pC6-2/caspase-6 system to purify glutathione-*S*-transferase-free recombinant fusion proteins expressed in *Escherichia coli*

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Glutathione-*S*-transferase (GST) fusion protein expression vectors are often employed for the expression and purification of proteins in *Escherichia coli*. GST is then removed by site-specific proteolysis using thrombin. However, the presence of internal thrombin cleavage sites in expressed proteins can severely affect the purification of intact proteins. Cysteine-dependent aspartate-specific proteases (caspases) are efficient enzymes with defined substrate specificity. Unlike most of the proteases used for the removal of affinity tags, caspases do not leave any amino acids at the amino-terminus of cleaved proteins. We have engineered the caspase-6 site VEMD in a pGEX vector to give the pC6-2 vector. The caspase-6 can be easily removed after cleavage. Here, we describe the detailed protocol for purifying proteins using our pC6-2/caspase-6 expression and purification system. The cleavage by caspase-6 occurs in <30 min and the entire procedure can be completed in 2 d.

INTRODUCTION

Glutathione-*S*-transferase (GST) fusion protein expression vectors are widely used for the high-level expression and subsequent purification of proteins in *Escherichia coli*[1,2]. Glutathione sepharose-bound GST fusion proteins can be eluted competitively using reduced glutathione, under conditions mild enough not to affect the antigenicity and activity of expressed proteins. However, GST is a 26-kDa relatively bulky tag that might affect the functional and structural properties of the protein under investigation, making its removal highly desirable. The pGEX vectors contain thrombin, factor Xa or PreScission protease recognition and cleavage sites downstream of GST. Among these, thrombin is widely used to remove the GST tag. However, the presence of internal thrombin cleavage sites in the expressed protein can cause difficulties when using this vector system for the expression and purification of intact proteins[3,4]. Additionally, relatively extended incubation times (4–16 h) are required to ensure complete cleavage by all three of the proteases listed above. Sensitive proteins might lose their biological activity during such prolonged incubation periods. Most of the proteases used for the removal of various affinity tags leave one or two amino acids at the amino (N)-terminus of the cleaved protein, which might affect its structural and functional properties. To circumvent these limitations in existing technology, we engineered an expression vector containing a caspase-6 cleavage site (**Fig. 1**). This site, which we termed as C6, is rarely found in naturally occurring proteins, cleaves the carboxy (C)-terminal to its short recognition sequence leaving no residual amino acids at the N terminus of the protein of interest and can yield cleaved product in a relatively short period (<1 h). The protease caspase-6 used for the removal of tags is expressed and purified as a histidine-tagged recombinant protein and can be easily removed using nickel affinity chromatography, otherwise it remains as a contaminant in the protein preparation (schematically depicted in **Fig. 2**).

Cysteine-dependent aspartate-specific proteases (caspases) are efficient enzymes with defined substrate specificity[5,6]. The recognition sequences for all caspases consist of four amino acids with aspartate at the last (P1) position[5,6]. Caspases have been cloned, affinity purified and characterized extensively[7]. We therefore reasoned and demonstrated that engineering a caspase site might be useful in the removal of tags. Special AT-rich binding protein 1 (SATB1) is a T-cell-enriched matrix-attachment region (MAR)-binding protein that is cleaved by caspase-6 at the ^{251}VEMD^{254} site early during thymocyte apoptosis[8]. As our laboratory is interested in understanding the role of SATB1 in the regulation of chromatin

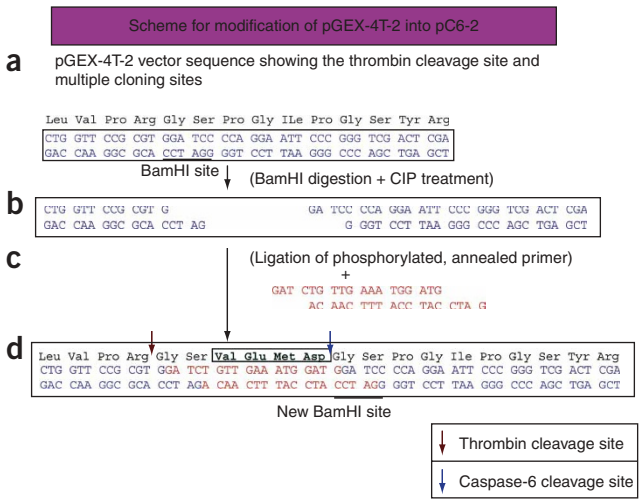

Figure 1 | Schematic representation of the strategy employed to construct the pC6-2 vector from the parent pGEX-4T-2 vector. (**a**) DNA sequences surrounding the MCS region of pGEX-4T-2 are depicted. (**b**) Restriction digestion of pGEX-4T-2 with BamHI generates the sticky ends as depicted. (**c**) Casp6F and Casp6R primers upon annealing generate the BamHI cohesive ends; therefore, they can be ligated directly into the BamHI digested vector. (**d**) The DNA sequence of the pC6-2 vector at the MCS region upon ligation of the Casp6F and Casp6R annealed oligonucleotides into the pGEX-4T-2 is depicted. One of the BamHI sites is lost but that at the MCS region is regenerated in the pC6-2 vector.

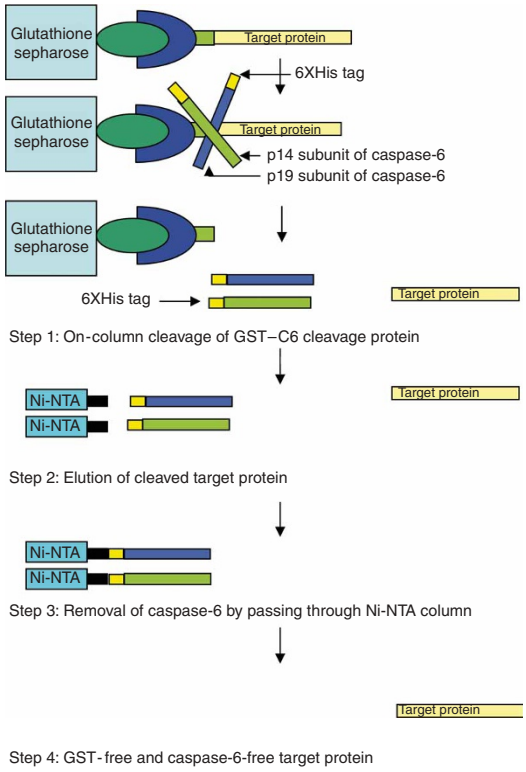

Figure 2 | Schematic representation of on-column cleavage of the GST–C6 fusion protein by caspase-6. Step 1 involves washing and equilibration with caspase buffer, after which the column bound GST–C6 fusion protein is incubated with affinity-purified caspase-6 to allow cleavage for 15–30 min. Step 2 involves elution of the caspase-6-cleaved protein using two to five volumes of buffer X; the eluted sample contains traces of caspase-6. Step 3 involves the removal of caspase-6 after passing the buffer-exchanged caspase-6-cleaved sample through an Ni-NTA column; caspase-6 is retained on the column. In Step 4, GST-free and caspase-6-free target protein is collected as flow-through.

architecture and gene expression, we chose this site from SATB1 for the design of the vector, although other variant caspase-6 recognition sequences (such as VEID) also work equally well.

We have shown that the caspase-6 cleavage site works in a more efficient and specific manner than the typically employed factor Xa or thrombin cleavage site. We engineered a caspase-6 site downstream of the thrombin cleavage site in the pGEX-4T-2 vector (Amersham Biosciences). Two oligonucleotides, casp6F (5′-GATCTGTTGAAATGGATG-3′) and casp6R (5′-GATCCATCC ATTTCAACA-3′), were designed[9] to generate a short synthetic DNA fragment upon annealing. This fragment includes the recognition site for caspase-6 and preserves a downstream BamHI site upon ligation into the BamHI digested pGEX-4T-2 expression vector. The oligonucleotides were designed according to the codon usage in *E. coli* (http://www.kazusa.or.jp/codon/E.html). The annealed oligonucleotide was phosphorylated at the 5′ end and ligated with the BamHI digested and dephosphorylated pGEX-4T-2 vector, which was then used to transform competent *E. coli* strain DH5α cells (Invitrogen). Transformants were screened by PCR using the casp6F primer and a pGEX-4T-2 vector-specific reverse primer[9]. After confirmation of the sequence of the multiple cloning site (MCS) and caspase-6 site, we named the modified pGEX-4T-2 vector as the pC6-2 vector (**Fig. 1**). The plasmid constructs for the cloning and expression of the cDNA of interest (pC6-2), and for the expression of caspase-6, are both available to research groups from non-profit organizations upon request.

TABLE 1 | List of naturally occurring proteins cleaved by caspase-6.

Protein	Reference
SATB1	8
Cytidylyltransferase-α (CCT-α)	23
Transcription factor-activating protein 2α (AP-2α)	24
Transmissible gastroenteritis coronavirus (TGEV) nucleoprotein (N)	25
Lamin A and C	26
Huntingtin	27
5-lipoxygenase	28
Periplakin	29
Notch 1 receptor	30
Tau	31
Guanylate cyclase-α1	32
Desmin	33

Only these proteins cannot be purified in full length by the pC6-2/caspase-6 system.

For several functional studies, we have purified various domains of SATB1 and the N-terminal region of p53 in milligram quantities in our laboratory. We exemplify the use of the pC6-2/caspase-6 system for the purification of two different regions of SATB1, comprising residues 1–204 and 255–763, in this protocol. The first region contains the PDZ domain of SATB1, which is required for the dimerization of SATB1 (ref. 8) and has been experimentally found to possess an internal thrombin site[9]. The second region harbors the MAR-binding domain (MD)[10] and homeodomain (HD)[11]. The system also works efficiently for sarcosyl-solubilized and refolded proteins[9]. We have designed primers with ClaI and SalI linkers, and cloned them into pGEX-4T-2; the new plasmid constructs are designated as pC6-2 ClaI and pC6-2 SalI. To accommodate these changes, the caspase-6 site had to be changed to VEID instead of VEMD, although both work equally well. Using these two restriction sites for cloning, one can eliminate any vector-derived amino acids at the N-terminal. A similar strategy has been employed by Feeney and colleagues[12] for engineering the caspase-3 cleavage site into GST fusions. The specific advantage of using caspase-6 is that, until now, only 12 naturally occurring proteins have been reported as its targets (listed in **Table 1**); by contrast, the list of targets for caspase-3 is many times larger, posing limits on its use in such systems. 6XHis-tagged caspase-6 can be easily purified in sufficient quantities for large-scale use[7]. Processed and purified active caspase-6 is composed of two subunits: p14 and p19 (**Fig. 3**). The cloning strategy described here can be employed to engineer a caspase-6 cleavage site in other affinity tag fusion vectors; hence, caspase-6 can be used for the removal of affinity tags other than GST (P.K.P. and S.G., unpublished data). Here, we describe a detailed protocol under three subheadings: subcloning of gene of interest, purification of caspase-6 and purification of pC6-2-expressed GST-free proteins. Examples of the small number of proteins purified using the pC6-2/caspase-6 system are shown in **Figure 4**. The protocol for the expression and purification of caspase-6 has been described in detail by Stennicke and Salvesen elsewhere[7].

MATERIALS

REAGENTS

- pC6-2 vector construct (Lac promoter-derived expression)
- pET23b-caspase-6 construct (T7 promoter-derived expression; for details, see ref. 7)
- Competent *E. coli* cells, such as XL1 blue (Stratagene, cat. no. 200249), DH5α (Invitrogen, cat. no. 18258-012) and BL21(DE3) (Novagen; efficiency 10^6–10^7 CFU per μg DNA)
- Transformation plate: Luria-Bertani (LB) agar plate (diameter, 14 cm) containing an appropriate antibiotic ▲ CRITICAL While adding antibiotic, the temperature of the media should not exceed 55 °C otherwise it might be inactivated.
- 2× TY broth (1.6% (wt/vol) Bacto tryptone, 1.0% (wt/vol) Bacto yeast extract and 0.5% (wt/vol) NaCl, adjusted to pH 7.0 with NaOH and autoclaved)
- IPTG (Sigma, cat. no. I6758)
- Milli Q water (Millipore) or equivalent grade water
- Buffer A: 100 mM NaCl and 100 mM Tris (pH 8.0)
- Buffer B: 500 mM NaCl, 20 mM imidazole and 100 mM Tris (pH 8.0)
- Reduced glutathione (Amersham Biosciences, cat. no. G4251), prepared as a 20-mM stock in water and stored frozen in aliquots at –20 °C
- RC DC protein assay kit (Bio-Rad, cat. no. 500-0122) to estimate proteins without interference from detergent or reducing agent
- LB broth containing 100 μg ml^{-1} ampicillin (Sigma)
- 6× SDS loading buffer
- Buffer X: 50 mM Tris HCl, 150 mM NaCl, 0.2% (vol/vol) Triton X100, 1 mM DTT and 2 mM PMSF ! CAUTION PMSF is highly toxic and should be handled with gloves and a mask
- Lysis buffer for pC6-2-expressed proteins: 50 mM Tris (pH 8.0), 150 mM NaCl, 0.2 mg ml^{-1} lysozyme, 0.2% (vol/vol) Triton X100 or 0.2% (wt/vol) sodium deoxycholate
- Protease inhibitor cocktail (Roche, cat. no. 11873580001) or individual protease inhibitors (see REAGENT SETUP)
- Glutathione Sepharose 4B beads (Amersham Biosciences, cat. no. 17-0756-01)
- Sodium *N*-lauryl sarcosine (sarcosyl; Amersham, cat. no. 21653) prepared as 3% (wt/vol) solution in water (for solubilization of the insoluble proteins in inclusion bodies)
- Caspase buffer: 20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% (wt/vol) CHAPS and 10% (wt/vol) sucrose (pH 7.2; for details, see ref. 6)
- QIAquick gel-extraction kit (Qiagen, cat. no. 28706)
- Denaturants, such as guanidine hydrochloride (GuHCl) or urea (from Amersham Biosciences)
- Calf intestinal alkaline phosphatase (CIP; New England Biolabs)
- T4 DNA ligase (New England Biolabs)
- Metal-chelating Sepharose (Amersham Biosciences or Qiagen)

EQUIPMENT

- Conical Erlenmeyer flasks (preferably baffled to increase aeration), beakers and measuring cylinders of various volume capacities (50, 100 and 250 ml, and 1 and 2 l)
- Two shaking incubators with cooling capacity (Thermo/New Brunswick)
- Bullet filter (0.22 μm; Amicon) or PVDF syringe-driven filter unit (0.22 μm; Millipore, cat. no. SLGV033RS)
- Freezer (set at –80 °C)
- Rotating shaker
- Plastic (Tarsons) or glass Petri dish (Borosil) of 14 cm diameter
- Protein gel-electrophoresis apparatus for analysis of protein quality and quantity (Protean III, BioRad)
- Sonicator (Sonics) with probes of various dimensions for working with various sample volumes ! CAUTION The sonicator chamber door should be closed while performing the sonication, as prolonged exposure to ultrasonic waves might lead to deafness.
- Sepharose Q column (Amersham Biosciences)
- RC-5B plus centrifuge and SS34 rotor (Sorvall)
- Econo-column, 2× 2 ml (BioRad)
- Prepacked 0.5 ml Ni-NTA column (Qiagen)
- PD 10 column (Amersham Biosciences)

REAGENT SETUP

Protease inhibitor cocktail or individual protease inhibitors Examples include 1 mM PMSF (Sigma; stock, 200 mM in methanol), 1 μg ml^{-1} aprotinin (Sigma; stock, 1 mg ml^{-1} in water), 10 μM antipain (Sigma; 1 mM in methanol), 10 μM leupeptin (Sigma; stock, 1 mM in water), 10 μM pepstatin (Sigma; stock, 1 mM in methanol), endonucleases (such as benzonase; Novagen) or DNAseI. Alternatively, viscosity can be reduced non-enzymatically by sonicating the lysates. Each protease inhibitor should be dissolved in specific solvent. A table of solvent usage for preparing stock solutions of various inhibitors is available online (http://www.serva.de/products/knowledge/061311.shtml). DTT helps in keeping protein in reduced condition and does not allow oxidation ▲ CRITICAL If DNaseI is used to reduce the viscosity, add $CaCl_2$ to the lysate at 5 mM.

IPTG Should be prepared fresh as 1 M stock in water (working concentration ranges between 20 and 500 μM ▲ CRITICAL The concentration of IPTG used can be optimized for the maximum induction and increased solubility of the protein of interest. The temperature of induction often determines the solubility of the recombinant protein; therefore, the induction of expression might be carried out at a lower temperature to enhance the solubility. The amount of culture to be used for expression depends on the specific requirement and the yield of purifiable fraction of protein, and can be determined in a pilot-scale experiment.

High grade water The quality of water should be good and we recommend Milli Q (Millipore) or equivalent grade water (resistance 18 MΩ) to be used for all procedures ▲ CRITICAL Poor quality of water might lead to degradation of protein during the purification process.

PROCEDURE

Subcloning of gene of interest into the pC6-2 vector

1| Restriction enzyme-digested fragments of DNA can be ligated in-frame with the GST-encoding region of the pC6-2 vector by employing routine molecular cloning techniques[13]. Here we present an example of such a procedure in which we subcloned two regions of SATB1 at the BamHI site in the pC6-2 vector.

2| The DNA sequence encoding 1–204 amino acids of SATB1 was amplified using 5′BamHI-F (5′-CAACGGATCCATGGATCATTTGAAC-3′) and 204BamHI-R (5′-CCATGGATCCGTCTTTCAAATCAG-3′) primers, with full-length human SATB1 cDNA cloned in pBluescript as a template (pAT1146)[14] under appropriate PCR conditions. The DNA sequence encoding the MD+HD region was amplified using S255-BamHI-F (5′-CACGGATCCAGTCTTTCTGAGCTATCC-3′) and 2290-BamHI-R (5′-CCATGGATCCGTCTTTCAAATCAG-3′) primers under appropriate PCR conditions.

3| The pC6-2 vector was digested with BamHI for 1.5 h, heat-inactivated at 80 °C for 20 min and dephosphorylated at the 5′ ends by treating with CIP at 37 °C for 1 h.
▲ CRITICAL STEP The plasmid DNA to be used for subcloning should be purified using Qiagen or an equivalent column, or by CsCl density gradient centrifugation to obtain mostly supercoiled DNA.

4| Both the PCR products were digested with BamHI for 1.5 h and heat-inactivated at 80 °C for 20 min.

5| The digested vector and PCR products were gel-purified using a QIAquick gel-extraction kit and ligated using T4 DNA ligase.

! CAUTION Agarose gel containing EtBr should be handled with gloves and should be discarded at a properly assigned place. EtBr is a mutagenic and carcinogenic agent.

6| The ligation mixture was used to transform the DH5α strain of *E. coli*. The orientation of the fragment in a clone was confirmed by automated DNA sequencing.

Purification of caspase-6

7| Transform 50 µl BL21(DE3) competent cells with 0.02 µg pET23b-caspase-6 construct and spread on LB agar containing 100 µg ml^{-1} ampicillin.

8| Inoculate one colony into 3 ml of 2× TY media supplemented with 50 µg ml^{-1} ampicillin and incubate at 37 °C with shaking (200 rpm) for 12 h.

9| Transfer 1 ml culture into 50 ml of 2× TY media supplemented with 50 µg ml^{-1} ampicillin for 2 h at 37 °C with shaking (200 rpm).

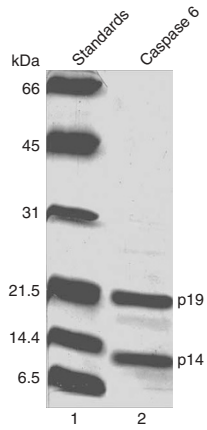

Figure 3 | SDS-PAGE analysis of affinity-purified caspase-6. An aliquot corresponding to 1 µg nickel affinity column-purified caspase-6 was resolved on a 15% SDS-polyacrylamide gel (lane 2) along with molecular mass standards (lane 1) and was visualized by staining with Coomassie brilliant blue. The two subunits of caspase-6 are indicated as p19 and p14.

10| After 2 h, transfer 25 ml culture into two 500-ml samples of 2× TY media in 2 l baffled Erlenmeyer flasks. Shake at 37 °C and 200 rpm until the cell density (A600) reaches 0.6–0.8.

11| Once the A600 has reached between 0.6 and 0.8, lower the temperature to 30 °C, induce expression by adding 0.02 mM IPTG and further incubate for 18 h.

12| Harvest cells after completion of induction by centrifugation at 3,000*g* and 4 °C for 10 min, and resuspend in 1:10 volumes of buffer A immediately.

■ **PAUSE POINT** The cell pellet can be stored at –80 °C for up to 1 month or can be processed for purification immediately. Alternatively, the cells can be resuspended in buffer A and stored frozen at –80 °C for next-day use.

13| Lyse the cells by three freeze–thaw cycles (dry ice/ethanol and 37 °C baths) followed by sonication for 5 min to disrupt the cells and shear the genomic DNA. The sonication time and power might vary depending on the instrument and sample (e.g., a large probe, 60% output power supply and 2 s pulse for 2–4 min using the Sonics instrument). Subsequently, clear the lysate by centrifugation for 45 min at 17, 000*g* and 4 °C, followed by filtration through a 0.22-µm bullet filter or PVDF millex syringe-driven filter.

▲ **CRITICAL STEP** After centrifugation the lysate should be cleared or it can clog the Ni-NTA column and adversely affect the flow. We recommend that the lysate is passed through a 0.22-µm filter.

14| Pack a 1-ml column of metal-chelating Sepharose according to the manufacturer's guidelines. Typically, a 1-ml column bed volume per liter of culture is recommended.

15| Apply the lysate to the column at a flow rate of no more than 75 ml per h. Wash off non-specifically bound material with 50 column volumes of buffer B (flow rate, 100 ml per h). Elute caspase-6 using 20 volumes of linear imidazole gradient (0–200 mM) in buffer A. Fractions are then analyzed on 15% SDS-PAGE and pooled according to the level of purity.

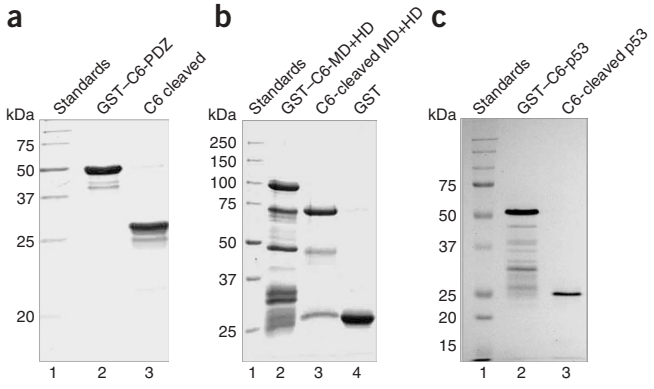

Figure 4 | Cleavage of column-bound fusion proteins releases those devoid of the GST tag. Proteins are resolved on 10–15% SDS-PAGE for 1–1.5 h (until the bromophenol blue dye front reaches the bottom) at 100 V in 1× SDS running buffer and visualized by staining with Coomassie brilliant blue. (**a**) Purification of the PDZ domain of SATB1. Lane 1, molecular mass standards; lane 2, reduced glutathione-eluted uncleaved GST–C6-PDZ fusion protein (top band); lane 3, caspase-6-cleaved PDZ eluted with buffer X. (**b**) Purification of the C-terminal MD+HD domain of SATB1. Lane 1, molecular mass standards; lane 2, uncleaved GST–C6-MD+HD fusion protein eluted with reduced glutathione (top band); lane 3, caspase-6-cleaved MD+HD eluted with buffer X; lane 4, GST left bound to the beads after caspase-6 cleavage and elution of MD+HD eluted with reduced glutathione. (**c**) Purification of the N-terminal 1–101 aa domain of p53. Lane 1, molecular mass standards; lane 2, uncleaved GST–C6-p53 fusion protein eluted using reduced glutathione (top band); lane 3, caspase-6-cleaved p53 eluted with buffer X.

Pure fractions can be pooled and dialyzed against storage buffer containing 20 mM Tris (pH 8.0), 150 mM NaCl and 10% (vol/vol) glycerol, and stored in aliquots at –80 °C for at least 2 years without significant loss of activity.
▲ CRITICAL STEP The flow rate is kept low to allow the proper binding of protein to the column. The flow-through can be loaded again onto the regenerated column.

16| Additional purification is rarely necessary; however, if desired, it can be accomplished by anion exchange following dilution of the sample fivefold into ion-exchange buffer. The diluted sample is applied to a Sepharose Q column, washed with 20 mM Tris-HCl (pH 8.0) until the A280 is <0.01 and then eluted with a gradient of NaCl (0–500 mM) in the same buffer.

17| Caspase-6 activity can be measured using a GST–C6 fusion protein as a substrate, or using several other methods described by Stennicke and Salvesen[7].

Purification of pC6-2-expressed GST-free proteins

18| Transform the XL1 blue strain of *E. coli* competent cells with the pC6-2-cloned construct using the heat-shock method and spread on an LB agar plate supplemented with 100 μg ml^{-1} ampicillin.
▲ CRITICAL STEP The colonies on the plate should be present in moderate numbers and should be well spaced.
? TROUBLESHOOTING

19| Inoculate a single colony in 5 ml LB media supplemented with 50 μg ml^{-1} ampicillin and incubate at 37 °C for 12 h (overnight) with shaking (200 rpm).

20| Transfer 1 ml culture into 250 ml pre-warmed LB media (37 °C) supplemented with 50 μg ml^{-1} ampicillin.

21| Induce protein expression with 0.2–0.5 mM IPTG at an A_{600} of 0.6–0.8 for 3–4 h.
▲ CRITICAL STEP The level of induction of expression is an important variable and requires standardization of multiple parameters, such as temperature, cell density and IPTG concentration.
? TROUBLESHOOTING

22| Pellet the cells by centrifugation at 4,000*g* and 4 °C for 10 min. The total induced culture should be aliquoted into two parts (one small volume of 5 ml and the remaining 245 ml) before centrifugation. Confirm the expression level and solubility of expressed protein by resolving the soluble extract from 5 ml induced cell pellet on SDS-PAGE. Steps 23 to 25 describe the preparation of soluble and insoluble fractions.
■ PAUSE POINT The cell pellet can be stored at –80 °C for up to 1 month or can be processed for purification immediately. Alternatively, cells can be resuspended in lysis buffer, kept on ice for 30 min to lyse the cell wall by lysozyme and stored frozen at –80 °C for next-day use.

23| Resuspend the cell pellet from 5 ml induced culture in 500 μl lysis buffer, keep on ice for 10–15 min and sonicate until the sample becomes clear (e.g., miniprobe, 30% output power supply and 2 s pulse for 1–2 min using the Sonics instrument). Centrifuge at 17,000*g* and 4 °C for 15 min.
▲ CRITICAL STEP The volume of lysis buffer used for resuspension of the pellet can be varied depending on the size of the pellet obtained after centrifugation of the induced sample. The cells should be properly resuspended in the buffer.

24| Transfer the supernatant (soluble fraction) to a new tube. Add 10 μl of 6× SDS loading buffer to 50 μl supernatant and boil the sample for 5 min at 100 °C.

25| Wash the pellet (insoluble fraction) by resuspending it in 1 ml buffer X and then pellet down at 17,000*g* and 4 °C for 15 min. The pellet can be dissolved in 600 μl of 2× SDS loading buffer and boiled for 20 min at 100 °C. Boiling for a longer time reduces the viscosity and renders the sample easy to load on gels.

26| Centrifuge the boiled samples from Steps 24 and 25 at 15,000*g* for 10 min at room temperature (25 °C). Prepare an SDS-polyacrylamide gel and resolve the proteins by electrophoresis.
▲ CRITICAL STEP Both the samples should not be viscous before loading. Generally, soluble samples are fine and only the insoluble fractions appear viscous. If this happens, then the boiling time can be increased or brief sonication can be performed to reduce the viscosity. The bottom particulate material should be avoided and only the top layer from the sample should be loaded in the wells. Although induction can be achieved at 37 °C to a greater extent, lowering the temperature helps to keep the metabolic activity of the cell low and also ensures that the proteins are in a more soluble form than in the inclusion bodies. Shortening the duration of induction also helps to increase the soluble fraction of protein.
? TROUBLESHOOTING

27| Fix the protein in the gel by treating the latter in fixing solution (50% (vol/vol) methanol and 10% (vol/vol) acetic acid) for 30 min with shaking. Stain for 15 min with a staining solution (50% (vol/vol) methanol, 10% (vol/vol) acetic acid and

0.25% (wt/vol) Coomassie brilliant blue) followed by two changes of the destaining solution (30% (vol/vol) methanol and 10% (vol/vol) acetic acid). Compare the protein profile of the soluble and insoluble fractions.
▲ CRITICAL STEP GST fusion protein should ideally be obtained in a soluble fraction, as purification under denaturing conditions is not possible with GST tags (unlike 6XHis tagged proteins). The insoluble GST fusion proteins can be purified only after the solubilization of insoluble fractions followed by refolding.
? TROUBLESHOOTING

28| After confirming that the protein is in a soluble fraction from Steps 23–27, proceed to purification using a larger volume of culture pellet. Resuspend the harvested cells (from the 245-ml induced cell pellet; Step 21) in 12 ml lysis buffer (10 ml lysis buffer per g of wet pellet can be used) and incubate on ice for 30 min or at room temperature for 15 min for adequate lysis of the cells. Lysis of the bacterial cell wall leads to an increase in viscosity.

29| The viscosity can be reduced by treatment with benzonase or DNaseI, or by sonication. We usually perform sonication until the lysate becomes clear.
▲ CRITICAL STEP Keep the sample on ice during sonication and avoid frothing. Sonication generates heat and therefore pulses should be given after a short period of incubation on ice. When using DNaseI, include 5 mM $CaCl_2$ in the lysate.

30| Prepare soluble cell lysate by the centrifugation of sonicated cleared lysate at 17,000*g* and 4 °C for 30 min.

31| Filter the lysate using a 0.22-μm PVDF syringe-driven filter. The filtered lysate can be used for binding with the glutathione Sepharose beads.
▲ CRITICAL STEP After centrifugation, the lysate should not be viscous.
? TROUBLESHOOTING

32| Alternatively, the solubilization of insoluble proteins can be achieved by the use of denaturants, such as guanidine-HCl (at 6 M), urea (at 8 M) or detergents such as sarcosyl (0.3% (wt/vol)). After solubilization into any of the above denaturants, dialysis should be performed following dilution of the denaturant. The protein should be refolded using an appropriate protocol, several of which are freely available online (http://refold.med.monash.edu.au)[15–17]. The use of specific detergents has been shown to assist the refolding of carbonic anhydrase[18,19] and lysozyme[20]. Sarkosyl has been used to solubilize and purify the *E. coli* heat-shock sigma factor[21]. We have observed the precipitation of proteins during the removal of GuHCl and urea; however, we were successful in purifying sufficient amount of proteins when 0.3% (wt/vol) sarcosyl was used for solubilization and subsequent 10-fold dilution with buffer X, followed by dialysis in the same buffer (with four changes of 10 volumes of buffer X each time). The presence of sarcosyl increases the solubility of GST fusion proteins but reduces their binding with glutathione beads[22]; when sarcosyl is removed or decreased to 0.01%, the binding of GST fusion proteins to glutathione beads increases (P.K.P. and S.G., unpublished observations) and therefore purification yields are improved.

33| Incubate 200 μl of glutathione Sepharose beads with 6 ml refolded and dialyzed lysate in buffer X of insoluble fraction or soluble lysate of fusion protein in two separate tubes. Perform binding for 30 min at 4 °C with proper mixing (Rota-spin). Pack the protein-bound beads in 2×2 ml Econo-columns and wash three times with 10 volumes each of buffer X. One sample should be used to elute the GST fusion protein by passing 500 μl of 20 mM reduced glutathione in buffer X. Note that the protein eluted here can be used for the protein estimation by the RC DC protein assay kit and the estimated value can be used to empirically determine the amount of caspase-6 required for on-column cleavage. The second column should be used to perform on-column caspase-6 cleavage in 500 μl caspase buffer after equilibrating the column with five volumes of caspase buffer.
▲ CRITICAL STEP Different molar ratios of purified caspase-6 enzyme to substrate GST–C6 fusion protein (1:50, 1:100, 1:150, 1:200, 1:300, 1:400, 1:500, 1:600, 1:800 and 1:1000) can be employed to optimize the maximum cleavage at the minimum enzyme concentration *in vitro*. We found that a 1:500 molar ratio of caspase-6:substrate (GST–C6-PDZ and GST–C6-MD+HD) was optimal. The amount of caspase-6 required for the on-column cleavage was ~1.4 times greater than that required for cleaving the eluted protein.

34| An empirically determined concentration of caspase-6 can be used to perform the cleavage of the target protein. The bead-bound fusion protein in caspase buffer can be transferred to a 1.7-ml conical microcentrifuge tube, and an appropriate amount of caspase-6 can be added and incubated further at room temperature with mixing on a rotating shaker. The caspase-cleaved protein can be eluted by passing an additional 2 ml buffer X (reduced glutathione should not be added). The caspase-6 used in the reaction can be removed by passing the eluted cleaved protein through a prepacked 0.5 ml Ni-NTA column after buffer exchange in buffer A using a PD 10 column. The flow-through from the Ni-NTA column is free from contamination of caspase-6 and can be buffer-exchanged in appropriate buffer using pre-equilibrated PD 10 columns.
▲ CRITICAL STEP To remove 6XHis-tagged caspase-6, it is essential to buffer-exchange the fusion protein and caspase-6 protein mixture either by dialysis or using PD 10 columns that are pre-equilibrated with buffer A. DTT in the caspase buffer can reduce the charge on nickel if used directly.
? TROUBLESHOOTING

● TIMING

Steps 1–5: 3–4 d
Step 6: 1 h followed by overnight incubation
Steps 7–11: 1 d
Steps 12–15: 5–8 h
Steps 16–17: (optional) 2 d
Step 18: 1 h followed by overnight incubation
Steps 19–22: 1 d
Steps 23–27: 5–6 h
Steps 28–31: 4–6 h
Step 33: 2 h
Step 34: 2–3 h

? TROUBLESHOOTING

See **Table 2** for troubleshooting advice.

TABLE 2 | Troubleshooting table.

Problem	Possible reasons	Solution
Step 18: no colonies on the plate	DNA concentration is too low	Increase DNA quantity
	Poor competent cells	Use freshly prepared or commercially available competent cells with good transformation efficiency
	Poor DNA quality	Check the DNA on agarose gel, prepare fresh plasmid DNA and purify using a Qiagen column or CsCl gradient
Step 18: high number or mat of colonies on the plate	DNA used for transformation is too high	Reduce the amount of DNA to be used for the transformation
	Antibiotic on the plate is inactivated or of low concentration	Make sure that the antibiotic is added when the temperature of the autoclaved LB agar is $<55\ ^{\circ}C$. Make sure that the antibiotic stock is prepared appropriately and has not expired
Step 21: poor induction	IPTG might be old or not used optimally	Confirm the expiry date, prepare fresh IPTG and standardize the concentration
	Bacterial cell density is not appropriate at the time of induction of expression	Induce at different A600 values ranging from 0.3 to 1.0
	Temperature of induction is not appropriate	Optimize the temperature for expression
Step 26: the protein is in the insoluble fraction	Insoluble inclusion body is formed	Try a lower temperature for the expression. Reduce the induction time. Inclusion body can be solubilized using a denaturant, such as 8 M urea, 6 M guanidine HCl or 0.2–0.4% (wt/vol) sarcosyl, followed by refolding before binding to the glutathione beads
Step 26: the insoluble sample is viscous before loading	Too much genomic DNA	Boil the sample for a longer time. Sonicate at a low setting to shear the genomic DNA
Step 27: streak in the gel instead of sharp bands	Mainly because of high molecular weight genomic DNA, might lead to difficulty in visualizing the band corresponding to the protein of interest	Sonicate the samples or treat with DNaseI during sample preparation and make sure that the sample appears clear before loading
Step 31: sample is viscous and might not allow filtration through the filter membrane	Volume of lysis buffer used for resuspension is less	Dilute with buffer X and centrifuge again

ANTICIPATED RESULTS

All clones generated using pC6-2 must be sequenced to verify the reading frame and nucleotide sequence (**Fig. 1**) to ensure the authenticity of the fusion protein.

Expression of caspase 6 is carried out at 30 °C for 18 h using 0.02 mM IPTG (Step 11). The yield of processed caspase-6 may vary from batch to batch and at times a differentially induced band may not be observed while comparing the SDS-PAGE profiles of uninduced and induced cells. Therefore, after induction we directly proceed for Ni-NTA column purification. Caspase-6 starts eluting in purer form at 150 mM imidazole. We pool those fractions which show cleaner and intense protein bands in 15% SDS-PAGE (**Fig. 3**).

Occasionally, the protein eluted from the glutathione Sepharose column after caspase-6 cleavage may show few additional bands (**Fig. 4b**). This is solely dependent on the nature of the protein expressed as fusion protein. In such cases, further processing may be required using gel filtration chromatography or any other affinity chromatography allowing purification of the full length protein.

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