J. Microbiol. Biotechnol. (2014), 24(5), 719–723
http://dx.doi.org/10.4014/jmb.1312.12034

jmb

Expression of the Pro-Domain–Deleted Active Form of Caspase-6 in *Escherichia coli*

Phil Young Lee[1], Jin Hwa Cho[1], Seung Wook Chi[1], Kwang-Hee Bae[2], Sayeon Cho[3], Byoung Chul Park[1], Jeong-Hoon Kim[1*], and Sung Goo Park[1*]

[1]*Medical Proteomics Research Center, KRIBB, Yusung, Daejeon 305-806, Republic of Korea*
[2]*Cell Function Regulation Research Center, KRIBB, Yusung, Daejeon 305-806, Republic of Korea*
[3]*College of Pharmacy, Chung-Ang University, Seoul 156-756, Republic of Korea*

Received: December 13, 2013
Revised: February 13, 2014
Accepted: February 26, 2014

First published online
February 27, 2014

**Corresponding authors*
S.G.P.
Phone: +82-42-860-4262;
Fax: +82-42-860-4593;
E-mail: sgpark@kribb.re.kr
J.-H.K.
Phone: +82-42-860-4264;
Fax: +82-42-860-4593;
E-mail: jhoonkim@kribb.re.kr

pISSN 1017-7825, eISSN 1738-8872

Caspases are a family of cysteine proteases that play an important role in the apoptotic pathway. Caspase-6 is an apoptosis effector that cleaves a variety of cellular substrates. The active form of the enzyme is required for use in research. However, it has been difficult to obtain sufficient quantities of active caspase-6 from *Escherichia coli*. In the present study, we constructed a caspase-6 with a 23-amino-acid deletion in the pro-domain. This engineered enzyme was expressed as a soluble protein in *E. coli* and was purified using affinity resin. *In vitro* enzyme assay and cleavage analysis revealed that the engineered active caspase-6 protein had characteristics similar to those of wild-type caspase-6. This novel method can be a valuable tool for obtaining active caspase-6 that can be used for screening caspase-6–specific substrates, which in turn can be used to elucidate the function of caspase-6 in apoptosis.

Keywords: Caspase-6, active form, *E. coli*, enzyme assay

Apoptosis, also known as programmed cell death, is an essential mechanism for selectively removing unwanted or damaged cells during development and normal tissue homeostasis [1, 2, 7, 10, 14]. Apoptotic signals are typically accompanied by the activation of caspases, which are cysteine proteases that show specificity toward aspartic acid residues of proteins. Abnormalities in apoptosis have been linked to a variety of diseases, including cancer, neurodegeneration, and autoimmune disorders. It has been widely accepted that inactivation of apoptosis by blocking upstream death signals or inhibition of caspase activity is central to cancer development and the resistance of cells to anticancer agents [4, 5, 11]. Therefore, the catalytic activities of caspases—both inhibition and activation—are of particular interest in developing therapeutic strategies for various diseases. A major requirement for the development of novel therapies targeting caspases is the availability of caspases in sufficient quantities. Caspases are produced as single-chain inactive zymogens, termed procaspases, which consist of a pro-domain, a large 20 kDa subdomain, and a small 10 kDa subdomain. Formation of active caspase requires the precise cleavage of the precursor and the subsequent accurate assembly into an active tetramer composed of two small and two large subdomains [6, 12]. The pro-domain of the initiator caspases contain domains such as a CARD domain or a death effector domain (DED) that enables the caspases to interact with other molecules that regulate their activation. The pro-domain of effector caspases is relatively short and can bind with other proteins regulating their activation [15]. Certain caspases seem to be auto-processed in recombinant hosts, resulting in marginal expression; this could be due to the cytotoxicity of the recombinant proteins [9]. Furthermore, the purified recombinant caspases can undergo spontaneous auto-cleavage(s) during storage. Therefore, efficient expression of either the precursor or the active caspase(s) in *E. coli* is

difficult. To date, several strategies for the expression of caspases in *E. coli* have been reported [8, 13]. Based on the report that the pro-domain of caspase-3 can regulate activation of precursor caspase-3, we tried expressing the pro-domain-deleted form of caspase-6 in *E. coli* [15].

Caspase-3 and caspase-7 are the major apoptosis mediators in cells and are closely related in sequence. These two caspases also have overlapping substrate specificities, with special preference for the DEVD motif. However, they localize to different subcellular locations during apoptosis, and mice deficient in either of these caspases show distinct phenotypes, implying the two caspases are markedly different [3]. Caspase-6 is similar to caspase-3 and -7; however, very little is known about this enzyme. Caspase-6 is an apoptosis effector that cleaves a variety of cellular substrates. The active form of the enzyme is required for use in research. However, it has been difficult to obtain sufficient quantities of the active caspase-6 from *Escherichia coli*. In the present study, we developed an efficient *in vitro* method for the expression of the active form of caspase-6 in *E. coli* and confirmed its activity.

For the expression of active caspase-6 (GenBank Accession No. NM_001226) in *E. coli*, a mutant caspase with a 23-amino-acid deletion at the *N*-terminal was constructed using PCR and was subcloned into the pET21a(+) vector using *Bam*HI and *Xho*I. For comparison of enzyme activity

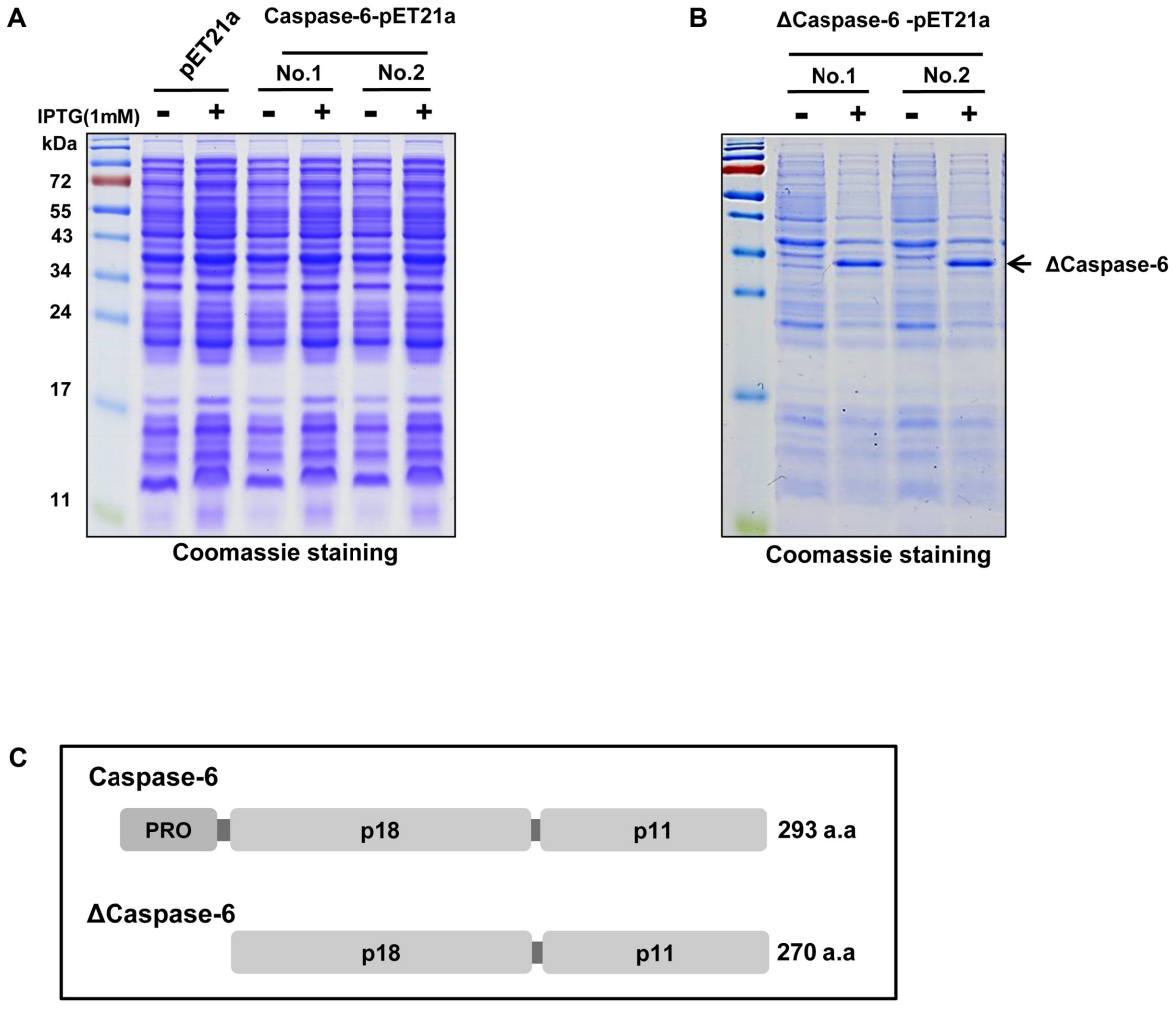

Fig. 1. Expression analysis of Δcaspase-6 in *E. coli.*

(**A**) Analysis of full-length caspase-6 expression in *E. coli.* (**B**) Pro-domain–deleted caspase-6 expression. (**C**) Domain structure of full-length caspase-6 and pro-domain–deleted caspase-6. The expressed band in the IPTG-treated lane was detected only with the pro-domain-deleted mutant.

and substrate specificity, an inactive mutant of caspase-6 was constructed by substituting an active-site cysteine residue with a serine using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA) according to the manufacturer's instructions. The oligonucleotides used for this mutation were Caspase-6 Forward C163S 5′-TTT ATC ATT CAG GCA AGT CGG GGA AAC CAG CAC-3′, and Caspase-6 Reverse C163S 5′-GTG CTG GTT TCC CCG ACT TGC CTG AAT GAT AAA-3′. The constructs were confirmed by sequencing at Solgent (Korea).

Recombinant Δcaspase-6 and the inactive mutant with a *C*-terminal His-tag were expressed in *E. coli* BL21 Codon Plus (DE3) RIL (Stratagene). In brief, the cells were lysed in the lysis buffer (50 mM NaH_2PO_4, 500 mM NaCl, pH 8.0) with 0.5% sarcosine and EDTA-free protease inhibitor (Roche, Germany). The lysate was clarified by centrifugation at 13,000 rpm and 4°C for 15 min, and the supernatant was incubated with Ni-NTA agarose beads (Qiagen, USA.). After washing, the proteins bound to the agarose beads were eluted with lysis buffer containing 250 mM imidazole. The protein concentration was determined using the Bradford (Bio-Rad, USA) protein assay. Fig. 1 shows that the deletion mutant was induced well, whereas the wild-type caspase-6 was not induced. Fig. 2 shows the results of the protein analysis (SDS-PAGE) at each step of the purification process.

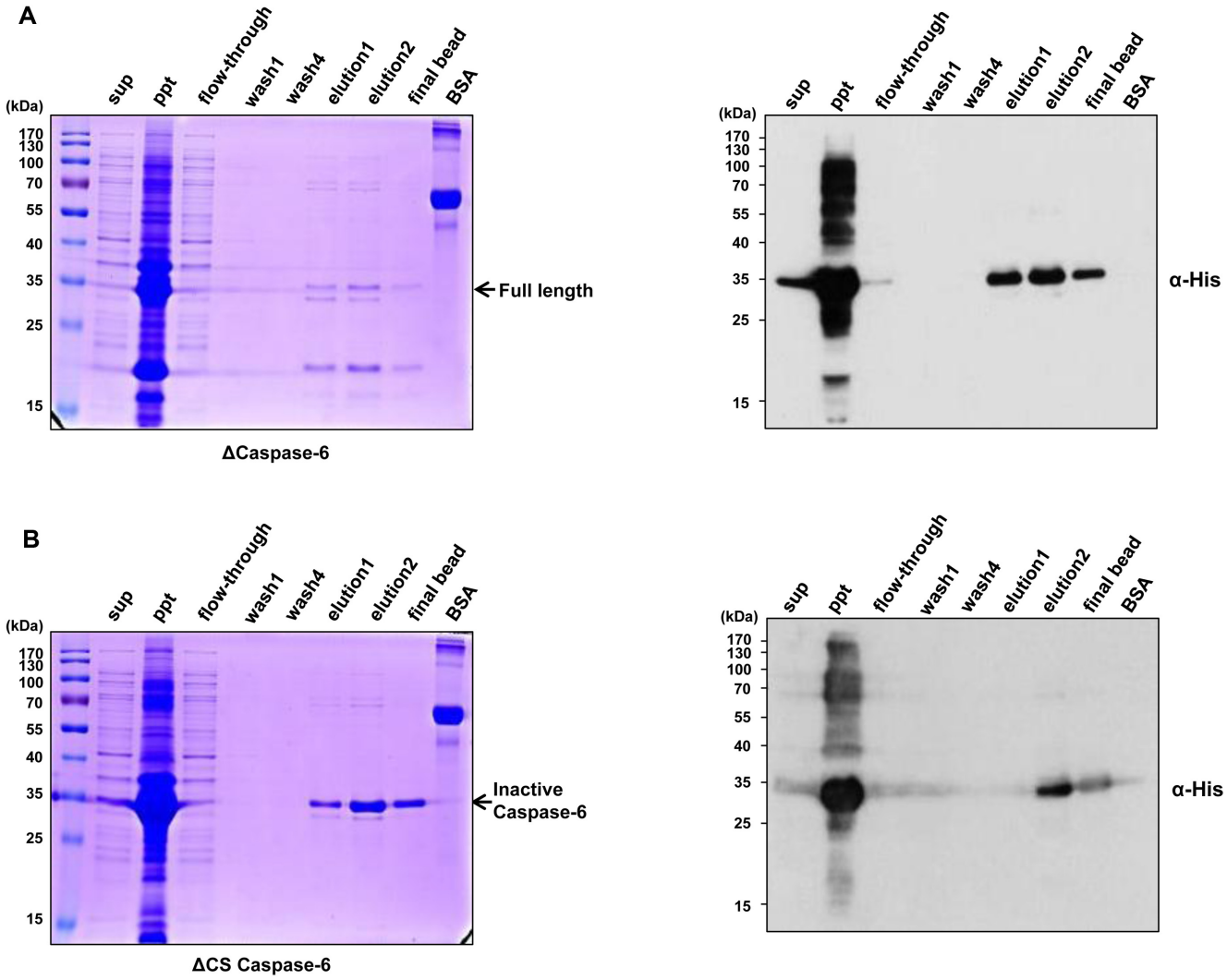

Fig. 2. SDS-PAGE analysis at each step of purification.

(**A**) Purification of Δcaspase-6 expressed in *E. coli*, using Ni-NTA agarose beads. (**B**) Purification of the inactive form caspase-6 from of *E. coli*. The cells were lysed in the lysis buffer and the lysate was clarified by centrifugation. The supernatant (sup) was used for caspase-6 purification. These samples including precipitate (ppt) were detected using western blotting with an anti-His antibody. The arrow indicates caspase-6.

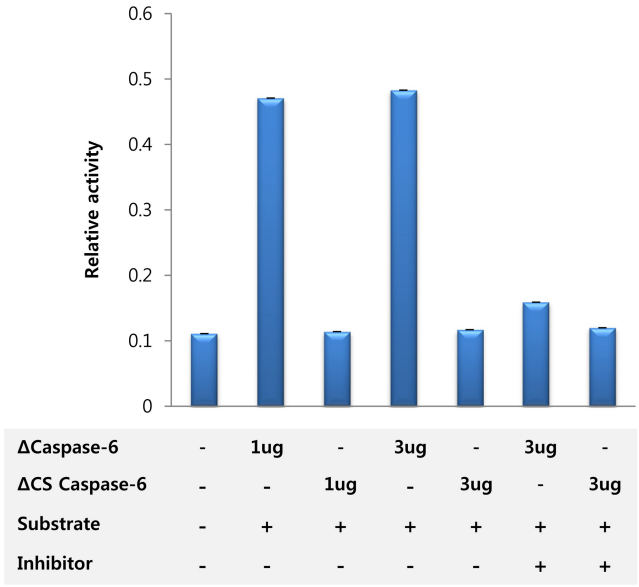

Fig. 3. *In vitro* enzyme activity test for Δcaspase-6: confirmation of Δcaspase-6 enzyme activity with a known substrate, Ac-VEID-pNA.

After purification, the activity of purified Δcaspase-6 and inactive Δcaspase-6 were tested using the substrate Ac-VEID-pNA (200 μM) and the inhibitor z-VEID-FMK (100 μM). Ac-VEID-pNA induced Δcaspase-6 activity, but did not induce the inactive mutant. The activity of caspase-6 was saturated at the amount of 1 μg of caspase-6. The activity of Δcaspase-6 was blocked by z-VEID-FMK (Fig. 3). These results clearly indicated that the active form of Δcaspase-6 had been expressed in *E. coli* and that the inactive mutant had no activity.

For confirmation of Δcaspase-6 activity, we conducted an *in vitro* digestion test using cell lysates. HeLa and KM12SM cells were lysed using NP40 lysis buffer (137 mM NaCl; 20 mM Tris-HCl, pH 8.0; 2 mM EDTA; 10% glycerol; 1% NP40) containing a protease inhibitor (Roche). The cell lysates were incubated with either recombinant Δcaspase-6 or the inactive variant at 37°C for 1 h. The reaction was stopped by the addition of Laemmli buffer, and the lysates were boiled at 100°C for 5 min. Two known substrates of caspase-6, lamin A and tubulin, were detected by western blotting. Lamin A and tubulin were cleaved only by Δcaspase-6 and not by the inactive mutant (Fig. 4).

In this study, we successfully expressed the active form of caspase-6 without the pro-domain in *E. coli*. This recombinant enzyme can be a useful tool for studying the mechanism and interactome of caspase *in vitro*, as well as for screening for novel caspase substrates.

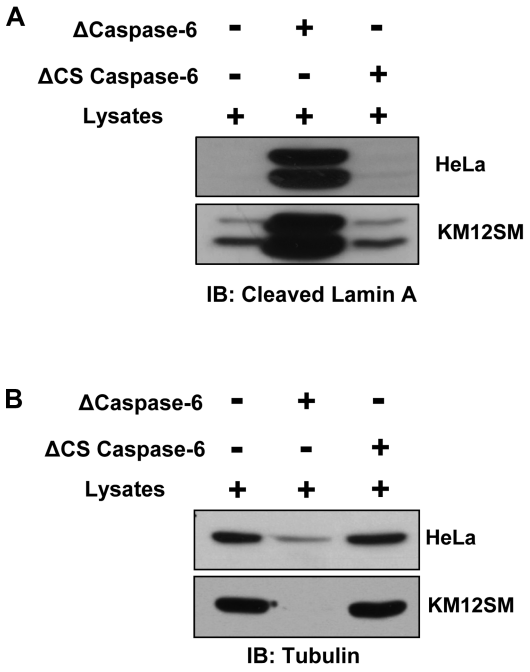

Fig. 4. *In vitro* cleavage assay.
(**A**) HeLa and KM12SM cell lysates (50 μg) were incubated with recombinant active Δcaspase-6 and inactive caspase-6 for 1 h at 37°C. Lamin A cleavage was detected by western blotting using an antibody against cleaved lamin A. (**B**) Tubulin cleavage was detected by western blotting using an anti-tubulin antibody.

Acknowledgments

This work was supported by KRIBB and research grants from the National Research Foundation of Korea (NRF-2011-0028172).

References

1. Brown JM, Attardi LD. 2005. The role of apoptosis in cancer development and treatment response. *Nat. Rev. Cancer* **5:** 231-237.
2. Ekici OD, Li ZZ, Campbell AJ, James KE, Asgian JL, Mikolajczyk J, *et al*. 2006. Design, synthesis, and evaluation of azapeptide Michael acceptors as selective and potent inhibitors of caspases-2, -3, -6, -7, -8, -9, and -10. *J. Med. Chem.* **49:** 5728-5749.
3. Jang M, Park BC, Kang S, Lee DH, Cho S, Lee SC, *et al*. 2008. Mining of caspase-7 substrates using a degradomic approach. *Mol. Cells* **26:** 152-157.
4. Lamkanfi M, Declercq W, Kalai M, Saelens X, Vandenabeele P. 2002. Alice in caspase land. A phylogenetic analysis of caspases from worm to man. *Cell Death Differ.* **9:** 358-361.

5. Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC. 1994. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* **371:** 346-347.
6. Lee AY, Park BC, Jang M, Cho S, Lee DH, Lee SC, *et al*. 2004. Identification of caspase-3 degradome by two-dimensional gel electrophoresis and matrix-assisted laser desorption/ ionization-time of flight analysis. *Proteomics* **4:** 3429-3436.
7. Letai AG. 2008. Diagnosing and exploiting cancer's addiction to blocks in apoptosis. *Nat. Rev. Cancer* **8:** 121-132.
8. Linton SD. 2005. Caspase inhibitors: a pharmaceutical industry perspective. *Curr. Top. Med. Chem.* **5:** 1697-1716.
9. Loetscher H, Niederhauser O, Kemp J, Gill R. 2001. Is caspase-3 inhibition a valid therapeutic strategy in cerebral ischemia? *Drug Discov. Today* **6:** 671-680.
10. Mattson MP. 2000. Apoptosis in neurodegenerative disorders. *Nat. Rev. Mol. Cell Biol.* **1:** 120-129.
11. Miura M, Zhu H, Rotello R, Hartwieg EA, Yuan J. 1993. Induction of apoptosis in fibroblasts by IL-1β-converting enzyme, a mammalian homolog of the *C. elegans* cell death gene *ced-3*. *Cell* **75:** 653-660.
12. Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, *et al*. 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **376:** 37-43.
13. Schimmer AD, Dalili S, Batey RA, Riedl SJ. 2006. Targeting XIAP for the treatment of malignancy. *Cell Death Differ.* **13:** 179-188.
14. Taylor RC, Cullen SP, Martin SJ. 2008. Apoptosis: controlled demolition at the cellular level. *Nat. Rev. Mol. Cell Biol*. **9:** 231-241.
15. Voss OH, Batra S, Kolattukudy SJ, Gonzalez-Mejia ME, Smith JB, Doseff AI. 2007. Binding of caspase-3 prodomain to heat shock protein 27 regulates monocyte apoptosis by inhibiting caspase-3 proteolytic activation. *J. Biol. Chem.* **282:** 25088-25099.