

Production of Polyclonal Antibodies Against Calpastatin Gene Domain I from Skeletal Muscle of the Kamphaeng Saen Beef Breed

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ABSTRACT

Calpastatin is an endogenous inhibitor of the calpain (EC.3.4.22.17, Ca⁺-dependent cysteine proteinase) proteolytic enzyme system, which plays a major role in the postmortem tenderization. Complementary strand DNA of calpastatin gene derived from domain I of *Longissimus dorsi* muscle from Kamphaeng Saen beef breed was amplified using the 1AF_1DR primer. Analysis of PCR results indicated the presence of product of approximately 402 bp. Nucleotide sequencing of the cDNA for bovine calpastatin corresponded with published domain I of calpastatin nucleotide sequences from bovine skeletal muscle and bovine heart calpastatin in Genbank (accession no. L14450 and NM_174003, respectively). PCR products were cloned into the plasmid pDrive cloning vector and subcloned into the plasmid pET28a expression vector. Recombinant calpastatin corresponding to domain I had the size approximately 21 kDa. The recombinant calpastatin used as immunogens applied to an immunized rabbit. Polyclonal antibodies, were raised in the rabbit against recombinant calpastatin, specifically recognized the calpastatin protein of approximately 130 kDa as determined by the Western immunoblot analysis. These results indicated that calpastatin domain I was presented within the recombinant calpastatin protein and direct subcutaneous of immunization of recombinant calpastatin into rabbit was effective in polyclonal antibodies production against calpastatin.

Key words: cloning, polyclonal antibodies, calpastatin gene, Kamphaeng Saen beef breed

INTRODUCTION

Consumers consider tenderness to be the most important component of the meat quality. The majority of the tenderness problems were improved by post-mortem aging and mechanical tenderization. The calcium-dependent proteolytic

systems, calpains, are responsible for proteolysis of the myofibrilla proteins whose degradation causes the weakening of the myofibrilla structure and, therefore, meat tenderization. One of the primary factors that influence calpain activity in post-mortem muscle is calpastatin activity (Koohmaraie, 1990).

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Calpastatin, an endogenous inhibitor of the calpain proteolytic system, has been found in all tissues that contain calpains and reside predominantly in phosphorylated-aggregated bodies, with the inhibitor protein being dephosphorylated and distributed in a Ca^{2+} -dependent manner (Koohmaraie, 1994; Doumit *et al.*, 1996; Huff-Loneragan and Lonergan, 1999; Betts *et al.*, 2003).

The primary structure of calpastatin is divided into four homologous C-terminal repeating inhibitory domains, domain I-IV, each containing 120-140 amino acid residues, and one non-homologous sequence on the NH_2 -terminal domain that lacks inhibitory function, domain L. Conserved residues among the four repetitive domains are not randomly distributed but clustered in three restricted regions in each domain (Maki *et al.*, 1989). Inside each repetitive domain is three conserved subdomains referred to as A, B, and C (Ma *et al.*, 1994).

Expression of truncated cDNAs encoding portions of the calpastatin polypeptide and assay of the expressed polypeptides has indicated that each of the domains, domain I-IV, can inhibit proteolytic activity of either μ -calpain or m-calpain. Ability of the individual domains to inhibit either μ -calpain or m-calpain has been reported to differ in the order, domain I > domain IV > domain III > domain II, from most to least effective (Goll *et al.*, 2003). The characterizations of partial calpastatin cDNAs from human, rabbit, pig, and cattle have proposed a translation initiation site that yields a predicted protein of 639-718 amino acids, depending on species (Cong *et al.*, 1998). Doumit *et al.* (1996) found that Western blot analysis showed the band of approximately 130 kDa in pre-rigor skeletal muscle.

Variation in calpastatin activity has been shown to be associated with the variation in the rate and extent of postmortem proteolysis and the tenderization in beef. Post-rigor calpastatin activity, which inversely proportional to post-

mortem tenderization, accounts for a greater proportion of the variation in tenderness of aged beef *Longissimus dorsi* (~40%) than any other single measure (Shackelford *et al.*, 1994).

Shackelford *et al.* (1994) and Wheeler *et al.* (1990) reported that the decreased of tenderness associated with *Bos indicus* breeding seems to be highly related to increased calpastatin activity. Furthermore, Pringle *et al.* (1997) shown that calpastatin activity increased 31% ($P < 0.01$) in activity as the percentage of Brahman breed increased from 0 to 100. Most of cattle in Thailand had higher percentages of *Bos indicus*. Kamphaeng Saen beef breed was an example of crossbred which established by the Department of Animal Science, Kasetsart University, Thailand, using the Charolais breed (*Bos Taurus*), top crossing to the Crossbred (Brahman x native cattle, *Bos indicus*).

Because of the significant functions of the calpastatin, therefore, the objectives of this study were: 1) to clone, express, and purify recombinant bovine skeletal muscle calpastatin domain I of the calpastatin gene from the Kamphaeng Saen beef breed and 2) to raise polyclonal antibodies against recombinant calpastatin, in order to investigate the expression of calpastatin gene domain I from the cattle in Thailand and product of specific polyclonal antibodies to detect calpastatin protein.

MATERIALS AND METHODS

Tissue for calpastatin RNA preparation

Muscle samples (10 g) were collected from the *Longissimus dorsi* muscle from 5 Kamphaeng Saen beef breed steers at 12th-13th interface within 30 minutes of exsanguinations. Muscle tissue was trimmed of fat and minced in 500 μl denaturing solution (4M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, and 0.5% sarcosyl) and stored at -80°C until used for RNA isolation.

RNA extraction and complementary strand DNA (cDNA) synthesis

Total RNA was isolated according to acid guanidine thiocyanate phenol chloroform RNA extraction method of Siebert and Chenchik (1993). The RNA template was used for cDNA synthesis. The iScript™ cDNA Synthesis Kit (Bio-Rad, USA) contained the unique blend of oligo (dT) and random hexamer primers was used for cDNA synthesis. The component of reaction was carried out in final reaction volume of 20 µl of the following reaction mixture: 4 µl 5x iScript reaction mix, 1 µl iScript reverse transcriptase, 13 µl nuclease-free water, and 2 µl RNA template. A mixed content of tube was incubated as follow: 25°C for 5 minutes, 42 °C for 30 minutes, and 85 °C for 5 minutes. Stored at -20°C until used for PCR amplification.

PCR procedure

To amplify bovine calpastatin gene, primers were designed using sequence base on the reported sequence (GenBank accession no. L14450), corresponding to domain I of calpastatin nucleotide sequence, a sense primer 1AF: TCAGACATGGACACTGCTCTGG and antisense primer 1DR: CT(C/T)TTCCACTTT(C/T)TTTTCTC. The PCR was carried out in a PTC-200 Peltier Thermal Cycler machine using the following conditions were 94 °C for 5 minutes for the first cycle and 94 °C for 30 seconds for denaturation, 57 °C for 1 minute for annealing, and 72 °C for 1 minute for extension, with total of 35 cycles. Ethidium bromide stained DNA fragments were visualized on 1.5% agarose gels. PCR products were sequenced by autosequencing. The nucleotide sequences were blasted to GenBank using BLAST2 sequences version BLASTN 2.2.9 program (NCBI).

Cloning, expression and purification of recombinant calpastatin

PCR product of bovine calpastatin was

ligated with pDrive cloning vector (Qiagen® PCR cloning kit, QIAGEN, USA) and transformed into *E. coli* JM109. The recombinant plasmid was cut with *EcoRI* restriction enzyme and inserted to the pET28a plasmid, which cut with *EcoRI* restriction enzyme and dephosphorylated by alkaline phosphatase. The expression host strain, *E. coli* BL21, was transformed and grown in LB medium containing 30 mg/mL kanamycin.

Fusion protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 0.4 mM. After 4 hours, cells were harvested by centrifugation. Cells were washed twice by 5 ml 1X PBS, lysed by 1 M urea, and fractionated by SDS-PAGE in Mini-Portean (Bio-Rad, USA) using 0.75 mm spacers. Twelve percent separating gels (30% acrylamide/0.8% bisacrylamide), with 4% stacking gels were used. A vertical strip containing molecular weight standards and portion of the sample lane was cut and stained with copper chloride staining to visualize the protein. A portion of the gel that contained the recombinant protein was cut and crushed in 0.1% sodium dodecyl sulfate (SDS) and agitated for 60 minutes at -80°C to passively elute the recombinant protein from the acrylamide gel after that sonicated 3 times of 1 minute. The slurry was clarified by centrifugation (14,000 rpm) for 2 minutes (modified from Doumit *et al.*, 1996). The supernatant was collected immediately and SDS was removed by precipitation with acetone about four volumes of the purified protein and incubated at -20 °C for 1 hour. After that, the SDS solubilized portion SDS was discarded by centrifuged 14,000 rpm for 10 minutes and resuspended the purified protein pellet by 50 µl distilled water.

Polyclonal antibodies production

A 5 ml pre-immunization blood sample of a rabbit was taken. The purity of recombinant calpastatin fraction (250 µg) was emulsified with an equal volume of complete Freund's adjuvant

(SIGMA, USA) and injected into the rabbit subcutaneously on the dorsal side. Subsequent immunizations were given with incomplete Freund's adjuvant (SIGMA, USA). After four immunizations at weekly intervals, the rabbit was bled at weekly intervals and tittered to assess the need for further booster injections. Rabbit sera was lyophilized and store at -20 °C until utilized. The fluid of rabbit serum was used in the Western blot immunoassay experiment. Polyclonal anti-recombinant calpastatin antibodies were raised in the Department of Pathology, Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen campus.

Preparation of calpastatin

Muscle samples (10 g) were homogenizes in two volumes of 100 mM Tris, 10 mM EDTA (pH 8.3). The homogenate was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was collected and heated at 95°C for 15 minutes then cooled on ice. Sample was clarified by centrifugation at 10,000 rpm for 10 minutes at 4°C. Supernatant containing heat-stable proteins, including calpastatin, were used for the Western blot immunoassay analysis (Geesink *et al.*, 2005).

Western blot immunoassay

Soluble proteins from heated muscle homogenates were dissolved by 12% SDS-PAGE. Proteins were eletrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Pall Life Sciences, USA) for 3 hour at 4°C and 300 mA in buffer containing 25mM Tris, 193mM glycine, and 15% methanol. To prevent nonspecific antibodies binding, membranes were incubated with blocking buffer (3% BSA in Tris buffer saline (TBS) containing 0.05% Tween 20, pH 7.5) for 1 hour at 37°C. Antibodies were adsorbed using lysate from induced *E. coli* BL21 containing plasmid pET-28a for 1 hour at 37 °C. Membranes were incubated with primary

antibodies diluted 1:1,600 for 1 hour at 37°C and goat anti-rabbit IgG HRP conjugate secondary antibodies (ZYMED, USA) diluted 1:1,000. Membranes were washed three times with TBS after each incubation. Antibodies binding was visualized by exposure to stableDAB[®] peroxidase substrate (AMRESCO, USA) (modified from Doumit *et al.*, 1996).

RESULTS

Amplification and nucleotide sequencing of bovine calpastatin

The RT-PCR products of total RNA from the *Longissimus dorsi* muscle using the 1AF_1DR primer had the size approximately 402 bp (Figure 1).

The nucleotide sequences of the PCR product indicated 402 bp of calpastatin domain I accompanied by the 134 amino acid residues (Figure 2).

The nucleotide sequences from five Kamphaeng Saen beef breed steers verified that

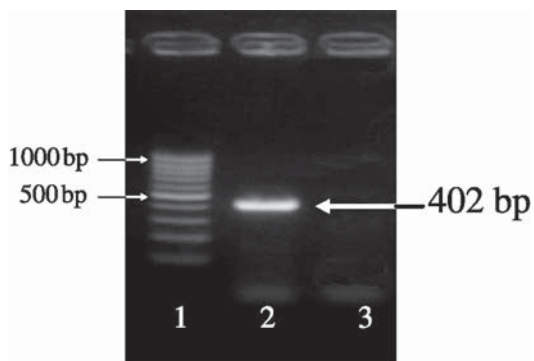


Figure 1 Agarose gels showed 402 bp cDNA encoded domain I of bovine calpastatin. The first lane corresponded to GeneRuler™ 100bp DNA ladder (FERMENTAS INC., USA). Lane 2 contained the RT-PCR products of total RNA from the *Longissimus dorsi* and lane 3 was negative control.

the nucleotide was most closely related to the published subdomain 1A-1D of calpastatin sequence accession no. L14450 and NM_174003 (98% and 96% of homology, respectively). However, there was a 21 bp nucleotide deletion found in the sequence of the Kamphaeng Saen beef breed when compared with L14450 but not NM_174003 (Figure 3). Furthermore, there was some divergence in nucleotide sequence among calpastatins from difference beef breeds. Comparing the amino acids with other reports indicated that domain I of Kamphaeng Saen calpastatin contains a 7 amino acid deletion (data not shown) corresponding to domain I of NM_174003.

Cloning, expression, and purification of recombinant calpastatin

The PCR products were cloned into the plasmid pDrive cloning vector and subcloned into the plasmid pET-28a expression vector.

Recombinant protein corresponding to calpastatin domain I was expressed in *E. coli* BL21 and induced by 0.4 mM IPTG. By 12% SDS-PAGE analysis, the size of recombinant calpastatin was approximately 21 kDa (Figure 4).

Western blot immunoassay

The supernatant containing heat-stable proteins, including calpastatin, were electrophoresed and transferred onto PVDF membrane. Normal rabbit serum diluted at 1: 1,600 and polyclonal antibodies against recombinant calpastatin domain I diluted at 1: 1,600 were used as sources of primary antibodies. Western blot immunoassay revealed that the recombinant calpastatin recognized a 130 kDa protein in heated pre-rigor skeletal muscle homogenates by polyclonal antibodies (Figure 5b), while no band of normal rabbit serum was deserved. (Figure 5a).

1	TCA GAC ATG GAC ACT GCT CTG GAT GAC TTA ATA GAC ACT TTA GGA	45
1	Ser Asp Met Asp Thr Ala Leu Asp Asp Leu Ile Asp Thr Leu Gly	15
46	GAA CCT GAA GAG ATG AAA GAA GAT AAC ACA ACA TAT ACC GGA CCG	90
16	Glu Pro Glu Glu Met Lys Glu Asp Asn Thr Thr Tyr Thr Gly Pro	30
91	GAA GTG TCG GAT CCA ATG AGT TCT ACC TAC ATA GAG GAA CTG GGT	135
31	Glu Val Ser Asp Pro Met Ser Ser Thr Tyr Ile Glu Glu Leu Gly	45
136	AAA AGA GAA TCC ACA CTT CCT CCA AAA TAT AAG GAA CTT CTG AAT	180
46	Lys Arg Glu Ser Thr Leu Pro Pro Lys Tyr Lys Glu Leu Leu Asn	60
181	AAA GAA GAA GGG ATC GCA GGG CCT CCT CCA GAC TCC TTG AAA CCC	225
61	Lys Glu Glu Gly Ile Ala Gly Pro Pro Pro Asp Ser Leu Lys Pro	75
226	CTG GGG CCC AAT GAT GCC ATC GAT GCC TTG TCA TCC GAC TTC ACG	270
76	Leu Gly Pro Asn Asp Ala Ile Asp Ala Leu Ser Ser Asp Phe Thr	90
271	TGC AGT TCC GCT ACA GCT GAT GCA AAG AAA ACT GAG AAA GAG AAA	315
91	Cys Ser Ser Ala Thr Ala Asp Ala Lys Lys Thr Glu Lys Glu Lys	105
316	TCT ACA GAA GAG GCT TTA AAA GCT CAG TCA GCT GGG GTG ATC AGA	360
106	Ser Thr Glu Glu Ala Leu Lys Ala Gln Ser Ala Gly Val Ile Arg	120
361	AGT GCT GCT CCA CCC AAA GAG AAA AGA AGA AAA GTG GAA GAG	402
121	Ser Ala Ala Pro Pro Lys Glu Lys Arg Arg Lys Val Glu Glu	134

Figure 2 Nucleotide sequences of domain I of calpastatin gene from the Kamphaeng Saen beef breed. The deduced amino acid sequences were shown below the derived nucleotide sequences.

	10	20	30	40	50	60	70	80	
Kamphaengsaen	TCAGACATGG	ACACTGCTCT	GGATGACTTA	ATAGACACTT	TAGGAGAAC	TGAAGAGATG	AAAGAAGATA	ACACAACATA	80
L14450									80
NM_174003									80
	90	100	110	120	130	140	150	160	
Kamphaengsaen	TACCGGACCG	GAAGTGTGG	ATCCAATGAG	TTCTACCTAC	ATAGAGGAAC	TGGGTAAAAG	AGAATCCACA	CTTCCTCCAA	160
L14450			...G.....					..C.....	160
NM_174003									160
	170	180	190	200	210	220	230	240	
Kamphaengsaen	AATATAAGGA	ACTTCTGAAT	AAAGAAGAAG	GGATCGCAGG	GCCTCCTCCA	GACTCCTTGA	AACCCCTGGG	GCCCAATGAT	240
L14450									240
NM_174003			G.....						240
	250	260	270	280	290	300	310	320	
Kamphaengsaen	GCCATCGATG	CCTTGTCAAT	CGACTTCACG	TGCAGTTCGG	-----	-----	-CTACAGCTG	ATGCAAGAGAA	299
L14450			C.....	C.....	TACAGCTGAC	TACCTGCAGT	C.....	...GC...G.	320
NM_174003			C.....	C.....	-----	-----	-----		299
	330	340	350	360	370	380	390	400	
Kamphaengsaen	AACCTGAGAAA	GAGAAATCTA	CAGAAGAGGC	TTTAAAAGCT	CAGTCAGCTG	GGGTGATCAG	AAGTGCTGCT	CCACCCAAAG	379
L14450									400
NM_174003								...C...	379
	410	420							
Kamphaengsaen	AGAAAAGAAG	AAAAGTGGAA	GAG						402
L14450			...						423
NM_174003	A...	G.....	A...						402

Figure 3 Nucleotide sequence alignment of subdomain 1A-1D of calpastatin gene from the Kamphaeng Saen beef breeds compared with the sequences from GenBank accession no. L14450 and NM_174003. Denote conservation by plotting identities to a standard as a dot (.) and deletion as a dash (-).

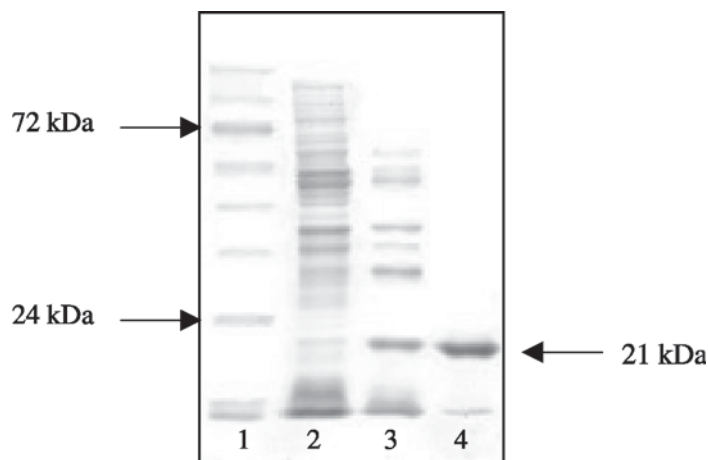


Figure 4 SDS-PAGE analysis of recombinant bovine skeletal muscle calpastatin. The first lane corresponded to PageRuler™ Prestained Protein Ladder (FERMENTAS INC., USA). Lane 2 was *E. coli* BL21 with plasmid containing gene but not induced. Lane 3 contained the recombinant protein expression. Lane 4 contained the purified recombinant protein.

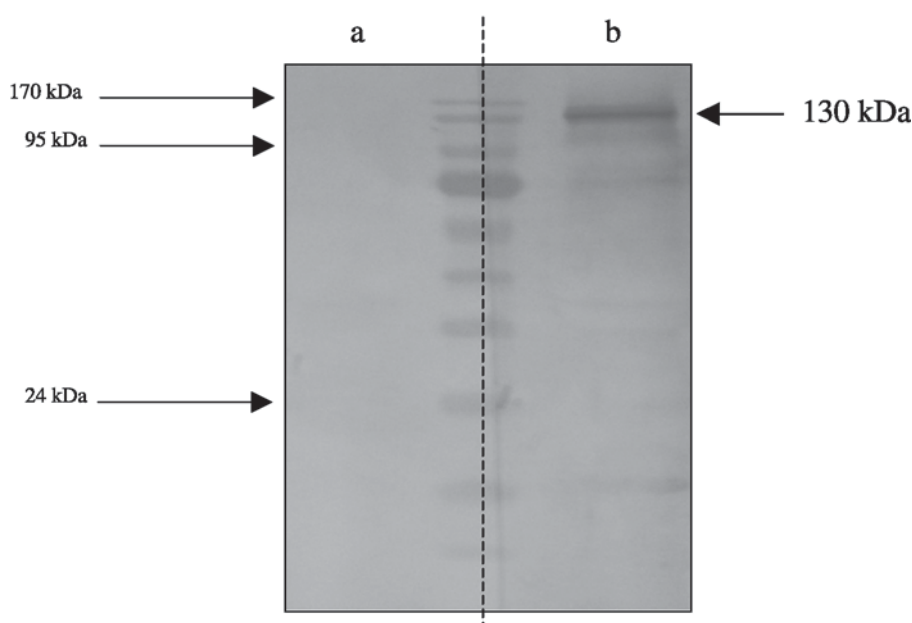


Figure 5 Western blot analysis shown activity of the recombinant protein. The PVDF membrane was divided into two portions at the middle of protein marker lane (PageRuler™ Prestained Protein Ladder, FERMENTAS INC., USA) after blotting. The membrane of a and b portions were probed with normal rabbit serum and the polyclonal antibodies against calpastatin domain I, respectively.

DISCUSSION

Amplification of the cDNA revealed a product of approximately 402 bp, consisted of domain I of skeletal muscle calpastatin gene from the Kamphaeng Saen beef breed, when used the 1AF_1DR primer. The nucleotide sequences of the 5 steers verified that the nucleotide sequences were most closely related to the published domain I of bovine skeletal muscle calpastatin cDNA accession no. L14450 and bovine heart calpastatin cDNA accession no. NM_174003 (Killefer and Koohmaraie, 1994; Cong *et al.*, 1998). A 21 bp nucleotide deletion of the Kamphaeng Saen beef breed resulted from alternative splicing (Cong *et al.*, 1998). This variability may be due to the genetic differences of cross-breeding between *Bos Taurus* and *Bos indicus* of the Kamphaeng Saen cattle and *Bos Taurus* of the accession no. L14450.

In this paper, we have amplified and cloned calpastatin cDNA from bovine skeletal muscle only domain I of calpastatin gene because a 27-residue peptide derived from subdomain IB of the repetitive domains of calpastatin, namely B27-WT, contained an influential inhibitor of calpain (Ma *et al.*, 1994; Betts *et al.*, 2003). However, a difference in the amino acid make up at the position 18 in subdomain IB was found. At the position, Proline was reported by Genbank (accession no. L14450) while in this study the Leucine was deserved instead (data not shown). In addition, mutation of any residues of amino acid in the subdomain IB resulted in inhibitory activity. It was anticipated that this difference may have a high relationship to the beef tenderness.

This study elucidated the expression and purification of recombinant bovine skeletal muscle calpastatin domain I suitable for the production

of polyclonal antibodies. Production and purification of the immunoassay revealed that the recombinant calpastatin recognized a 130 kDa protein in heated pre-rigor skeletal muscle homogenates by polyclonal antibodies against calpastatin domain I, while no band of normal rabbit serum was observed.

The result is concurrent with Doumit *et al.* (1996) who reported that Western blot analysis showed the same value for the molecular mass of muscle calpastatin when probed with polyclonal rabbit anti-domains 2, 3, 4, and the 3'untranslated region of bovine recombinant calpastatin antibodies. These incidents related to the reported of Doumit and Koohmaraie (1999) who found that the monoclonal antibodies raised in mouse against recombinant bovine skeletal muscle calpastatin interact an undergraded ~130 kDa protein in lamb *longissimus* muscle. However, the polyclonal antibodies against calpastatin domain I does not recognized the calpastatin from pork and chicken (data not shown).

CONCLUSION

Bovine skeletal muscle calpastatin domain I cDNA from the Kamphaeng Saen beef breed has been cloned, sequenced, expressed, and purified. The results showed the recombinant protein was calpastatin domain I. The purity of the recombinant calpastatin corresponding to domain I can be used for a rabbit immunogen. Polyclonal rabbit anti-recombinant antibodies specifically recognized the calpastatin protein of approximately 130 kDa as determined by the Western immunoblot analysis. This investigation provides the information of the calpastatin gene expression in the Kamphaeng Saen beef breed.

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