An Improved Purification Protocol for Heart and Skeletal Muscle Calpastatin Reveals Two Isoforms Resulting from Alternative Splicing

G. H. Geesink, D. Nonneman, and M. Koohmaraie[1]

Roman L. Hruska U.S. Meat Animal Research Center, ARS, USDA, P.O. Box 166, Clay Center, Nebraska 68933-0166

Employment of a new protocol for efficient purification of calpastatin revealed the presence of two forms of calpastatin in porcine heart with apparent molecular weights of 125 and 145 kDa. Purification from bovine muscle resulted in a single species with an apparent molecular weight of 125 kDa. The presence of multiple species of calpastatin in porcine heart does not appear to be an artifact of the purification procedure since Western blotting revealed the presence of two types of calpastatin in ovine and porcine heart, but only one type in bovine heart and bovine, ovine, and porcine muscle. The origin of the two species in porcine heart was examined by RT-PCR and direct sequencing of calpastatin cDNA. This analysis revealed that porcine skeletal muscle exclusively produces transcripts lacking exon 3, while porcine heart produces transcripts that include or lack exon 3, consistent with the presence of two isoforms of the protein. The 125-kDa form of porcine calpastatin therefore appears to be a result of alternative splicing of the calpastatin transcript. The biological significance of the heart specific isoform is not clear; however, its ability to inhibit μ- or m-calpain does not differ considerably. The present purification protocol yielded 4.9 and 1.8 mg calpastatin per kg tissue from porcine heart and bovine skeletal muscle, respectively. © 1998 Academic Press

Key Words: calpastatin; heart; skeletal muscle.

Calpastatin is a specific inhibitor of the ubiquitous calcium dependent proteinases μ- and m-calpain. Since its identification in bovine cardiac muscle (1), calpastatins with widely varying molecular weights have been purified from a number of tissues. The initial confusion regarding the molecular weight of calpastatin was due to several factors: (i) calpastatin is very sensitive to proteolytic degradation, and some proteolytic fragments of calpastatin retain inhibitory activity (2–4); (ii) because of its unusual amino acid composition, the molecular weight is overestimated using SDS–PAGE (5); and (iii) the protein is highly asymmetrical, leading to an overestimation of its molecular weight using gel filtration (2). Analysis of cDNAs encoding the calpastatins from rat, pig, rabbit, cattle, and humans has shown that the most prominent form found in all tissues except erythrocytes has a predicted molecular weight of 72–77 kDa (depending on species) but migrates anomalously on SDS–PAGE with an apparent molecular weight of 115–130 kDa (6, 7). These calpastatins are composed of four repetitive regions (domains 1–4), each of which can inhibit calpain activity, and a unique N-terminal region: domain L. The lower molecular weight calpastatin found in most mammalian erythrocytes appears to be a truncated form of the larger calpastatin, lacking domains L and 1 (8). Using reverse transcriptase polymerase chain reaction (RT-PCR), Lee *et al.* (9) detected three calpastatin cDNAs, one having full-length domains 1 and L and two with deletions in either exon 3 or in both exons 3 and 5. Human tissues and cell lines displayed different patterns of expression of these RT-PCR products. Subsequently, using Western blotting, Arnold *et al.* (10) detected two forms of calpastatin in porcine heart, but only one in porcine muscle. Using RT-PCR, it was found that most of the cardiac calpastatin mRNA contained exon 3, whereas exon 3 was deleted in the most prominent skeletal muscle calpastatin mRNA. However, chromatography of the muscle extracts to separate calpains and calpastatin in this study led to extensive fragmentation of calpastatin (10). Furthermore, Arnold *et al.* (10) did not determine the basis for different forms of calpastatin in porcine heart. Here we

Summary of Total Protein Yields, Total Calpastatin
Activity, and Specific Calpastatin Activity at Each Step
of the Purification of Calpastatin from Bovine Muscle

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)
Crude extract	13,810	—	—	—
After TCA treatment	3,000	2,223	0.74	—
After heat treatment	610	1,904	3.12	4.2
Affigel Blue	15.4	1,043	67.7	91.5
Mono Q	1.8	863	479	648
S-300	0.9	815	906	1224

report a purification method yielding large quantities
of undegraded calpastatin from skeletal and cardiac
muscle. Also, we demonstrate the basis for different
forms of calpastatin in porcine heart.

MATERIALS AND METHODS

Purification of calpastatin. Bovine sternomandibularis muscle
was obtained at a local slaughterhouse, transported to the Roman L.
Hruska Meat Animal Research Center (MARC), and processed
within 45 min postmortem as described below. Porcine heart was
obtained from pigs slaughtered at MARC. Bovine sternomandibu-
laris muscle (500 g) or porcine heart (1 kg) were trimmed of visible
fat and connective tissue and homogenized in 3 vol of extraction
buffer [100 mM Tris/HCl, pH 8.3, 10 mM EDTA, 0.05 % (v/v) 2-mer-
capthoethanol (MCE), 100 mg/L ovomucoid, 2 mM phenylmethylsul-
fonyl fluoride (PMSF), 6 mg/L leupeptin, 4°C]. Minced tissue was
homogenized with a Waring blender, three times at high speed for
30 s, with a 30-s cooling period between bursts. The homogenate was
centrifuged at $17,700g_{max}$ for 15 min at 4°C. The supernatant was
filtered over glass wool, and 15% (w/v) trichloroacetic acid (TCA) was
added. TCA-precipitated protein was pelleted by centrifuging at
$17,700g_{max}$ for 10 min at 4°C. The pellets were suspended in 400 mL
0.1 M Tris base and the pH was adjusted to pH 7.5 with 1 M NaOH.
This suspension was dialyzed overnight against three changes of 12
L of TEMA (20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 0.1% MCE, 1 mM
NaN_3). The suspension was clarified by centrifuging at $17,700g_{max}$.
The supernatant was heated in a microwave to 85°C, kept in a
waterbath at 90°C for 10 min, and cooled on ice. Precipitated mate-
rial was pelleted by centrifuging at $17,700g_{max}$, and the supernatant
was filtered over a Whatman No. 1 filter, adjusted to pH 7.5 with
solid Tris base, and loaded on an Affi-Gel blue column (Bio-Rad, 2.6 x
40 cm, 1 mL/min). The column was washed with 1 column volume
TEMA, and bound protein was eluted with a linear gradient of 0–150
mM KCl or 0–500 mM KCl in 800 mL of TEMA for muscle and heart,
respectively. Calpastatin containing fractions were collected, concen-
trated by ultrafiltration to about 15 mL using an Amicon YM10
membrane, diluted to 50 mL with TEMA to reduce the ionic strength
of the sample, and loaded on a mono-Q HR 5/5 column (Pharmacia)
at 1.25 mL/min. The column was washed with 1 column volume
TEMA and eluted with a linear gradient of 0–300 mM KCl in TEMA.
Calpastatin containing fractions were collected and concentrated to
less than 2 mL using Centriprep 10 or Centriprep 30 concentrators
(Amicon), loaded on a Sephacryl S-300 Hiload 16/60 column (Phar-
macia) at 0.5 mL/min, and eluted with TEMA at 0.5 mL/min.

Calpain and calpastatin activities. Calpain and calpastatin
activities were quantified as described (8). Purified bovine μ- and
m-calpain were used to determine the specific activities of the
purified calpastatins. Purified μ-calpain was obtained from sol-
uble muscle extract after successive chromatography over DEAE–
Sephacel (Pharmacia), phenyl–Sepharose (Pharmacia), butyl–
Sepharose (Pharmacia), Mono-Q (Pharmacia), and Sephacryl S-300
(Pharmacia). Purified m-calpain was obtained from soluble muscle
extract after successive chromatography over DEAE–Sephacel
(Pharmacia), phenyl–Sepharose (Pharmacia), Reactive red 120–aga-
rose (Sigma), and DEAE–TSK (Tosohaas).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Slab
SDS–PAGE was done according to Laemmli (12) using 7.5 and 12.5%
acrylamide gels.

Western blotting. Proteins from SDS–PAGE were transferred
electrophoretically to nitrocellulose membrane according to the pro-
cedure described by Towbin et al. (13). After transfer, Western blots
were processed as described by Koohmaraie et al. (14) using a poly-
clonal antibody raised against recombinant calpastatin as described
by Doumit et al. (15).

RNA isolation and cDNA synthesis. Total RNA was isolated from
porcine muscle according to Chomczynski and Sacchi (16) and Puis-
sant and Houdebine (17). Briefly, muscle tissue was homogenized
using a polytron (0.5–1 g/10 ml) in a solution of 4 M guanidinium
isothiocyanate, 10 mM Tris, and 1% β-mercaptoethanol, pH 7.6. The
homogenate was then extracted with phenol–chloroform–isoamyl
alcohol, ethanol precipitated, and resuspended in sterile DEPC-
treated water.

First strand cDNA was synthesized from 2 μg total RNA using
500U M-MLV reverse transcriptase, 10 mM dNTPs, and 2 μM
poly(T) primer, following the suppliers directions (Clonetech's, RT-
PCR Advantage kit). These reactions were incubated for 60 min at
42°C and used directly for RT-PCR after ethanol precipitation.

PCR amplification. Oligonucleotide primers were designed from
published porcine cDNA sequence in exons 2 and 6 (GenBank Acces-
sion No. M20160; nucleotides 101–124 and 344–368). They were
synthesized by the MARC DNA Core Facility on a Beckman 1000M
automated synthesizer (Beckman Industries, Inc.). The primers used
for amplification and sequencing were as follows: forward: (5'→3')
tct gct tca acg agc aag tct tcc reverse: (5'→3') ctt tat gag cac gct tgc
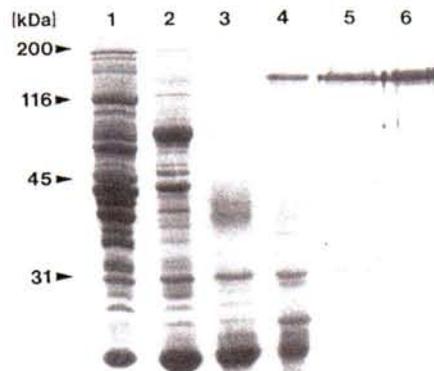tcc ctg c.

FIG. 1. SDS–PAGE of samples from various stages of the bovine
muscle calpastatin purification. Lane 1, crude extract; lane 2, solu-
bilized TCA-precipitate; lane 3, soluble fraction after heat treatment;
lane 4, pooled Affigel blue chromatography fractions; lane 5, pooled
Mono Q chromatography fractions; lane 6, pooled S-300 chromatog-
raphy fractions. Molecular weight markers are indicated on the left
side of the gel.
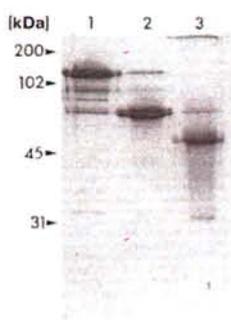
FIG. 2. SDS–PAGE of samples from a purification of calpastatin from bovine muscle in which TCA treatment was ommitted. Lane 1, partially pure intact calpastatin; lane 2, degraded calpastatin which binds to Affigel blue; lane 3, degraded calpastatin which does not bind to Affigel blue.

PCR amplifications were 50 μl in 10 mM Tris–HCl, pH 8.3, at 25°C, 50 mM KCl, 2.5 mM MgCl, 200 μM dNTPs, 1 μM oligonucleotide primer, and 2.5 U *Taq* DNA polymerase/reaction. Reactions were cycled at an initial denaturing temperature of 94°C for 30 s, followed by 40 cycles at 94°C for 30 s, 57°C annealing temperature for 30 s, and 72°C extension for 45 s. A 5-min extension period at 72°C was incorporated after the last cycle of PCR.

Sequencing of PCR products. Direct dideoxy sequencing was performed as described by Shibuya *et al.* (18). Briefly, PCR amplicons were purified by electrophoresis in 3.6% polyacrylamide gel in 1× Tris–borate–EDTA, pH 8.0 (TBE), running buffer. PCR products were excised from the gel, eluted, and ethanol precipitated. DNA sequencing was performed on purified products following ABI's recommendations for the Rhodamyne Big Dye reagents with a single primer and cycled using an initial denaturation of 96°C for 30 s, followed by 25 cycles of 96°C denaturation for 30 s, 50°C annealing of primer for 60 s, and 60°C extension for 4 min. Sequencing reactions were then ethanol precipitated and submitted to the MARC DNA Core facility for analysis using an ABI 377 automated sequencing apparatus.

RESULTS AND DISCUSSION

Bovine muscle. A summary of the purification procedure is given in Table I and Fig. 1. The goal was to increase the yield of intact calpastatin. The most significant step in achieving this goal is probably the TCA treatment, since it eliminates contaminating protease activity which is often a problem during the purification of calpastatin (2–4). Successive TCA and heat treatment is superior to heat treatment alone since heating large samples results in at least several minutes of exposure to temperatures at which proteases are optimally active. Each of the successive steps resulted in significant purification of calpastatin. TCA- and heat treatment eliminated over 96% of contaminating proteins, whereas successive chromatography, using Affi-Gel blue, anion exchange, and gel filtration, resulted in an additional 267-fold purification.

The importance of inactivation of protease activity in an early stage is illustrated in Fig. 2. The calpastatin fraction in this purification from bovine muscle was subjected to heat treatment after ammonium sulfate precipitation of the crude extract and chromatography over DEAE–Sephacel (Pharmacia). At this stage of the purification, about 50% of the activity was not bound by Affi-Gel blue (Fig. 2, lane 3), which was loaded at an ionic strength equivalent to 50 mM KCl, and an estimated 25% of the activity was present in a 70-kDa form (Fig. 2, lane 2), which was partly separated from intact calpastatin using gel filtration. These lower molecular weight forms did react with our antibody against calpastatin (data not shown), but were not detected on Western blots of crude extracts (Figures 4 and 8, lane
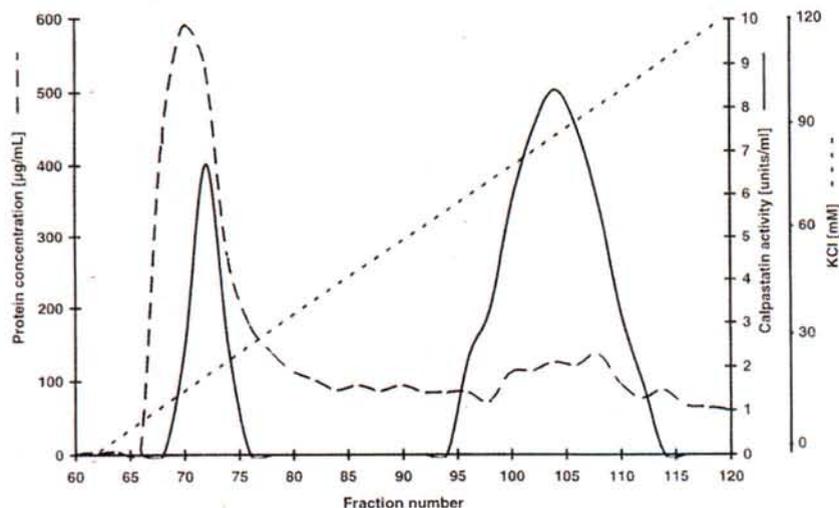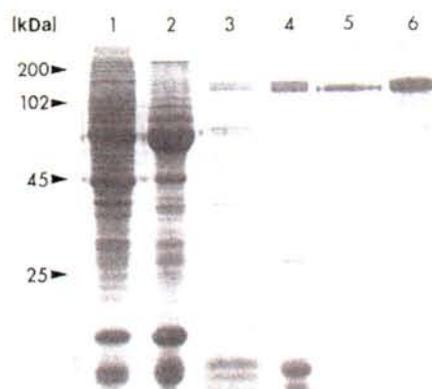

FIG. 3. Affigel blue chromatography of bovine muscle calpastatin after TCA- and heat treatment of the crude extract. Fractions 94 to 114 were pooled for further purification.
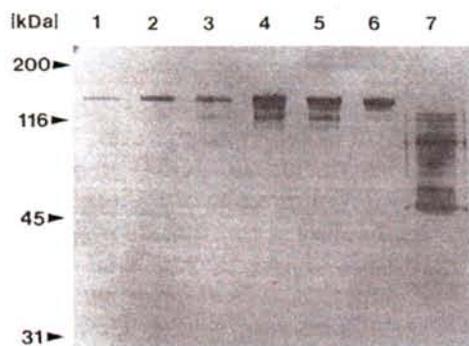
FIG. 4. Western blot against calpastatin in samples from the various stages of the bovine muscle calpastatin purification. Lane 1, crude extract; lane 2, solubilized TCA-precipitate; lane 3, soluble fraction after heat treatment; lane 4, pooled Affigel blue chromatography fractions; lane 5, pooled Mono Q chromatography fractions; lane 6, pooled S-300 chromatography fractions; lane 7, degraded calpastatin which eluted early off the Affi-Gel blue column (Fig. 3). Molecular weight markers are indicated on the left side of the gel.

FIG. 5. SDS–PAGE of samples from various stages of the porcine heart calpastatin purification. Lane 1, crude extract; lane 2, solubilized TCA-precipitate; lane 3, soluble fraction after heat treatment; lane 4, pooled Affigel blue chromatography fractions; lane 5, pooled Mono Q chromatography fractions; lane 6, pooled S-300 chromatography fractions. Molecular weight markers are indicated on the left side of the gel.

4), indicating that these are degradation products generated during the purification.

Binding of calpastatin to Affi-Gel blue probably involves domain L since the truncated form present in erythrocytes, which lacks part of domains 1 and domain L, fail to bind to this column material at an ionic strength equivalent to 120 mM NaCl (19). As a result, a large part of degraded calpastatin, which likely lacks domain L, elutes very early (Figs. 3 and 4), thus effectively separating intact calpastatin from degraded calpastatin. In the purification of calpastatin from bovine heart as described by Mellgren et al. (20), calpastatin bound to Affi-Gel blue in the presence of 0.12 M NaCl. In the present purification, however, calpastatin started to elute from this column at a lower ionic strength (Fig. 3), and no additional activity was detected when flushing the column with 0.5 M KCl. The recovery of cal-

pastatin after TCA precipitation could not be determined because of the presence of calpains and leupeptin in the crude extract. However, Mellgren and Carr (2) recovered 100% of calpastatin activity when TCA precipitation was used in a later stage of the purification. Assuming 100% recovery after TCA treatment, the present purification method resulted in 37% recovery of calpastatin activity.

Porcine heart. A summary of the purification procedure is given in Table II and Fig. 5. In contrast to bovine muscle calpastatin, porcine calpastatin eluted in two major peaks off the Affi-Gel blue column (Fig. 6). The first peak eluted at a similar ionic strength as bovine muscle calpastatin, whereas the second peak eluted at an ionic strength similar to that of bovine heart calpastatin as reported by Mellgren et al. (20). After gel filtration, two closely spaced bands were observed. These bands were partially separated on a Affi-Gel blue column (1 × 10 cm) eluted with a shallow gradient (15 column volumes) from 0 to 300 mM KCL in TEMA (Fig. 7). The low-molecular-weight form eluted earlier from this column than the high-molecular-weight form. Assuming 100% recovery after TCA treatment, the purification resulted in 27% recovery of the initial calpastatin activity. In contrast to the purification of bovine muscle calpastatin, heat treatment resulted in a 64% reduction of activity. Possibly, the higher molecular weight form is less heat resistant than the lower molecular weight form. Western blotting of the soluble muscle extract indicated that the higher molecular weight form was more abundant (Fig. 8). However, after heat treatment, the lower molecular weight form seems more abundant (Fig. 5).

TABLE II

Summary of Total Protein Yields, Total Calpastatin Activity, and Specific Calpastatin Activity at Each Step of the Purification of Calpastatin from Porcine Muscle

| Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)
Crude extract	34,283	—	—	—
After TCA treatment	8,647	17,259	2.0	—
After heat treatment	183	7,563	41.3	20.7
Affigel Blue	41.8	6,563	155	77.6
Mono Q	11.0	5,044	424	212
S-300	4.9	4,666	952	476
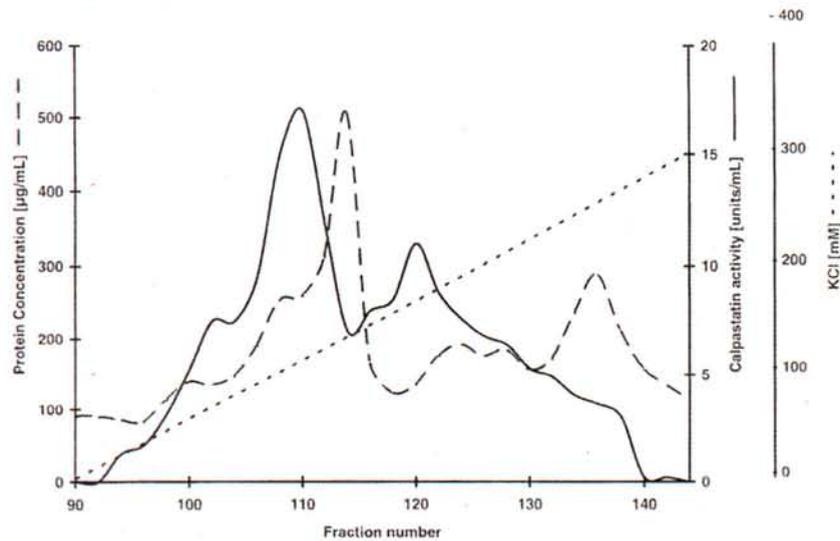
FIG. 6. Affigel blue chromatography of porcine heart calpastatin after TCA- and heat treatment of the crude extract. Fractions 93 to 140 were pooled for further purification.

To test whether the lower molecular weight form is a degradation product of the higher molecular weight form, whole tissue extracts or soluble extracts of ovine, porcine, and bovine heart and skeletal muscle were probed for calpastatin using Western blotting. The ovine and porcine samples were homogenized and extracted within 15 min postmortem and the bovine extracts within 45 min postmortem. As shown in Fig. 8, heart extracts (lanes 2, 4, and 6) contained a high-molecular-weight form of calpastatin which was not detected in muscle. The lower molecular weight form was detected in ovine and porcine heart but not in bovine heart.

Purified bovine m- and μ-calpain (Fig. 2, lanes 1 and 2) were used in titrations to determine the specific activities of the different porcine heart calpastatins. Specific activities are based on amounts that inhibited between 25 and 75% of the activities of μ- or m-calpain, i.e, the linear part of the inhibition curve. One microgram of the high-molecular-weight (HMW) calpastatin inhibited 3.8 μg of m-calpain and 2.8 μg of μ-calpain.

However, 1 μg of the low-molecular-weight (LMW) form inhibited 6.4 μg of m-calpain and 6.7 μg of μ-calpain. Therefore, it appears that HMW and LMW calpastatin differ in their ability to inhibit the calpains. Lee et al. (9) demonstrated that deletion of exon 3 occurs in domain L which does not impart inhibitory activity. Both forms of calpastatin have four repetitive, inhibitory domains. Therefore, the results of this study that show that HMW and LMW calpastatin inhibit calpain differentially is not plausible and requires further experimentation. However, the function of domain L is not known and, thus, it is possible that exon 3 may have an effect on the inhibitory activity of calpastatin.

RT-PCR of porcine cardiac and skeletal muscle. To determine if alternative splicing of calpastatin transcripts contributed to the size differences of pig heart and skeletal muscle calpastatin found by Western blotting, we used RT-PCR of total RNA extracted from these tissues (Fig. 9). PCR amplification of cDNAs isolated from porcine heart and skeletal muscle using primers in exons
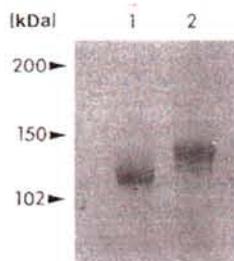
FIG. 7. SDS–PAGE of low- and high-molecular-weight porcine heart calpastatin following partial separation on Affigel blue. Molecular weight markers are indicated on the left side of the gel.

FIG. 8. Western blot against calpastatin. Lane 1, ovine skeletal muscle (soluble fraction); lane 2, ovine heart (whole homogenate); lane 3, porcine skeletal muscle (soluble fraction); lane 4, porcine heart (soluble fraction); lane 5, bovine skeletal muscle (soluble fraction); lane 6, bovine heart (soluble fraction).
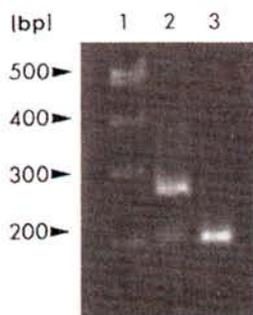
FIG. 9. RT-PCR amplification of porcine heart and skeletal muscle cDNAs. Lane 1, size markers; lane 2, heart; lane 3, skeletal muscle.

2 and 6 showed two different products for porcine heart and a single product from porcine muscle, consistent with earlier analyses (10). The PCR product found in skeletal muscle was identical in size to the smaller product found in porcine heart cDNA. Direct sequencing revealed that the larger product of porcine heart cDNA retained exon 3 of the calpastatin coding sequence, while the other two smaller PCR products of heart and muscle lacked exon 3. Exon 3 encodes for a region of domain L of the calpastatin protein, the function of which is still unknown. Since calpastatin exons are maintained in 0 phase with its introns, alternative splicing would only result in deletion of specific exon coding sequences and not a complete disruption of the amino acid sequence following exon skipping. The observed alternative splicing preserves the proper reading frame, such that the carboxy end of the protein is intact.

Recently it has been reported that the bovine calpastatin gene contains a preferred alternative initiation signal in an upstream exon from the exon 2 ATG that confers an additional 68 amino acids to the bovine calpastatin protein (22). Whether this sequence exists in the porcine calpastatin gene remains to be seen. This sequence has a predicted molecular mass of 7.1 kDa. Porcine calpastatin exon 3 encodes for an amino acid sequence of a predicted mass of 2.5 kDa. Together, these alterations in the translation of porcine calpastatin account for a size difference of 9.6 kDa. Since both of these coding regions are very basic in nature, which may cause anomolous migration on SDS–PAGE (5), the aforementioned alterations may explain the 20-kDa size difference between the two porcine heart calpastatins as estimated by SDS–PAGE.

The question remains, however, why cardiac and skeletal muscle differ in their expression of calpastatin isoforms. The phenomenon of differential splicing of mRNAs has been demonstrated in several tissues for a number of genes (23), yet the reason(s) for this phenomenon is still speculative at best. Alternative splicing of transcripts may not always result in expression of different protein isoforms if one transcript is preferentially translated. The results presented here, documenting the presence of pro-

tein isoforms associated with the alternative transcripts is, therefore, important in demonstrating the value of calpastatin as a candidate gene to study the biological question of differential splicing of mRNAs and the functions assigned to these gene products.

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