near the translation start site of bovine α-TTP (5GSP2 :AGCAGGAAGGCGTCGCTGA-GCAGCCACCCG), the universal primer A mix provided in the RACE Kit, and LA taq DNA polymerase (TAKARA Bio).

For 3'-RACE, 2 μg of poly (A) RNA isolated form bovine liver was reverse transcribed using the 3'-RACE CDS primer A and PowerScript reverse transcriptase from the RACE Kit. PCR was performed using the gene specific primer (BaTTP-3GSP: CGAGACCCCACCGGCAGCAA-AGTTCT), a universal primer from the RACE Kit, and Ex taq DNA polymerase (TAKARA Bio). The amplified DNA fragment was checked by agarose gel electrophoresis, cloned, and sequenced, as described above.

For amplification of bovine α-TTP cDNA between the regions amplified by 5'-RACE and 3'-RACE, RT-PCR was performed by utilizing Qiagen One Step PCR kit (Qiagen) and gene specific primers (BaTTP-sense (GGACCACCGCAGGAGAGG) and BaTTP-antisense (CTTGGTGGCCCGTGG-AAATAACTA), designated by the sequence of PCR product of 5'-RACE and 3'-RACE, respectively).The amplified DNA fragment was cloned, and subjected to sequencing, as described above.

2.5 Preparation of the Polyclonal Antibody against Bovine α-TTP

A polyclonal antibody was raised against a synthesized peptide corresponding to amino acid positions 255 to 273 of bovine α-TTP (255>CQEWTNFIMKSENYLSSIS<273). Every 6-7 days, for a total of six times, the peptide was injected intradermally into the back of an adult rabbit. For each injection, approximately 100 μg of the peptide was homogenized with Freund's complete adjuvant (first injection) or Freund's incomplete adjuvant (second to sixth injection). One week after the last injection, whole blood was collected from the animal. Antibodies specific for the peptide were purified from serum derived from the blood by affinity

chromatography using a column with immobilized peptide antigen.

2.6 Western Blot Analysis

Each bovine tissue (0.1g) was homogenized on ice in buffer (7M urea, 2M thiourea, 4% CHAPS). The homogenized tissue was centrifuged (20,000 × g, 4°C, 10 min) twice, and the supernatant of the second centrifugation was used as protein samples. Protein samples (50 μg) were electrophoresed and transferred to a nylon membrane (Hybond-N^+, Amersham). The membrane was blocked with Block Ace (DS Pharma Medical), incubated with anti-α-TTP rabbit polyclonal antibody (5.0 μg/mL) and incubated for 1 hour with anti-rabbit IgG-conjugated with horseradish peroxidase (0.25 μg/mL) (SIGMA-ALDRICH). For a control, the membrane was incubated with anti-GAPDH goat polyclonal antibody (2.0 μg/mL) (SANTA CRUZ BIOTECHNOLOGY) and anti-goat IgG-conjugated with horseradish peroxidase (0.1 μg /mL) (SANTA CRUZ BIOTECHNOLOGY). Signals were detected by chemical luminescence using an ECL Western Blotting kit (Amersham).

2.7 Immunohistochemical Analysis

Bovine lung tissue was fixed in 4% (v/v) formaldehyde, embedded in paraffin, and sectioned (1 μm). The sections were incubated at 4°C overnight in anti-α-TTP rabbit polyclonal antibody and then incubated with peroxidase-labeled anti-rabbit serum (Histofine Simple Stain MAX-PO(R), Nichirei Biosciences, Tokyo). The colorimetric reaction was carried out with 0.3 mg/mL of diaminobenzidine and 0.003% H_2O_2, and nuclei were counterstained with hematoxylin.

3. RESULTS AND DISCUSSION

We succeeded in amplifying and sequencing a full-length α-TTP cDNA from bovine liver (GenBank accession: LC223005). The cDNA contains 2740 nucleotides and the open reading frame contains 846 bp encoding 282 amino acids with 88%,83%,83% and 98% identity with the human [6], rat [9], mouse [14] and sheep [15] orthologs, respectively (Fig. 1). The sequence is identical to the sequence based on the whole-genome sequence data from domestic cow (GenBank accession number: NM001206676). The domain containing the α-helix and β-sheet, which are thought to form the α-tocopherol binding site [16], are well conserved among bovine, rat, human, mouse and sheep α-TTP. Bovine α-TTP has an additional 5 amino acids (GEEVT) at the C terminus, which rat, human, mouse α-TTPs do not have. Sheep α-TTP has similar 5 amino acid C-terminal sequence (GEEVI) [15]. A protein BLAST search revealed that many *Cetartiodactyla* animals, such as water buffalo, bison, goat, deer, antelope, yak, camel, dolphin, whale, and killer whale, have similar C-terminal sequences, while other mammals (primates, rodents, bat, leopard, mole and *Perissodactyla* animals, such as horse, donkey and rhinoceroses) do not have them. These results suggest that the C-terminal sequences of α-TTP are specific for *Cetartiodactyla* animals.

We performed RT-PCR using RNA extracted from various tissues of cows to examine α-TTP expression. α-TTP mRNA expression was strongest in liver, while the second-strongest signal was observed in lung (Fig. 2A). The same pattern was observed in all three cows (data not shown) and was confirmed by Northern blot analysis (Fig. 2B). We also examined α-TTP protein expression by western blot in one of the cows. Like α-TTP mRNA, the protein was strongly expressed in liver and less strongly expressed in lung (Fig. 3). In contrast, expression of α-TTP mRNA and protein is very weak or absent in human and rodent lungs [6-10], suggesting that α-TTP plays a more important role in bovine lung. The reason why α-TTP is expressed more strongly in bovine lung than in human and rodent lungs is unclear. Further studies of the expression of α-TTP in the lungs other ruminants might shed some light on this issue.

In lung tissue from the same cow described in Fig. 3, immunostaining with anti-α-TTP antibody was observed in alveolar walls, which consist of alveolar cells (Fig. 4A), epithelial cells of small bronchi (Fig. 4B), and endothelial cells of pulmonary blood vessels (Fig. 4C). The surfaces of lung alveoli are covered with a lipid monolayer called alveolar surfactant. The surfactant is produced in alveolar cells (type II), accumulated in the lamellar body in the cell, and secreted to the lung alveolar lumen. The main constituents of the surfactant are phospholipids and cholesterol, but α-tocopherol is also present as an anti-oxidant agent [17]. Our results thus suggest that, in the bovine lung, α-TTP serves to recruit α-tocopherol to alveolar surfactant to protect the lung tissue from oxidative stress.

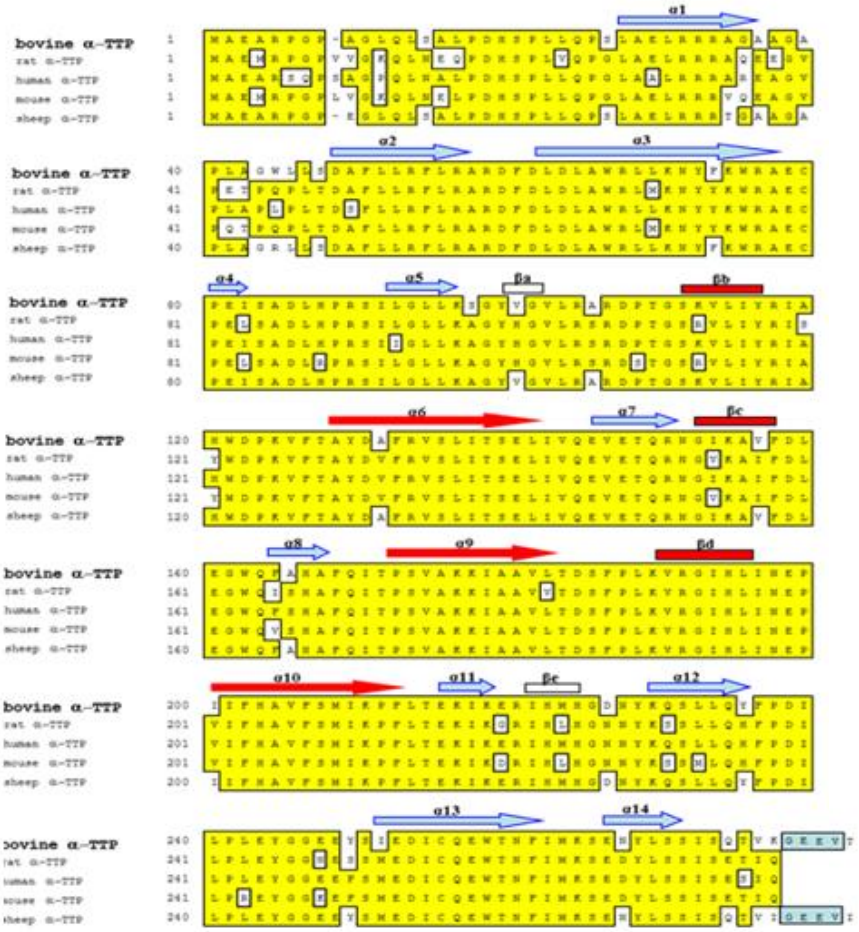

Fig. 1. Alignment of the predicted amino acid sequence of bovine, rat, human, mouse and sheep α-TTP orthologs

Bovine α-TTP was aligned with Alignment was performed with rat [9], human [6], mouse [14] and sheep [15] α-TTPs. Alignment was performed with GENETYX software (GENETYX CORPORATION). Conserved amino acids are shaded. Secondary structure assignments are based on crystal structure of human α-TTP described [16]

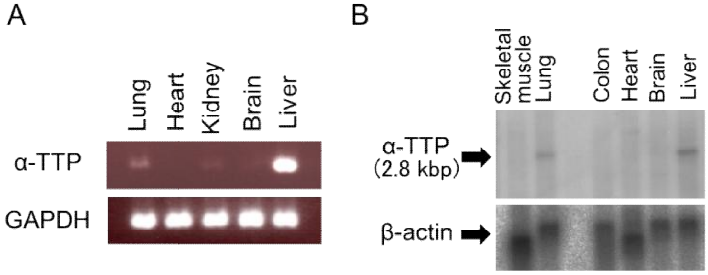

Fig. 2. Representative tissue-specific expression of α-TTP mRNA in a Japanese black cow

A: RT-PCR analysis. Poly (A)$^+$ RNA obtained from bovine tissues was subjected to RT-PCR analysis using gene specific primers for bovine α-TTP and GAPDH as described in materials and methods.

B: Northern blot analysis. 2.3 μg of poly(A)$^+$ RNA from the indicated bovine tissues were separated on a 1% agarose gel containing formaldehyde, and blotted to nitrocellulose membrane. The blot was hybridized with a ^{32}P-labeled fragment of bovine α-TTP cDNA. The lower panel shows the same blot hybridized with a β-actin probe for control

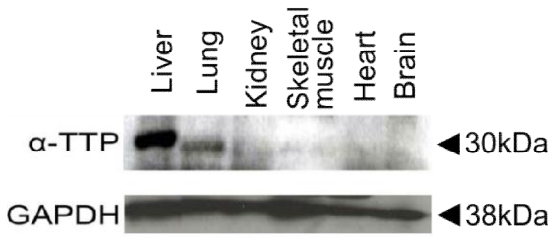

Fig. 3. Western blots showing expressions of α-TTP and GAPDH (control) in different tissues of a Japanese black cow

The antibodies were a rabbit polyclonal antibody raised against an oligopeptide derived from bovine α-TTP and an anti-GAPDH polyclonal antibody for control

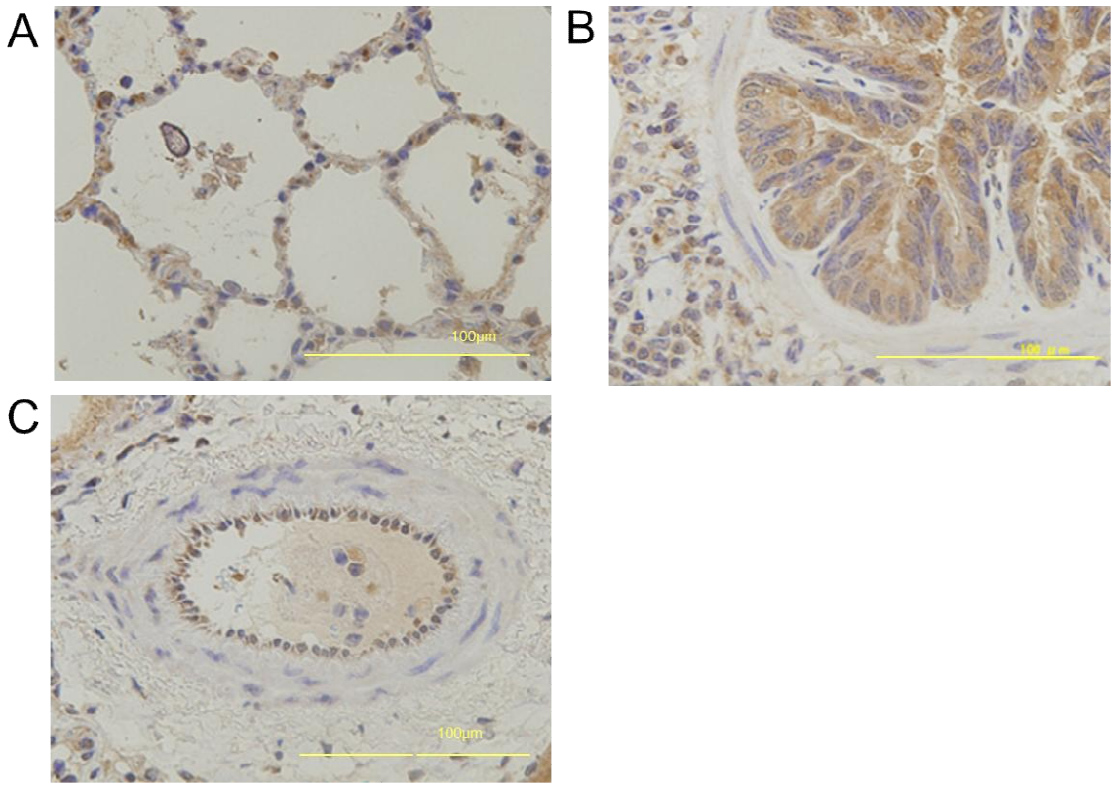

Fig. 4. Immunostaining of lung tissue from the cow with anti-bovine α-TTP polyclonal antibody

Immunohistochemically stained regions appear dark brown from oxidized diaminobenzidine, while nuclei appear blue from counterstaining with hematoxylin. A: Alveolar cells. B: Small bronchi. C: Cross-section of a pulmonary blood vessel. Scale bars, 100 μm

4. CONCLUSION

We isolated a full-length cDNA for bovine α-tocopherol transfer protein (α-TTP). The expression level of α-TTP mRNA and protein are highest in liver and second-highest in lung. Immunostaining showed that α-TTP of bovine is expressed in lung alveolar cells, suggesting that α-TTP is involved in supplying vitamin E to lung alveolar surfactant.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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