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Overexpression, Purification, and Characterization of Human m-Calpain and Its Active Site Mutant, m-C105S-Calpain, Using a Baculovirus Expression System

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Recombinant human m-calpain was produced in a soluble form at a level of 20 mg/liter of Sf-9 cell culture by the coexpression of recombinant human m-calpain large (m80K) and small (30K) subunits using a baculovirus expression system. The expressed m-calpain was purified by sequential column chromatographies on DEAE-Toyopearl, gel-filtration, and Mono Q by the same method used to purify native m-calpain. The recombinant m-calpain had a specific activity of 691 U/mg and a K_a value (Ca^{2+} requirement for 50% caseinolysis activity) of 0.4 mM, which are essentially identical to those of native rabbit m-calpain. A mutant m-calpain large subunit, m-C105S-80K, where the active-site cysteine-105 is converted to serine by site-directed mutagenesis, was coexpressed with 30K in Sf-9 cells, purified, and characterized. m-C105S-calpain does not degrade casein nor an artificial tetra-peptide substrate, succinyl-Leu-Leu-Val-Tyr-MCA. Further, it shows no autolytic activity with Ca^{2+}. This is the first report of the large-scale production of a fully active m-calpain species in the baculovirus system.

Key words: baculovirus, m-calpain, mutant, overexpression, Sf-9 cell.

Calpain is an intracellular calcium-dependent protease widely expressed in the cytosol of higher animals and, thought to play an important role in various Ca^{2+}-regulated biological functions in cells (*1-5*). There are two major calpains, μ-calpain and m-calpain, which are activated at μM and mM orders of Ca^{2+} *in vitro*, respectively. They are found ubiquitously in animal tissues and purified as a heterodimer comprising of a large distinct catalytic 80K subunit and a small common regulatory 30K subunit.

The large-scale production of target proteins is essential to study structure-function relationships. The overexpression of active calpain species for such purposes has just started in recent years. Recombinant active rat m-calpain has been produced using an *Escherichia coli* expression system (*6*), and recombinant active human μ-calpain has been produced in a baculovirus expression system (*7*). The establishment of an overexpression system makes it possible to analyze protein structures by X-ray crystallography, and to study various properties, mainly by mutagenesis. Moreover, novel calpain species known only at the mRNA level, such as p94 (*8*), nCL-2, and nCL-2′ (*9*), can be studied at the protein level. In this paper, we report the overproduction of recombinant m-calpain as an active form in a baculovirus expression system, as well as its purification and characterization.

MATERIALS AND METHODS

Materials—Casein was purchased from Merck (Germany). Native m-calpain was purified from rabbit skeletal muscle by sequential column chromatographies on DE52, gel-filtration, and MonoQ by the method described by Inomata *et al.* (*10*). Succinyl-Leu-Leu-Val-Tyr-methylcoumarine amide (Suc-LLVY-MCA) was purchased from Peptide Institute (Osaka). pBlueBac III and linear *Autographa californica* nuclear polyhedrosis virus (AcMNPV) were purchased from Invitrogen (San Diego, CA, USA). Restriction enzymes were purchased from Takara Shuzo (Osaka).

Preparation of a Baculovirus Transfer Vector with Human m-Calpain Large Subunit—The human m-calpain large subunit (hm80K) cDNA in pTV119 (*11*) was digested with *Nco*I and *Hin*dIII endonucleases. The hm80K fragment was isolated and cloned into *Nco*I and *Hin*dIII-cut pBlueBac III to make pBlueBac III/hm80K.

Preparation of a Baculovirus Transfer Vector with Human m-C105S-Calpain Large Subunit—To make a recombinant human m-calpain large subunit with an active center serine in place of cysteine, an oligonucleotide with a mutated serine in its active center and a restriction enzyme

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Abbreviations: SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; m-C105S-calpain, m-calpain whose active center cysteine is changed to serine; m80K, m-calpain large subunit; 30K, calpain small subunit; AcMNPV, *Autographa californica* nuclear polyhedrosis virus; MOI, multiplicity of infection; Suc-LLVY-MCA, succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide.

site (*Pvu*II site) (CTAGGTGACAGCTGGCTGCTG) was synthesized by a DNA synthesizer. Mutation of the active site cysteine residue of human m80K was performed by Kunkel's method (*12*). The introduction of the mutated sequence was verified by DNA sequencing. The resulting vector (pTV119/hm-C105S-80K) was subcloned into pBlueBac III in the same way as for the construction of pBlueBac III/hm80K. The recombinant hm80K virus and hm-C105S-80K virus were expected to have seven additional residues (MGRRDRS) at the N-terminus, but these additional residues appeared not to affect the enzymatic activity.

Preparation of a Baculovirus Transfer Vector with Human Calpain Small Subunit—The human calpain small subunit (h30K) cDNA in pBR322 (*13*) was digested with *Sma*I and *Pst*I endonucleases. The h30K fragment was isolated and cloned into *Pst*I-cut, Klenow fragment-treated, and *Hin*dIII-cut pBlueBac III.

Cell Culture—Sf-9 cells (an ovarian cell line of the fall armyworm *Spodoptera frugiperda*) were obtained from Dr. T. Nishino (Nippon Medical School). The cells were maintained in an IPL-41 insect medium (GIBCO-BRL, USA), pH 6.1, supplemented with 0.35 g/liter $NaHCO_3$, 2% tryptose phosphate broth (GIBCO-BRL), 9% heat-inactivated fetal bovine serum (Filtron Pty., Australia), 50 μg/ml gentamicin sulfate (Wako Pure Chemical Industries, Tokyo), and 0.1% Pluronic F-68 (GIBCO-BRL) at 27°C with constant shaking (110–140 rpm).

Preparation of Recombinant Virus—Sf-9 cells were cotransfected with inserted pBlueBac III and linear wild-type AcMNPV in IPL-41 without fetal bovine serum for recombination *in vivo*. A single blue plaque containing recombinant baculovirus was isolated from white plaques containing wild-type virus by plaque assay. High titer stock was obtained by infection of Sf-9 cells. From three clones (pBlueBac III/hm80K, pBlueBac III/hm-C105S-80K, pBlueBac III/h30K), three recombinant baculoviruses were obtained.

Expression of Recombinant Human m-Calpain and Its Mutant—Sf-9 cells were cultured in IPL-41 supplemented with 9% fetal bovine serum, 2% tryptose phosphate broth, 0.1% Pluronic F-68, and 50 μg/ml gentamicin. Cells were maintained at 27°C. Exponentially growing cultures were co-infected with m80K or m-C105S-80K virus and 30K virus at a multiplicity of infection (MOI) = 1 at room temperature with occasional rocking for 1 h at a density of 1×10^7 cells/ml. The cells were further incubated for 44–47 h at a density of 1×10^6 cells/ml at 27°C.

Purification of Recombinant Human m-Calpain and m-C105S-Calpain—The cells were harvested by centrifugation ($300 \times g$), and the pellets were suspended in buffer A [20 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 10 mM 2-mercaptoethanol]. The cell suspension was subjected to a French Press at 900 p.s.i. at 4°C. The cell lysate was then ultracentrifuged at $100,000 \times g$ for 1 h. The supernatant was loaded onto a DEAE-Toyopearl anion-exchange chromatography column (1.3×9.5 cm, Tosoh), and eluted with a linear gradient from 0 to 500 mM NaCl at a flow rate of 2 ml/min. Active fractions were pooled and loaded onto a gel filtration column after concentration by ultrafiltration. The Superdex 200 column (1.6×60 cm, 1 ml/min; Pharmacia) was connected to an FPLC system (Pharmacia), and m-calpain was eluted with buffer A containing 0.15 M NaCl. The active fractions were loaded onto a MonoQ anion-exchange column (1.3×10 cm, Pharmacia) and elution was performed using a linear gradient from 0 to 500 mM NaCl at a flow rate of 2 ml/min.

Protein Determination—Protein concentrations were determined by the method of Bradford (*14*) with bovine serum albumin as a standard.

Enzyme Activity Assays—Calpain activity was assayed using alkali-denatured casein (*15*) or Suc-LLVY-MCA (*16*) as a substrate.

SDS-PAGE—Samples were added to an SDS-PAGE loading buffer (62 mM Tris-HCl, pH 6.8, 143 mM 2-mercaptoethanol, 2% SDS, 0.005% bromophenol blue, 10% glycerol), boiled for 5 min, and analyzed in 10% polyacrylamide gels (*17*). The gels were stained with Coomassie Brilliant Blue R250.

Autolysis of m-C105S-Calpain—m-C105S-calpain was incubated in the presence of 5 mM Ca^{2+} for various periods, and aliquots were withdrawn for analyses.

Trypsin Digestion—Reaction mixtures (8 μl) containing 0.2 μg trypsin and 10 μg m-calpain or m-C105S-calpain in 50 mM Tris-HCl, pH 7.5, were allowed to digest at 25°C for 0, 5, 30, or 90 min. The reactions were stopped by the addition of the same volume of SDS-sample buffer.

RESULTS

Expression of Recombinant m-Calpain—Initially we ligated a full-length cDNA fragment of the human 80K subunit with baculovirus transfer vector, pBlueBac III, which contains the polyhedrin promotor and β-galactosidase gene for blue-plaque selection as shown in Fig. 1A. The resulting construct (pBlueBac III/hm80K) was then used to obtain a recombinant virus (AcMNPV/hm80K) formed *in vivo* by homologous recombination between the pBlueBac III/hm80K vector and linear wild type AcMNPV DNA. A single blue plaque containing the target virus was picked up and amplified. The purity of the virus is, therefore, expected to be guaranteed. In the same way we prepared recombinant viruses of human m-C105S-80K and 30K. As shown in Fig. 1B, hm80K and hm-C105S-80K have an additional seven amino acid residues at the N-terminus. Expression of recombinant m-calpain was accomplished by co-expression of the catalytic m80K subunit and 30K subunit at MOI 1. Western blotting analysis (data not shown) of the sample obtained from the DEAE-Toyopearl column chromatography step showed 30K to be expressed at a higher level than 80K. Caseinolysis activity was detected in fractions in which both 80K and 30K subunits were present. It has been reported that both m80K and μ/m80K have a full protease activity in the absence of regulatory 30K when renatured *in vitro* (*18*). A baculovirus expression system is useful for examining this previous *in vitro* result *in vivo*. The m80K virus was used to infect Sf-9 cells without the 30K virus. The supernatant of Sf-9 cells recovered 47 h after infection was loaded onto DEAE-Toyopearl and eluted with a 0 to 0.5 M NaCl gradient. m80K was detected in the fractions eluting at 270 mM NaCl, as judged from the Western blotting analysis, but no caseinolytic activity could be detected. We examined the N-terminal structure of the m80K expressed with or without 30K. The presumed first methionine was eliminated and a sequence starting from the second residue, *i.e.*,

GRRDRSM, was detected by an amino acid sequencer in both cases.

Purification of Recombinant m-Calpain—Purification of recombinant m-calpain was accomplished by sequential column chromatographies on DEAE-Toyopearl, gel-filtration, and Mono Q in the same way as for native m-calpain. The SDS-PAGE gel pattern shown in Fig. 2 indicates that m-calpain was efficiently purified at the DEAE-Toyopearl step. From 50 ml of infected suspension culture, 0.9 mg m-calpain was purified. The results of m-calpain purification are summarized in Table I together with those of its active-site mutant. Based on the results of gel filtration and SDS-PAGE analysis, the preparation was found to contain a heterodimer consisting of m80K and 30K subunits. The final m-calpain preparation has a specific activity of 691 U/mg (Table I). This value is essentially identical to or even higher than that of rabbit m-calpain (*19*). As shown in Fig. 3, the Ca^{2+} sensitivity of the recombinant human m-calpain is essentially the same as that of native rabbit m-calpain. Recombinant human m-calpain shows a half maximum caseinolytic activity (K_a) at 0.4 mM Ca^{2+}, while native rabbit m-calpain has a K_a value of 0.6 mM Ca^{2+}.

Expression of Recombinant m-C105S-Calpain—Next we constructed a human m-C105S-80K virus and used it to infect Sf-9 cells together with the 30K virus at a MOI of 1. m-C105S-calpain was purified as a heterodimer in the same way as m-calpain, as shown in Table I. The final yield from 50 ml of infected suspension culture was estimated to be 0.8 mg, which was nearly identical to that of the native form. Purified recombinant m-C105S-calpain, however, did not cleave casein or the artificial tetra-peptide Suc-LLVY-MCA. The autolysis of m-C105S-calpain was also

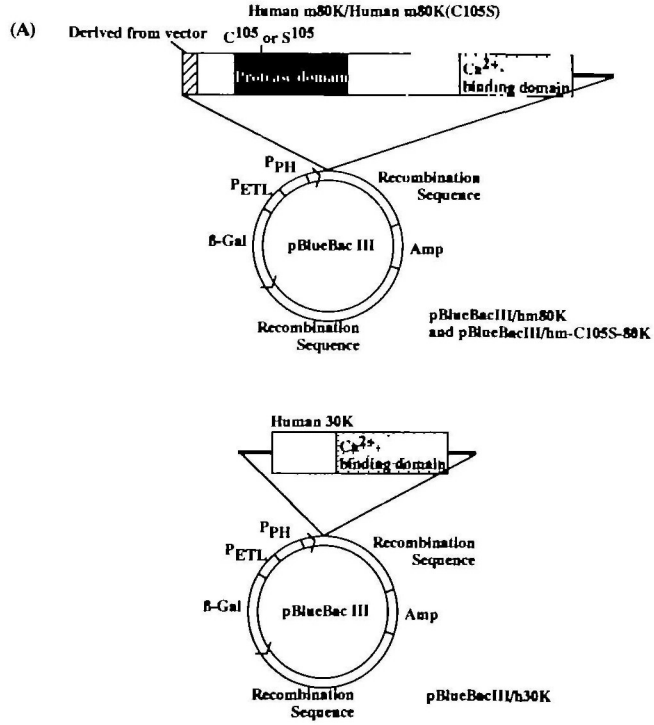

Fig. 1. **Representation of recombinant plasmid transfer vectors.** (A) The human m80K, human m-C105S-80K, and human 30K cDNAs were inserted into a baculovirus transfer vector plasmid, pBlueBac III. (B) Presumed amino acid sequence of the N-terminal region of the expressed proteins, 80K and m-C105S-80K for (a) and 30K for (b). The box indicates the additional seven amino acid residue sequence at the N-terminus derived from the vector.

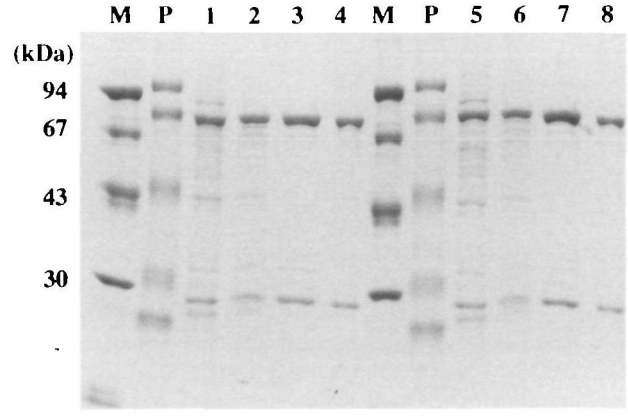

Fig. 2. **Purification of recombinant m-calpain and m-C105S-calpain.** Expressed m-calpain was purified by sequential column chromatographies on DEAE-Toyopearl, gel-filtration, and Mono Q. A pooled fraction at each purification step was analyzed by SDS-PAGE and the gel was stained with Coomassie Brilliant Blue R250. M, low molecular markers; P, prestained low molecular markers; 1-4, recombinant m-calpain; 5-8, recombinant m-C105S-calpain; 1, 5, pooled fractions from the DEAE-Toyopearl column; 2, 6, pooled fractions from the gel filtration column; 3, 7, pooled fractions from the Mono Q column; 4, 8, native rabbit m-calpain as a control.

TABLE I. **Purification of recombinant m-calpain and m-C105S-calpain.** Recombinant human m-calpain was purified from Sf-9 cells co-infected with recombinant m80K and 30K viruses 48 h after infection. Human m-C105S-calpain was purified from Sf-9 cells co-infected with m-C105S-80K and 30K viruses 48 h after infection.

	Volume (ml)	Total protein (mg)	Total activity (U)	Yield (%)	Specific activity (U/mg)	Purification (fold)
m-Calpain						
DEAE-Toyopearl	5.2	5.20	1680	100	323	1.0
Gel-filtration	10.0	1.90	1140	68	600	1.9
Mono Q	4.0	0.92	636	38	691	2.1
m-C105S-calpain						
DEAE-Toyopearl	5.2	5.70	–	–	–	–
Gel-filtration	10.0	2.20	–	–	–	–
Mono Q	3.0	0.78	0	–	0	–

Starting material was 5×10^7 infected Sf-9 cells (50 ml culture).

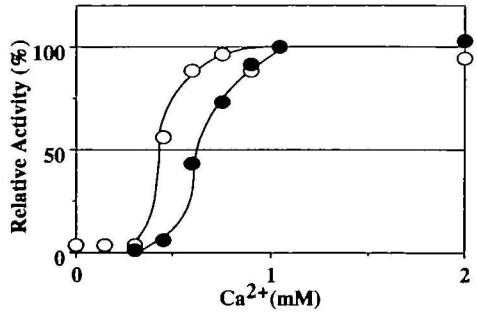

Fig. 3. **Ca^{2+} sensitivity of recombinant human m-calpain.** Recombinant m-calpain was assayed at 30°C in the presence of various concentrations of Ca^{2+} as described in "MATERIALS AND METHODS." ○, recombinant human m-calpain; ●, native rabbit m-calpain as control.

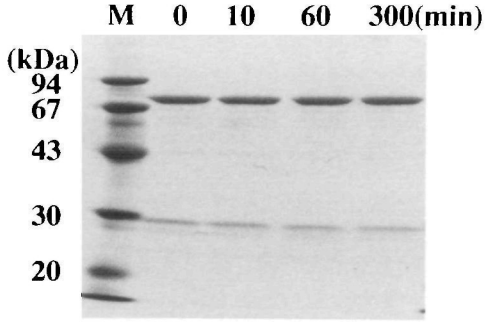

Fig. 4. **Autolysis of m-C105S-calpain.** Recombinant m-C105S-calpain was incubated at 30°C in the presence of 5 mM Ca^{2+} as described in "MATERIALS AND METHODS." Samples were withdrawn at intervals and analyzed by SDS-PAGE.

examined in the presence of 5 mM Ca^{2+}. As shown in Fig. 4, SDS-PAGE indicated that neither m-C105S-80K nor 30K was degraded after a 5-h incubation with 5 mM Ca^{2+}. Taken together, the results indicate that m-C105S-calpain is totally inactive.

To obtain additional information about the conformational difference between m-calpain and m-C105S-calpain, both were digested with trypsin and analyzed by SDS-PAGE. As shown in Fig. 5, the degradation patterns were almost identical, suggesting that the conformations of both preparations in the absence of Ca^{2+} are essentially the same.

DISCUSSION

In this work we accomplished the overexpression of a fully active recombinant m-calpain using a baculovirus expression system. It has recently been reported that the other type of calpain, human μ-calpain, was produced using the same system and that the purified μ-calpain was fully active (*7*). These results clearly indicate that the baculovirus expression system is very useful for the large-scale production of fully active μ- and m-calpains and their various mutants. Such overexpression is indispensable for analyzing the structure, structure-function relationship, and physiological functions of calpain, which usually require large amounts of sample.

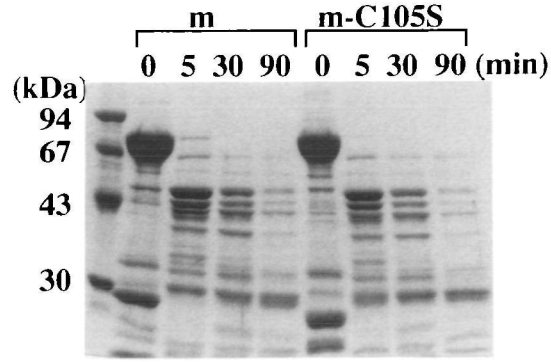

Fig. 5. **Degradation of m-calpain and m-C105S-calpain by trypsin.** m-Calpain and m-C105S-calpain incubated with trypsin for the indicated periods at 30°C were subjected to SDS-PAGE.

Native human m-calpain was purified from skeletal muscle and characterized (*20*). The K_a value was 2.8 mM Ca^{2+} and the specific activity was 340 U/mg under slightly different assay conditions. In this work, native human m-calpain, rather than native rabbit m-calpain, should be used for comparison, but since it is difficult to obtain human material, we used rabbit m-calpain instead. Recombinant human m-calpain has properties similar to those of native rabbit m-calpain with respect to Ca^{2+} sensitivity (Fig. 3), caseinolytic activity and fragmentation pattern following trypsin digestion (Fig. 5). The recombinant protein had an optimal pH of 7.8 in Tris-acetate buffer (data not shown). Considering the species difference, it can be concluded that the m-calpain prepared in a baculovirus-based expression system is essentially the same as native m-calpain from the same source.

We overexpressed m80K without 30K and partially purified it (data not shown) by chromatography on DEAE-Toyopearl and gel-filtration columns. We detected the expressed m80K in fractions eluting from DEAE-Toyopearl by Western blot analysis, but no casein degradation activity was detected. Moreover, the m80K eluted in the void volume from the gel-filtration column, which probably means that it forms large aggregates. Although the additional N-terminal seven amino acid residues might have some effect on refolding, the effect should not be significant because the same m80K construct expressed with 30K showed full enzyme activity. Presumably 30K is necessary for refolding enzymatically active 80K, although 80K alone can express full proteolytic activity under the proper conditions (*18*). In the case of μ-calpain, 80K expressed without 30K has only weak activity (~40%) compared to the heterodimeric enzyme against succinyl-Leu-Tyr-4-methoxy-2-naphthylamine (Suc-Leu-Tyr-MNA) (*7*), supporting the hypothesis that 30K is necessary for refolding μ80K to the proper conformation with full enzyme activity.

We produced an active-site mutant of m-calpain, m-C105S-calpain, where the active-site cysteine was replaced with serine. If this serine mutant is active, calpain can be converted to a serine protease and, if not, the mutant can be used to examine the effect of Ca^{2+} on m-calpain, because it should be stable in the presence of Ca^{2+}. In the case of papain, the active-site serine mutant is partly active (*21*) and a similar serine mutant of ER60, a cysteine proteinase in the endoplasmic reticulum, is also active (*22*). The m-

calpain serine mutant, however, showed no proteolytic activity. No autolysis occurred in the presence of Ca^{2+}, *i.e.*, both the m80K and 30K subunits were completely stable even during prolonged incubation with Ca^{2+}. m-C105S-calpain is, therefore, a promising candidate with which to analyze the structure of m-calpain in the presence of Ca^{2+}. Recently, the structure of the Ca^{2+}-binding domain of the small subunit overexpressed in *E. coli* was reported (*23, 24*). The results revealed that the Ca^{2+}-binding domain forms a homodimer in the absence of Ca^{2+} and that the fifth, most C-terminal EF-hand structure is responsible for the dimerization. This result suggests that both fifth EF-hand structures of the large and small subunits are involved in the formation of the dimeric structure of calpain. m-C105S-calpain is useful for analyzing the structure of the Ca^{2+}-binding domains of 80K and 30K and thus it will help to elucidate the activation mechanism of calpain in the presence of Ca^{2+}, because mutant calpain is stable at 4°C, while native calpain degrades very rapidly even at 4°C.

The success in expressing fully active m- and μ-calpains (*7*) should be followed by a similar study involving tissue-specific calpains such as p94, nCL-2, and nCL-2′. Tissue-specific calpains are very important in elucidating the physiological function of calpain, because they are believed to be involved in specific roles in the tissues in which they are expressed. At least five tissue-specific calpains have been identified but only at the cDNA level. The only one identified at the protein level is a skeletal muscle-specific calpain, p94. None has yet been isolated from tissues and their enzymatic and proteinaceous properties have not been analyzed. The baculovirus expression system will be useful for the expression, purification, and characterization of tissue-specific calpains and studies along this line are now in progress.

Graham-Siegenthaler *et al.* reported the expression of the rat m80K subunit as a soluble form in *E. coli*, but the expressed m80K showed no any caseinolytic activity (*6*). We also examined the same *E. coli* system for the expression of human m80K with and without 30K, but the proteins were produced as inclusion bodies that could not be renatured under the various conditions thus far tried.

REFERENCES

1. Mellgren, R.L. (1987) Calcium-dependent proteases: an enzyme system active at cellular membranes? *FASEB J.* **1**, 110-115
2. Wang, K.K.W., Villalobo, A., and Roufogalis, B.D. (1989) Calmodulin-binding proteins as calpain substrates. *Biochem. J.* **262**, 693-706
3. Suzuki, K., Sorimachi, H., Yoshizawa, T., Kinbara, K., and Ishiura, S. (1995) Calpain: Novel family members activation, and physiological function. *Biol. Chem. Hoppe-Seyler* **376**, 523-529
4. Johnson, P. (1990) Calpains (intracellular calcium-activated cysteine proteinases): Structure-activity relationships and involvement in normal and abnormal cellular metabolism. *Int. J. Biochem.* **22**, 811-822
5. Sorimachi, H., Ishiura, S., and Suzuki, K. (1997) Structure and physiological function of calpains. *Biochem. J.* **328**, 721-732
6. Graham-Siegenthaler, K., Gauthier, S., Davies, P.L., and Elce, J.S. (1994) Active recombinant rat calpain II. *J. Biol. Chem.* **269**, 30457-30460
7. Meyer, S.L., Bozyczko-Coyne, D., Mallya, S.K., Spais, C.M., Bihovsky, R., Kawooya, J.K., Lang, D.M., Scott, R.W., and Siman, R. (1996) Biologically active monomeric and heterodimeric recombinant human calpain I produced using the baculovirus expression system. *Biochem. J.* **314**, 511-519
8. Sorimachi, H., Imajoh-Ohmi, S., Emori, Y., Kawasaki, H., Ohno, S., Minami, Y., and Suzuki, K. (1989) Molecular cloning of a novel mammalian calcium-dependent protease distinct from both m- and μ-types. *J. Biol. Chem.* **264**, 20106-20111
9. Sorimachi, H., Ishiura, S., and Suzuki, K. (1993) A novel tissue-specific calpain species expressed predominantly in the stomach comprises two alternative splicing products with and without Ca^{2+}-binding domain. *J. Biol. Chem.* **268**, 19476-19482
10. Inomata, M., Nomoto, M., Hayashi, M., Nakamura, M., Imahori, K., and Kashima, S. (1984) Comparison of low and high calcium requiring forms of the calcium-activated neutral protease (CANP) from rabbit skeletal muscle. *J. Biochem.* **95**, 1661-1670
11. Imajoh, S., Aoki, K., Ohno, S., Emori, Y., Kawasaki, H., Sugihara, H., and Suzuki, K. (1988) Molecular cloning of the cDNA for the large subunit of the high-Ca^{2+}-requiring form of human Ca^{2+}-activated neutral protease. *Biochemistry* **27**, 8122-8128
12. Kunkel, T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **82**, 488-492
13. Ohno, S., Emori, Y., and Suzuki, K. (1986) Nucleotide sequence of a cDNA coding for the small subunit of human calcium-dependent protease. *Nucleic Acids Res.* **14**, 5559
14. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254
15. Imajoh, S., Kawasaki, H., and Suzuki, K. (1986) Limited autolysis of calcium-activated neutral protease (CANP): Reduction of the Ca^{2+}-requirement is due to the NH_2-terminal processing of the large subunit. *J. Biochem.* **100**, 633-642
16. Sasaki, T., Kikuchi, T., Yumoto, N., Yoshimura, N., and Murachi, T. (1984) Comparative specificity and kinetic studies on porcine calpain I and calpain II with naturally occurring peptides and synthetic fluorogenic substrates. *J. Biol. Chem.* **259**, 12489-12494
17. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
18. Yoshizawa, T., Sorimachi, H., Tomioka, S., Ishiura, S., and Suzuki, K. (1995) A catalytic subunit of calpain possesses full proteolytic activity. *FEBS Lett.* **358**, 101-103
19. Tsuji, S. and Imahori, K. (1981) Studies on the Ca^{2+}-activated neutral proteinase of rabbit skeletal muscle. I. The characterization of the 80K and the 30K subunits. *J. Biochem.* **90**, 233-240
20. Suzuki, K., Ishiura, S., Tsuji, S., Katamoto, T., Sugita, H., and Imahori, K. (1979) Calcium activated neutral protease from human skeletal muscle. *FEBS Lett.* **104**, 355-358
21. Clark, P.I. and Lowe, G. (1978) Conversion of the active-site cysteine residue of papain into a dehydro-serine, a serine and a glycine residue. *Eur. J. Biochem.* **84**, 293-299
22. Urade, R., Oda, T., Ito, H., Moriyama, T., Utsumi, S., and Kito, M. (1997) Functions of characteristic Cys-Gly-His-Cys (CGHC) and Gln-Glu-Asp-Leu (QEDL) motifs of microsomal ER-60 protease. *J. Biochem.* **122**, 834-842
23. Blanchard, H., Grochulski, P., Li, Y., Arthur, J.S.C., Davies, P.L., Elce, J.S., and Cygler, M. (1997) Structure of a calpain Ca^{2+}-binding domain reveals a novel EF-hand and Ca^{2+}-induced conformational changes. *Nat. Struct. Biol.* **4**, 532-538
24. Lin, G., Chattopadhyay, D., Maki, M., Wang, K.K.W., Carson, M., Jin, L., Yuen, P., Takano, E., Hatanaka, M., DeLucas, L.J., and Narayana, S.V.L. (1997) Crystal structure of calcium bound domain VI of calpain at 1.9 Å resolution and its role in enzyme assembly, regulation, and inhibitor binding. *Nat. Struct. Biol.* **4**, 539-547