Cloning, expression, and polymorphism of the porcine calpain10 gene

Xiuqin Yang¹, Di Liu¹²*, Hao Yu¹, Lijuan Guo¹, and Hui Liu¹

¹ College of Animal Science and Technology, Northeast Agricultural University, Harbin 150030, China
² Agricultural Academy of Heilongjiang Province, Harbin 150086, China
³ Institute of Animal Husbandry and Veterinary Medicine, Jilin University, Changchun 130062, China

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Calpains are calcium-regulated proteases involved in cellular functions that include muscle proteolysis both ante- and post-mortem. This study was designed to clone the complete coding sequence of the porcine calpain10 gene, CAPN10, to analyze its expression characteristics and to investigate its polymorphism. Two isoforms of the CAPN10 gene, CAPN10A and CAPN10B, were obtained by reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends methods combined with in silico cloning. RT-PCR results indicated that CAPN10 mRNA was ubiquitously expressed in all tissues examined and, with increasing age, the expression level increased in muscles at six different growth points. In the same tissues, the expression level of CAPN10A was higher than that of CAPN10B. In addition, three single nucleotide polymorphisms were detected by the PCR-single-stranded conformational polymorphism method and by comparing the sequences of Chinese Min pigs with those of Yorkshire pigs. C527T mutation was a missense mutation and led to transforming Pro into Leu at the 176th amino acid. The results of the current study provided basic molecular information for further study of the function of the porcine CAPN10 gene.

Keywords porcine; CAPN10 gene; cloning; expression; polymorphism

Calpains, implicated in signal transduction, nerve development, muscle growth, cell proliferation, apoptosis, and differentiation, constitute a large family of intracellular Ca²⁺-dependent cysteine neutral proteases. Calpains are believed to be related to physiological as well as pathological conditions such as ischemia, senile dementia, arthrophlogosis, and cancer [1–3].

In livestock animals, calpains play an important part in muscle growth and development, myoblast fusion, and differentiation [4]. Calpains were also shown to decompose key myofibrillar and associated proteins that maintain the structure of skeletal muscle such as titin, nebulin, and desmin [5,6]. It is the early post-mortem cleavage of these proteins that ultimately leads to the tenderization of meat. So in post-mortem skeletal muscles of meat animals, the proteolytic activity of the calpains has the primary influence on meat tenderness [7,8].

A number of calpains have been identified and their primary structures determined by cDNA cloning in mammals. They can be classified on the basis of structure into typical and atypical calpains. Typical calpains (calpain1, 2, 3, 8, 9, 11, and 12) have a four-domain structure in the catalytic subunit that is comprised of domain I (autolytic activation), domain II (cysteine catalytic site), domain III (“electrostatic switch”), and domain IV (calmodulin-like calcium binding sites). Atypical calpains (calpain5, 6, 7, 10, and 15) do not have calmodulin-like EF-hand sequences in their domain IV, and some even lack domain IV. Calpain10 (CAPN10), whose calmodulin domain was replaced by a divergent T domain containing no calcium-binding EF-hand structure, was recently discovered. It is ubiquitously expressed in many human and mouse tissues [9,10]. Genetic variation in this gene is associated with an increased risk of type 2 diabetes mellitus in humans [9]. The investigations on the roles of calpains in meat tenderization have been focused on the ubiquitous calpain1 and 2, and the muscle-specific calpain3 [11–15]. A few studies have also shown that CAPN10 might also be a candidate gene for meat tenderization. Ilian et al [16] studied the changes in CAPN10, tenderization level, and desmin in sheep longissimi and found that calpain10 proteins were strongly correlated with the rate...
of tenderization. But the number of published reports on CAPN10 is limited and further efforts should be made to reveal its role in meat tenderization.

The human CAPN10 gene has 15 exons spanning 31 kb. The splicing mechanisms of CAPN10 are very complicated. The human CAPN10 has eight transcript variants (a–h), some of which also lack the protease domain [9], and the mouse and rat CAPN10 each have two transcript variants. But the pig CAPN10 has not been reported.

In this study, two isoforms of pig CAPN10, CAPN10A and CAPN10B, were cloned using reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) methods combined with in silico cloning. Their expression was characterized, and the polymorphism in the coding region was analyzed in the Prosite database (hCAPN10). Some of these also lack the protease domain [9], and the mouse and rat CAPN10 each have two transcript variants. But the pig CAPN10 has not been reported.

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Materials and Methods

Animals, tissue collection, RNA isolation, and DNA extraction

Three Yorkshire pigs at each growth times (1-d-old, 21-d-old, 90-d-old, 180-d-old, 270-d-old, 360-d-old) with similar bodyweight were purchased from Zhongzhi Breeding Station (Harbin, China) and slaughtered by electrical stunning and severance of the carotid arteries for the cloning and expression analysis of the CAPN10 gene. Stomach, kidney, spleen, lung, heart, liver, large intestine, small intestine, gonad, uterus, fat, and muscle were harvested, flash-frozen in liquid nitrogen and stored at −70 °C. Total RNA was extracted from various tissues using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions.

The Chinese local breed Min pigs were used to investigate single nucleotide polymorphisms (SNPs) of the CAPN10 gene by the PCR-SSCP method. Genomic DNA was extracted from the ear using phenol/chloroform.

Primers used for cloning and expression

Using BLAST (http://www.ncbi.nlm.nih.gov/BLAST), electronic hybridization was carried out with human CAPN10 cDNA as a probe in the pig genome database. A few expressed sequence tags with significant similarity were found and a contig was constructed using Seqman II (Lasergene version 6; DNASTAR, Madison, USA). According to the contig, two pairs of primers, A1 and A2, were designed. In order to obtain the whole coding sequence of pig CAPN10, according to the sequences of PCR products using A1 and A2 primers, three additional primers were designed for 3′-RACE and 5′-RACE. For RT-PCR measurement of CAPN10 mRNA, primer pair C1 was designed for CAPN10 and primer pair C2 was designed for β-actin (GenBank accession No. U07786). The primer sequences and their positions in the cDNA sequence of pig CAPN10A or β-actin are listed in Table 1.

cDNA cloning of pig CAPN10

Total RNA was extracted from pig liver tissue. RT was carried out using 1 µg total RNA as a template with Superscript II and oligo(dT) primers (Invitrogen). PCR was carried out with 1 µl RT reaction mixture in a 25 µl final volume including 1×PCR reaction buffer, 200 µM each dNTP, 1 U Taq DNA polymerase, and 0.2 µM each forward and reverse primer. The thermal profiles were 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 60 °C (primer A1)/58 °C (primer A2) for 1 min, 72 °C for 1.5 min, and an extension at 72 °C for 7 min.

For cloning the ends of pig CAPN10 cDNA, 3′-RACE was carried out using 3′-Full RACE core set (version 2.0; TaKaRa, Dalian, China) according to the manufacturer’s instructions, and the specific outer and inner primers were A2 and B1, respectively; the 5′-RACE reaction was carried out according to the protocol of the 5′-RACE system for rapid amplification for cDNA Ends (version 2.0; Invitrogen), and the specific outer and inner primers were B2 and B3, respectively.

The products of RT-PCR and RACE were electrophoresed on a 1% agarose gel with ethidium bromide staining and purified by a Gel extraction mini kit (Watson Biotechnologies, Shanghai, China). Then the purified PCR products were cloned into pMD18-T (TaKaRa) vector and sequenced by the Biosia Co. (Shanghai, China).

Sequence analysis

Overlapping fragments amplified by RT-PCR, 3′-RACE, and 5′-RACE were assembled by the DNAMAN package (version 5.2.2; L ynnon Biosoft, Quebec, Canada). The open reading frame was found using the ORF Finder (http://www.ncbi.nlm.nih.gov/orf/ orf.html). The conserved domain was analyzed in the Prosite database (http://expasy.org/prosite/). An unrooted phylogenetic tree was constructed using the ClustalW program that calculates distances based on progressive multiple alignment and uses the neighbor-joining method for tree construction.
Measurement of $CAPN10$ mRNA

The expression profile of 12 tissues from 180-d-old Yorkshire pigs and muscles from six growth stages were determined by RT-PCR assays. Primer pair C1 (Table 1) was designed according to the pig $CAPN10A$ cDNA sequence and human $CAPN10$ genomic sequence to make the amplicon span introns, preventing amplification of any contaminating genomic DNA. The gene expression values were normalized using the pig $\beta$-actin gene amplified with primer pair C2 (Table 1). One microgram of total RNA extracted from each tissue was reversely transcribed into first-strand cDNA in a 20 µl volume with an oligo(dT) primer according to the specifications of the BcaBEST RNA PCR kit (version 1.1; TaKaRa). One microliter of the resultant cDNA product was subjected to PCR in a 25 µl volume with the same components as for primer pair A1. The PCR conditions were 94 ºC for 3 min followed by 34 cycles ($CAPN10$)/23 cycles ($\beta$-actin) of 94 ºC for 30 s, 59 ºC for 30 s, 72 ºC for 30 s, and primer extension at 72 ºC for 7 min. In order to precisely compare the expression level in muscles, the linear increasing experiment was carried out in muscle to ensure that the PCR products for both $CAPN10$ and $\beta$-actin were evaluated during the exponential phase, and the 34/23 cycles were selected. The images were scanned and quantified by the Laboratory Imaging and Analysis System (UVP, Upland, USA). The ratio of the intensities of $CAPN10$ versus $\beta$-actin in the same muscle represented the relative expression level of the target gene. The experiments were repeated three times. SAS (version 8.02; SAS, Cary, USA) and Excel (version 2003; Microsoft, Washington, USA) were used for data analysis and histogram plotting.

Measurement of two isoforms of $CAPN10$

To compare the mRNA level of two isoforms of pig $CAPN10$ in the same tissue, competitive RT-PCR was carried out in a 25 µl volume using primer pair A2 and 1 µl cDNA mixture synthesized by RT as carried out in semi-quantitative RT-PCR. The PCR products were separated on 2% agarose gel.

Development of PCR-SSCP assays and screening the population

Primer pair PF (5'-CCTGGTGACCTCAGCTTG-3') and PR (5'-ACCAGAGCCCTAATCACTAGA-3') was designed to amplify a 174 bp fragment in the coding sequences region according to the pig $CAPN10A$ cDNA. The PCR reactions were the same as for primer pair A1 except that the annealing temperature was 57 ºC and the template was 25 ng genomic DNA. The PCR products were used for SSCP to investigate sequence polymorphisms of the $CAPN10$ gene. The SSCP procedure were as follows: 3 µl PCR products were mixed with 8 µl loading buffer (98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 10 mM EDTA, and 10% glycerol) in a tube, denatured in 98 ºC for 10 min, placed on ice for 5 min, then electrophoresed for 16 h at 10 V/cm on a 16% polyacrylamide gel. Silver staining method was developed to display the bands [17]. The homozygous individuals with different genotypes were cloned and sequenced by Bioasia Co..

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequences (5’→3’)</th>
<th>Positions of primers in the cDNA sequence of $CAPN10A$ or $\beta$-actin gene</th>
<th>Fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>S: TGTGGCAGTTTGGACGCT</td>
<td>439</td>
<td>823</td>
</tr>
<tr>
<td></td>
<td>A: GCCACGTACAGCTCGCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>S: GCCTAGGAAAAGCGCTATG</td>
<td>1114</td>
<td>910/1020</td>
</tr>
<tr>
<td></td>
<td>A: GAAAGGCTACCCGTTAGCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>S: GCCTCCTTTATGGCGGTGAT</td>
<td>2091</td>
<td>361</td>
</tr>
<tr>
<td>B2</td>
<td>A: CCGAGACAAGGAAAGCGTG</td>
<td></td>
<td>852</td>
</tr>
<tr>
<td>B3</td>
<td>A: AGTCCACCGGCGCTCT</td>
<td>631</td>
<td>631</td>
</tr>
<tr>
<td>C1</td>
<td>S: CACGCTTCTTTCGGTCTCG</td>
<td>833</td>
<td>833</td>
</tr>
<tr>
<td></td>
<td>A: identical to A1 antisense</td>
<td></td>
<td>1261</td>
</tr>
<tr>
<td>C2</td>
<td>S: CGGGGACTCTGCCAGACTACCT</td>
<td>181</td>
<td>412</td>
</tr>
<tr>
<td></td>
<td>A: GGCCGTGATCTCCTTCG</td>
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</table>

Table 1 Primers for the cloning and expression of pig calpain10 ($CAPN10$)

A, antisense; S, sense.
Results

Sequence of two isoforms of pig CAPN10 cDNA
Specific products were obtained from each primer pair. The PCR had two different products using primer pair A2 and the sequence analysis showed that they belonged to different transcript variants of pig CAPN10. After assembling the sequence of RT-PCR and RACE products by the DNAMAN package (version 5.2.2), two isoforms of pig CAPN10 were obtained. The cDNA of CAPN10A is 2452 bp long containing a 116 bp 5′-untranslated region (UTR), the complete coding region of 2004 bp, and a 332 bp 3′-UTR with a typical polyadenylation signal (AAATAA). Conceptual translation predicted a protein of 667 amino acids with a theoretical molecular mass of 74 kDa and an isoelectric point of 7.96. The cDNA of CAPN10B results from a 110 bp deletion from the sequence of CAPN10A and is identical to the remainder of CAPN10A throughout the coding region and the UTR. The deletion sequence is from bases 1934 to 2043 in CAPN10A cDNA. The open reading frame of CAPN10B is 1923 bp with a different stop codon from CAPN10A, encoding a protein of 640 amino acids with a theoretical molecular mass of 71 kDa and an isoelectric point of 7.89. These sequence data have been submitted to the GenBank database under accession No. DQ647669 for CAPN10A and DQ647668 for CAPN10B.

Amino acid sequence analysis of the two isoforms of CAPN10 revealed that pig CAPN10 could be divided into the canonical four-domain structure typical of calpains: I, II, III, and the divergent C-terminal domain T containing no calcium-binding EF-hand structures. Domain II contained the Cys, His, and Asn residues found in the active sites of other calpain catalytic subunits. Domain T showed no significant similarity to the calmodulin-like Ca2+-binding domain IV of the traditional calpains (Fig. 1).

Molecular phylogenetic analysis of CAPN10
The cDNA sequences of calpain10 for human (GenBank accession No. NM023083), mouse (GenBank accession No. C005681), rat (GenBank accession No. B13362), chicken (GenBank accession No. M422752), dog (GenBank accession No. XM843215), and cattle (GenBank accession No. XM001256354), downloaded from GenBank (http://www.ncbi.nlm.nih.gov/), were used to determine the sequence identity among these vertebrates. The identity was between 61.8% and 95.5% in the coding sequences, and between 60.2% and 96.8% in deduced amino acid sequences. Molecular genetic trees were constructed using the methods of neighbor-joining for coding...
sequences (Fig. 2). The tree shown in Fig. 2 suggests that pig is more closely related to cow than dog, human, or mouse and the result is consistent with the phylogenetic tree of the vertebrate using other molecular markers.

**Expression analysis of CAPN10**

As shown in Fig. 3, CAPN10 mRNA was expressed in all tissues studied with abundant transcript in stomach, kidney, spleen, lung, heart, liver, and large intestine, with the lowest level in fat. The expression level in muscles from pigs at six different growth points was gradually increased, with the exception of muscle from 1-d-old pigs (Fig. 4).

The expression of the two isoforms of CAPN10 at the mRNA level was compared by competitive RT-PCR and the results are shown in Fig. 5. In all tissues examined, the expression level of CAPN10A was higher than that of CAPN10B.

**PCR-SSCP analysis of CAPN10**

The PCR-SSCP method was developed successfully for screening individual Min pigs. The polymorphism resulted in three genotypes, defined as AA, AB, and BB (Fig. 6). Sequencing results showed that there were two silent mutations, T567C and G573A, in the coding sequences region of the CAPN10 gene (GenBank accession No. DQ647668) between the sequences of AA and BB. Comparison with the sequences submitted to GenBank revealed the third mutation, C527T (Fig. 7). In the cloning of the CAPN10 gene, several Yorkshire pigs were used and cytosine at the position of 527 bp was verified, so there were three mutations in the region. The C527T

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**Fig. 2** Phylogenetic tree drawn with the neighbor-joining method based on calpain10A coding sequences

**Fig. 3** Expression of calpain10 in different tissues of 180-d-old Yorkshire pigs  
M, marker; 1, stomach; 2, kidney; 3, spleen; 4, lung; 5, heart; 6, liver; 7, large intestine; 8, small intestine; 9, gonad; 10, uterus; 11, fat; 12, muscle.

**Fig. 4** Expression of calpain10 in muscles from pigs at different growth stages  
M, marker; 1, 1-d-old; 2, 21-d-old; 3, 90-d-old; 4, 180-d-old; 5, 270-d-old; 6, 360-d-old. Different letters indicate significant difference (P<0.05).
mutation was a missense mutation and led to transforming Pro into Leu at the 176th amino acid of the mutant protein.

**Discussion**

In this study, using molecular biology techniques and *in silico* cloning, two isoforms of pig CAPN10 were cloned. This is the first report on the CAPN10 gene in pig. Sequence analysis showed that pig CAPN10A has a similar splicing pattern to human CAPN10A and one of the mouse/rat isoforms, whereas CAPN10B is distinct from any human/rat/mouse isoforms. This further showed that CAPN10 is extremely intricate terms of structure and function. The domain architecture of CAPN10 is unique compared to the conventional calpain1 and 2 in that the calmodulin-like domain is replaced by a divergent T domain with no EF-hand structures. This implies that the mechanism of CAPN10 regulation by calcium is distinct from that of calpain1 and 2. Homology analysis showed that CAPN10 was highly conserved among animals, supporting the hypothesis of extensive conservation between the CAPN10 genes among vertebrate species, and implied that CAPN10 could exert many functions during animal growth and development.

As expected, based on its ubiquitous distribution in rat tissues [10], RT-PCR revealed that CAPN10 transcripts were present in all tissues studied, confirming the essential roles of the calpains in pig and indicating their necessity for housekeeping functions [18]. However, their relative amounts in different tissues from 180-d-old pig was unevenly distributed, supporting the notion that calpains are highly regulated genes. Our results also showed that the highest level of expression was in large intestine (Fig. 3), whereas in the adult human, heart showed the highest level of expression [9], and in young rat, the highest level of expression was in brain [10]. Considering these facts, the expression pattern of CAPN10 at the mRNA level appears to be different between species.
In mammals, calpains are known to play a significant role in muscle protein turnover by mediating the degradation of myofibrillar proteins: desmin, filament, C-protein, tropomysin, tropinin T, troponin I, titin, nebulin, vimentin, gelsolin, and vinculin [19–21]. Proteolysis of these proteins affects muscle deposition in the living body, and impacts whole-muscle food texture, leading to meat tenderization in the early post-mortem period. The expression analysis at the mRNA level in muscles at six different growth points showed that the relative amount of the CAPN10 gene was present at its lowest level in muscle from 21-d-old pig and gradually increased to its highest level in 360-d-old pig. With increasing age, the capability of muscle deposition decreases, whereas the relative expression level in 360-d-old pig. With increasing age, the capability of muscle deposition decreases, whereas the relative expression level in 360-d-old pig. With increasing age, the capability of muscle deposition decreases, whereas the relative expression level in 360-d-old pig. With increasing age, the capability of muscle deposition decreases, whereas the relative expression level in 360-d-old pig.

When a gene is identified, there may be more than one polymorphism within it. An SNP occurring in the coding region (cSNP) could affect the expression level and protein structure, whereas a non-synonymous mutation that changes the amino acid is likely to have an effect on phenotype, making the cSNP more functional for marker-assisted selection [22,23]. In this study, three cSNPs were detected by PCR-SSCP and aligning sequences of Yorkshire and Min pigs, which laid the foundation for further study on the association of polymorphism with traits. The Pro176Leu mutation existed in a relatively conservative region of the catalytic domain and sequence analysis showed that, by aligning the sequence with that of chicken, human, mouse, rat, cattle, and dog from GenBank, Pro did not appear in that site in other species (Fig. 8). It will be interesting to investigate whether the mutation was specific to pig and influenced the function of CAPN10.

References

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