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Efficient expression and purification of recombinant human m-calpain using an *Escherichia coli* expression system at low temperature

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Shoji Hata*, Mika Ueno, Fujiko Kitamura and Hiroyuki Sorimachi

Department of Advanced Science for Biomolecules, Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kami-kitazawa, Setagaya-ku, Tokyo 156-8506, Japan

*Shoji Hata, Department of Advanced Science for Biomolecules, Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kami-kitazawa, Setagaya-ku, Tokyo 156-8506, Japan. Tel: +81 3 5316 3124, Fax: +81 3 5316 3163, email: hata-sj@igakuken.or.jp

Calpain belongs to the superfamily of Ca^{2+}-regulated cysteine proteases, which are indispensable to the regulation of various cellular functions. Of the 15 mammalian calpain isoforms, μ- and m-calpains are the best characterized. Both μ- and m-calpain are ubiquitously expressed and exist as heterodimers, containing a distinct 80-kDa catalytic subunit (CAPN1 and CAPN2, respectively) and the common, 30-kDa regulatory subunit (CAPNS1). To date, various expression systems have been developed for producing recombinant calpains for use in structural and physiological studies, however *Escherichia coli* systems have proven incompatible with large-scale preparation of calpain, with the exception of rat m-calpain. Here, we have established a highly efficient method to purify active recombinant human m-calpain using an *E. coli* expression system at low temperature (22°C). This was achieved by co-expressing CAPN2 with a C-terminal histidine-tag, and CAPNS1, lacking the first Gly-repeated region at the N-terminal. After three sequential passes through a chromatographic column, ~5 mg of human m-calpain was homogenously purified from 1 l of *E. coli* culture. Proteins were stable for several months. This is the first report of efficient, large-scale purification of recombinant human m-calpain using an *E. coli* expression system.

Keywords: bacterial expression/Gly-rich region/high yield/human m-calpain/large-scale purification.

Abbreviations: EDTA, di-sodium dihydrogen ethylenediamine tetraacetate dihydrate; GR, glycine-rich; PCR, polymerase chain reaction; PEF, penta-EF-hand; TED, Tris-Cl/EDTA/dithiothreitol.

Calpain (Clan CA-C2, EC 3.4.22.17) belongs to a family of intracellular Ca^{2+}-regulated cysteine proteases, whose members share homology in the protease domain in almost all eukaryotes and a few bacteria. In the human genome, there are 15 calpain genes with ubiquitous or tissue-specific expression. Calpains play pivotal roles in biological processes, including the cell cycle, apoptosis and myoblast fusion, through the limited proteolytic cleavage of diverse substrates, although their physiological functions remain obscure. A number of pathological conditions, including muscular dystrophies, lissencephaly, cataracts, neurodegenerative disorders and gastric ulcers have been associated with aberrant calpain action (*1*, *2*).

Two representative mammalian calpains, μ- and m-calpain, are ubiquitously expressed and have been extensively characterized. Both μ- and m-calpain exist as heterodimers, comprising the distinct 80-kDa catalytic subunits CAPN1 (previously called μCL, standing for μ-calpain large subunit) and CAPN2 (mCL, m-calpain large subunit), respectively, and a common 30-kDa regulatory subunit, CAPNS1, and require μM and mM levels of Ca^{2+}, respectively, for their activation *in vitro*. CAPN1 and CAPN2 consist of a regulatory N-terminal anchor helix, followed by a protease CysPc domain, a C2-like Ca^{2+}/phospholipid-binding C2L domain and a penta-EF-hand PEF domain. CAPNS1 comprises a structurally flexible, Gly-rich GR domain containing two Gly-repeated regions, and a PEF domain (Fig. 1). The PEF domains of the catalytic and regulatory subunits interact with each other through their C-terminal EF-hand motifs, forming a heterodimer. In the absence of Ca^{2+}, μ- and m-calpains are catalytically inactive, because the CysPC domain is separated into two protease core domains, PC1 and PC2, preventing active site formation. The binding of Ca^{2+} to the PC1, PC2, C2L, and PEF domains, induces structural changes that allow the domains to form a single protease core, involving subunit dissociation and/or N-terminal autolysis in both subunits including degradation within the GR domain (*3*–*6*).

Large-scale systems in *Escherichia coli* or insect cells have been used to purify full-length or truncated, recombinant μ- and m-calpain for structural–functional studies, involving 3D analyses. However, a persistent problem has been that recombinant full-length calpains, including μ- and m-calpain, aggregate as insoluble inclusion bodies within *E. coli* cells. Large-scale purification of recombinant full-length calpain using an *E. coli* expression system, has only been successful for rat m-calpain, by co-expression of CAPN2 and CAPNS1 lacking the GR domain at 30°C using compatible expression vectors (*7*, *8*). Recombinant human m-calpain can be extracted as a soluble protein using insect cells, but it accumulates as an insoluble

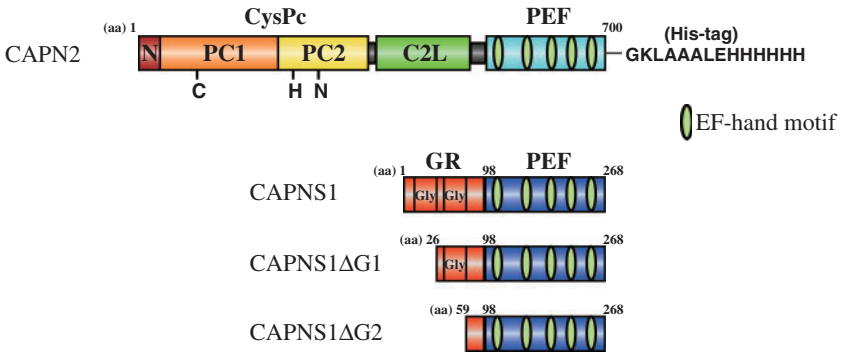

Fig. 1 Schematic illustration of the human CAPN2, CAPNS1, CAPNS1ΔG1 and CAPNS1ΔG2 constructs used in this study.

inclusion body in *E. coli* cells (*9*). In this study, we have established a method for high-level expression and purification of human m-calpain in a soluble and active form, using an *E. coli* system at a low temperature.

Materials and Methods

Reagents and antibodies

Chemical reagents were purchased from Sigma Inc. (MO, USA), Wako Pure chemicals Inc. (Osaka, Japan), Kanto chemicals Inc. (Tokyo, Japan) and Nakarai Tesque Inc. (Kyoto, Japan). Anti-His rabbit polyclonal antibody and anti-CAPNS1 mouse monoclonal antibody (clone P1) were purchased from Santa Cruz Biotechnology Inc. (CA, USA) and American Type Culture Collection (VA, USA), respectively.

Expression vectors

The cDNAs encoding human CAPN2, CAPNS1, CAPNS1ΔG1 and CAPNS1ΔG2 (for structures, see Fig. 1) were amplified by PCR using *pfu* turbo polymerase (Agilent Technologies Inc., CA, USA), and the sequences were verified after subcloning into compatible vectors for bacterial co-expression. Human CAPN2 was ligated into the pET24b(+) vector (Novagen, Darmstadt, Germany) to produce proteins fused with a C-terminal His-tag (CAPN2/pET), and human sequences CAPNS1, CAPNS1ΔG1 or CAPNS1ΔG2 were ligated into the pACpET24 vector (*7*) to produce proteins without peptide tags (CAPNS1/pACpET, CAPNS1ΔG1/pACpET or CAPNS1ΔG2/pACpET, respectively). The pACpET24 vector was constructed by replacing an 849-bp *Bam*HI-*Hind*III fragment, excised from the pACYC177 vector (New England Biolabs. Inc., MA, USA) with a 170-bp, *Bgl*II-*Hind*III fragment from the pET24b(+) vector. The pACpET24 vector contains an ampicillin-resistant gene, the T7 promoter, and the A15 replication origin.

Protein expression and purification

CAPN2/pET was co-transformed into *E. coli* BL21(DE3) (Novagen) with CAPNS1/pACpET, CAPNS1ΔG1/pACpET, or CAPNS1ΔG2/pACpET, and the transformants were stored as frozen glycerol stocks at −80°C. For protein expression and purification, frozen transformants were precultured at 27°C overnight, in 30 ml of LB medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin (LB+Amp+Kn), and then cultures were diluted into 1 l of LB+Amp+Kn, and grown at 27°C with vigorous shaking until A_{600} reached 0.8–1.0. Protein expression was induced by adding 0.2 mM isopropyl-thiogalactoside (IPTG), for 6 h at 22°C, with vigorous shaking.

Purification was performed at 4°C unless otherwise indicated. Harvested cells were washed once with ice-cold phosphate buffered saline, resuspended in 50 ml of ice-cold TED buffer (20 mM Tris–Cl (pH 7.5), 1 mM EDTA, and 1 mM dithiothreitol (DTT)) containing 0.3 mM PMSF, and lysed with a French Press (Ohtake Works, Tokyo, Japan). The cell lysate was ultracentrifuged at 55,000*g* for 30 min, and the recovered supernatant was filtered through a 0.22 μm-pore filter. The supernatant was applied to a DEAE-Toyopearl (Toyosoda Inc., Yamaguchi, Japan) anion exchange column (26 mm diameter, 124 mm length) equilibrated with TED buffer. The column was washed with TED buffer and the protein eluted with a linear gradient of 0–0.4 M NaCl in TED buffer, in 10 column volumes. Active fractions (total of 25 ml) were pooled, and 5 M NaCl and 1 M $MgCl_2$ added to final concentrations of 0.4 M and 5 mM, respectively, and filtered as described above. The pooled fractions were applied to a HisTrap HP (16 mm diameter, 25 mm length) affinity column (GE healthcare Inc., Buckinghamshire, England) equilibrated with buffer A (20 mM Tris–Cl (pH 7.5), 0.4 M NaCl, and 1 mM DTT) containing 4 mM imidazole. The column was washed with 10 column volumes of the same buffer, and protein was eluted with a linear gradient of 4–250 mM imidazole in buffer A, in 10 column volumes into collection tubes containing EDTA, to a final concentration of 1 mM. Active fractions (total of 15 ml) were immediately dialyzed against TED buffer overnight, pooled and centrifuged at 20,000*g* for 20 min to remove debris. The dialyzed sample was applied to a MonoQ HR10/10 anion-exchange column (GE healthcare), equilibrated with TED buffer, and after washing the column with TED buffer, the protein was eluted with a linear gradient of 0–0.55 M NaCl in TED buffer, in 5 column volumes. The purity of protein was verified by SDS–PAGE. The peak fractions (1 ml/fraction) were stored at 4°C until use. For gel filtration analysis, 0.7 μg of the final peak fractions were applied to a HiLoad 16/10 Superdex 200 column (GE healthcare) equilibrated with TED buffer containing 150 mM NaCl.

Activity assay for calpain

The proteolytic activity of calpain was measured using casein (Sigma), as previously described, with some modifications (*10*). Aliquots of the fractions eluted from columns were incubated at 30°C for 20 min, in reaction buffer (100 mM Tris–Cl (pH 7.5), 3 mg/ml casein, and 20 mM 2-mercaptoethanol) with 5 mM $CaCl_2$ or 5 mM EDTA, in total volume of 50 μl. After incubation, the reactions were stopped by the addition of 150 μl of 7% (v/v) trichloroacetic acid, incubated on ice for 30 min, and centrifuged at 15,000 rpm for 15 min at 4°C. A_{280} of the supernatant was measured using a spectrophotometer (SmartSpec-3000, Bio-Rad Laboratories Inc., CA, USA). One unit of calpain caseinolytic activity was defined as a Ca^{2+}-dependent increase in A_{280} of 1.0, in 1-hour, in a 1-ml scale reaction. The proteolytic activity was also measured using a fluorogenic substrate, succynyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide (Suc-LLVY-MCA) (Peptide Institute Inc., Osaka, Japan), with some modifications (*11*). The final peak fraction was incubated with 10–500 mM substrate for 10 min at 30°C, in 100 mM Tris–Cl (pH 7.5), 5 mM $CaCl_2$, 20 mM β-mercaptoethanol and 0.2% 3-[(3-cholamidopropyl)dimethyl-ammonio]propanesulfonic acid (CHAPS) in a total volume of 100 μl. Reaction was stopped by adding 100 μl of 10% SDS and 1.2 ml of 0.1 M Tris–Cl (pH 9.0). Release of 7-amino-4-methylcoumarin was monitored by a spectrofluorophotometer (RF-1500, Shimadzu, Kyoto, Japan), with excitation and emission wavelengths at 380 nm and 460 nm, respectively. The kinetic parameters (Km and Kcat) were calculated from Lineweaver-Burk plots.

Casein zymography

Casein zymography was performed as previously described (*12*). One μg of the final peak fraction, 1 μg of recombinant human m-calpain purified by Sf9/vaculovirus expression system and 25 μg of non-transfected COS7 cell lysate were added to Native-PAGE loading buffer (250 mM Tris–Cl, pH 6.8, 50% glycerol, 1 mM DTT, and

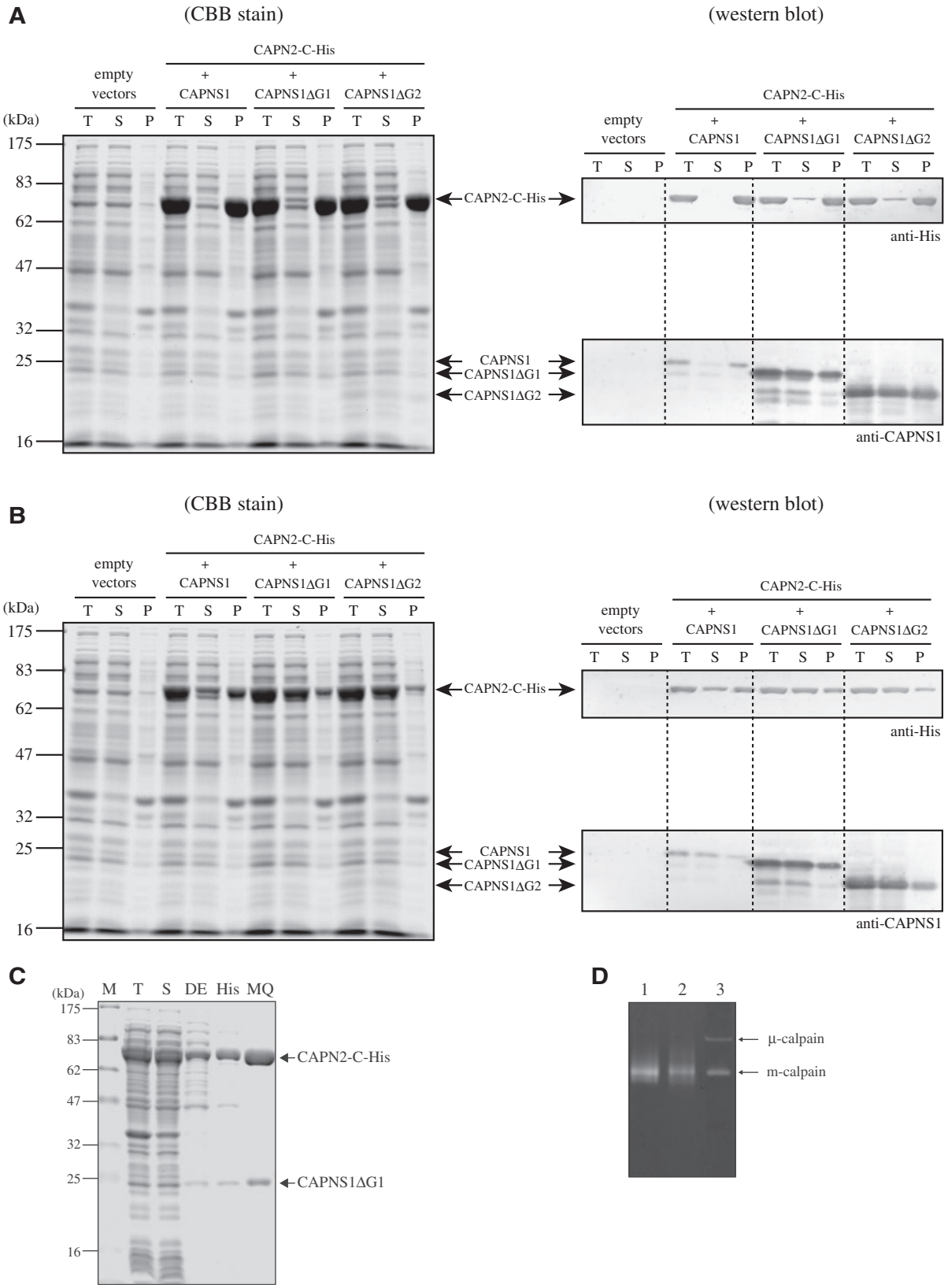

Fig. 2 Expression and purification of recombinant human m-calpain. CAPN2 was co-expressed with CAPNS1, CAPNS1ΔG1 or CAPNS1ΔG2 at 30°C (**A**) or 22°C (**B**). Each *E. coli* sample was lysed, fractionated and subjected to SDS–PAGE (CBB staining, left panels) and western blot analysis (right panels). (**C**) CAPN2 and CAPNS1ΔG1 were co-purified by sequential column chromatography, using DEAE-Toyopearl, HisTrap and MonoQ columns. Active peak fractions at each purification step were analysed by SDS–PAGE. M, protein marker; T, total lysate; S, soluble lysate; P, insoluble fraction; DE, His and MQ, active peak fractions from the DEAE-Toyopearl, HisTrap and MonoQ columns, respectively. (**D**) Casein zymography of the active peak fraction eluted from MonoQ column. One microgram of the peak fraction (lane 1), 1 μg of recombinant human m-calpain purified by the Sf9/vaculovirus expression system (lane 2), and 25 μg of non-transfected COS7 cell lysate (lane 3) were subjected to casein zymography as described in 'Material and Methods' section.

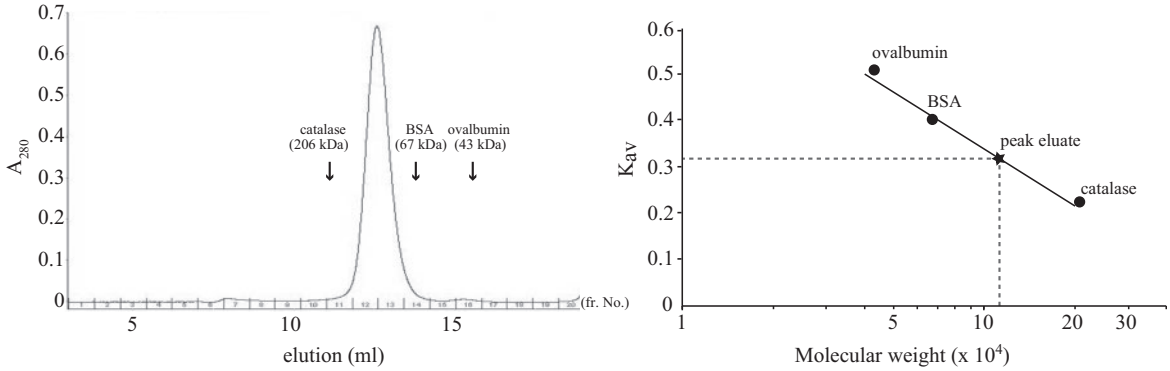

Fig. 3 Elution profile of purified CAPN2 and CAPNS1ΔG1 from a HiLoad 16/10 Superdex 200 gel filtration column. (Left) The solid line represents A_{280} of the column eluate. (Right) Molecular weight determination of the peak eluate. Values of partition coefficient (K_{av}) were calculated, and the K_{av} values of marker proteins show linear correlation with the logarithmic values of their molecular weights. Molecular weight of the peak eluate was estimated to be 11.0×10^4 from the regression line, which was almost identical to the theoretical total molecular weight of CAPN2 with the His-tag and CAPNS1ΔG1 (107,745).

0.005% bromophenol blue), and run on a 12% polyacrylamide gel containing 2 mg/ml casein at 125 V for 3 h. After electrophoresis, the gel was incubated overnight in Ca^{2+} incubation buffer (50 mM Tris–Cl, pH 7.0, 5 mM $CaCl_2$ and 10 mM DTT), stained with Coomassie Brilliant Blue (CBB) R250, and destained with 10% acetic acid and 30% methanol.

SDS–PAGE and western blotting

SDS–PAGE was performed as previously described (*13*). Samples were added to SDS–PAGE loading buffer (62 mM Tris–Cl (pH 6.8), 143 mM 2-mercaptoethanol, 2% SDS, 0.005% bromophenol blue and 10% glycerol), boiled for 5 min, and analysed on 10 or 12% polyacrylamide gels. The gels were stained with CBB R250. For western blotting, proteins were subjected to SDS–PAGE, transferred to PVDF membranes (Immobilon-P, Millipore Inc., MA, USA), and probed with appropriate antibodies. Signals were detected using a POD immunostain kit (Wako Pure Chemicals Inc.).

Results and Discussion

Expression of recombinant human m-calpain

It was previously reported that fully active, recombinant rat m-calpain was successfully produced at 30°C by bacterial co-expression of CAPN2 with a C-terminal His-tag, and 21 K, CAPNS1 lacking the N-terminal GR domain (amino acid (aa) 1–86) and using the two compatible expression vectors, pET24b(+) and pACpET24, with distinct antibiotic-resistant genes, and replicons (*7*, *8*). We applied this strategy to human m-calpain, although it was reported that, in *E. coli* cells, recombinant human m-calpain formed inclusion bodies that could not be renatured (*9*).

When CAPN2/pET and CAPNS1/pACpET were used in bacterial co-expression, expression levels of CAPNS1 were very low, and the CAPN2 protein was almost insoluble under the tested conditions (Fig. 2A and B). After DEAE column chromatography, the soluble lysate gave CAPN2-enriched, but not active fractions (data not shown). This suggests that the two Gly repeat regions in the GR domain caused a deleterious interaction with the host's post-translational machinery, as in the case of recombinant rat m-calpain containing full-length CAPNS1 (*8*). Therefore, instead of a CAPNS1/pACpET construct, we used truncated forms of CAPNS1, CAPNS1ΔG1/pACpET and CAPNS1ΔG2/pACpET. As shown in Fig. 1, CAPNS1ΔG1/pACpET lacks one Gly repeat (aa 1–25), while CAPNS1ΔG2/pACpET lacks both Gly repeats (aa 1–58), in the GR domain. As a result, the yields and solubility of not only CAPNS1ΔG1 and CAPNS1ΔG2 but also CAPN2 showed similar improvement at a lower growth temperature (22°C), after adding IPTG. In addition, the C-terminal His-tag fused to CAPN2 was not truncated during protein expression (Fig. 2A and B). We therefore co-expressed CAPNS1ΔG1, the closest to native form, with CAPN2, for large-scale purification.

Purification of recombinant human m-calpain

As described in 'Material and Methods' section, purification of co-expressed CAPN2+CAPNS1ΔG1 was performed by sequential column chromatography, using DEAE-Toyopearl, HisTrap and MonoQ columns. At each column step, the eluted fractions were monitored for calpain activity by caseinolytic assay. Calpain activity was detected in the fractions containing 200–250 mM NaCl from the DEAE column, 50–125 mM imidazole from the HisTrap column, and 450 mM NaCl from the MonoQ column. SDS–PAGE analysis of the peak active fractions eluted from each column, indicates that CAPN2 and CAPNS1ΔG1 were co-eluted and almost homogeneously purified (Fig. 2C). The final preparation gave a clear band at the same position as control samples in casein zymography (Fig. 2D). Gel filtration analysis of the final preparation showed a single elution peak at a position estimated to be 110 kDa, which is almost identical to theoretical total molecular weight of CAPN2 with the His-tag and CAPNS1ΔG1 (Fig. 3). These results indicate that human CAPN2 and CAPNS1ΔG1 formed a heterodimer without high molecular weight aggregates or dissociated molecules. The results of purification are summarized in Table I. From 1 l of *E. coli* culture, we obtained 5.8 mg of purified recombinant human m-calpain, with a yield of 51.1%, which was higher than that of recombinant human m-calpain obtained from a Sf9/baculovirus expression system (*9*). This protein was stable for several months. The final preparation showed a half maximal activity at ~0.5 mM Ca^{2+}, and a specific activity of 334 U/mg for casein, and Km and Kcat values for Suc-LLVY-MCA were

Table I. Purification of recombinant human m-calpain.

Fraction	Volume (ml)	Total proteins (mg)	Total activity ($U \times 10^3$)	Specific activity (U/mg)	Yield (%)	Purification ratio (fold)
Total	50	625	3.78	6	100	1
Sup	45	464	3.51	8	92.9	1.30
DEAE	25	42.5	3.47	82	91.7	13.6
HisTrap	16.4	13.8	2.30	166	60.8	27.7
MonoQ	3	5.80	1.93	334	51.1	55.6

Sup, soluble lysate.

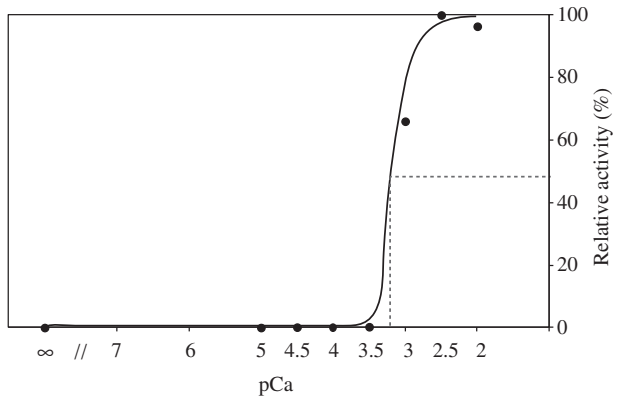

Fig. 4 Ca^{2+}-requirement of the purified recombinant human m-calpain. Purified recombinant human m-calpain was incubated at 30°C in the presence of varying concentrations of Ca^{2+} as described in the 'Materials and Methods' section. Activity was standardized by defining the value of pCa = 2.5 as 100%. The Ca^{2+} concentration for half maximal activity was ~0.5 mM.

determined as 0.322 mM and 0.066 sec^{-1}, respectively (Fig. 4). These values are similar to those reported previously for recombinant or native m-calpain (*9–11*, *14*), indicating that the N-terminal 25 amino acid truncation of CAPNS1 did not affect enzymatic properties of intact calpain, such as heterodimerization, specific activity, and the Ca^{2+}-requirement.

In conclusion, we have established a highly efficient bacterial expression and purification system for recombinant human m-calpain, a system that is easier to manipulate than the Sf9/baculovirus expression system. Our methodology has the potential to assist researchers with structural and physiological studies. Sustained activation of μ- and m-calpains is associated with various pathological states, making these proteases an obvious potential therapeutic target to reduce or prevent a spectrum of human diseases. Great efforts are underway to develop selective calpain inhibitors, and the use of human calpains rather than mammalian orthologues would be of great benefit in this process. In addition, our expression system and constructs may provide resources for further analysis into the physiological significance of the hydrophobicity exhibited by Gly-repeats in the calpain GR domain.

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Conflict of interest

None declared.

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