Overcoming protein instability problems during fusion protein cleavage

C. Dian*,†, P. Baráth*, R. Knaust*, S. McSweeney†, E. Moss‡, M. Hristova§, V. Ambros§, and D. Birse*¶
*The Structural Biochemistry Unit, Department of Biochemistry and Biophysics, Stockholm University, Stockholm, Sweden; †European Synchroton Radiation Facility (ESRF), Grenoble, France; ‡Cellular and Developmental Biology, Fox Chase Cancer Center, Philadelphia, USA; §Department of Biology, Dartmouth College, Hanover, New Hampshire, USA
¶Address correspondence to birse@dbb.su.se

PROTEOMICS

We describe a strategy to illustrate differences in the performance of various proteolytic enzymes used for on-column GST-fusion protein cleavage. The strategy uses a 5 ml GSTrap™ FF column connected in series to a 1 ml auxiliary GSTrap FF column, Ni^{2+}-charged HiTrap Chelating HP, or HiTrap™ Benzamidine FF (high sub). Using this approach, on-column cleavage performance of PreScission™ Protease, recombinant polyhistidine-tagged factor Xa, and thrombin was compared and evaluated under varying reaction conditions using various GST-fusion proteins as substrates.

Introduction

For the systematic processing of biomolecules for structural, functional, and drug discovery projects, rapid and efficient protein production methodologies must be developed and integrated with current and evolving technologies. Specifically, purification and on-column fusion protein cleavage methods have been developed to isolate recombinant, untagged proteins in a single chromatographic step. To date, one of the most effective methods in terms of simplicity, flexibility, robustness, efficiency, and fusion protein purity is to use a GST-fusion partner in conjunction with glutathione affinity media and apply an on-column cleavage strategy (1, 2, 4).

An inherent problem with all fusion protein systems is that the fusion partner (affinity tag) is often difficult to remove. Most systems rely on proteolytic cleavage to separate the fusion partner from the target protein. Several problems may be encountered during proteolytic cleavage, including:

- spurious, non-specific proteolytic attack of the fusion protein;
- the need for elevated temperatures for efficient cleavage, which can denature or cause aggregation of the fusion protein;
- incomplete cleavage, which reduces the yield and/or introduces heterogeneity to the purified protein;
- the need for additional steps to separate the cleaved fusion protein from the fusion tag, deactivate and remove the protease, and exchange buffer or desalt.

The method described in this study uses GST fusion proteins produced from the GST Gene Fusion System◊ vectors pGEX-6P, pGEX-5X, and pGEX-2T which encode optimal recognition sites for PreScission Protease, factor Xa, and thrombin, respectively. The performance of PreScission Protease, factor Xa, and thrombin was compared by screening their enzymatic stability, efficiency in removing the GST-fusion partner, and proteolytic specificity on cleavage of a diverse range of fusion proteins.

Preparation of GST-fusion proteins and binding to GSTrap FF column

The genes encoding various proteins were subcloned into the expression vectors pGEX-6P-1 (coding for a PreScission Protease cleavage site), pGEX-5X-1 (coding for a factor Xa proteolytic cleavage site), and pGEX-2T (coding for a thrombin proteolytic cleavage site) using standard molecular biology methods (3). The proteins chosen for this study are a series of developmental regulatory proteins selected from *Caenorhabditis elegans, Mus musculus,* and *Homo sapiens* and are denoted p28, p40, p52, p71, and p105.

Fusion protein expression and preparation of clarified cell lysates was essentially performed as described previously (4). Clarified cell lysates were loaded onto two, pre-equilibrated GSTrap FF 5 ml columns connected in series to ÄKTA™ explorer chromatography system. Column loading and preparation for on-column cleavage was performed as described previously (4).

On-column cleavage with PreScission Protease, factor Xa, or thrombin

PreScission Protease, recombinant polyhistidine-tagged factor Xa, and thrombin, (2 units enzyme/mg of bound fusion GST-fusion protein) were diluted in the appropriate cleavage buffer equal to 90% of the volume of the two 5 ml GSTrap FF columns. The enzymes were injected into the columns at a flow rate of ~5–7 ml/min using a syringe. Following injection, the column was placed in a closed flow status and the system was incubated online for 3–18 h at 4–22 °C according to the proteolytic enzyme used.

Elution of cleaved fusion protein

Prior to elution, an auxiliary 1 ml HiTrap column was connected downstream of the 5 ml GSTrap FF proteolytic cleavage columns, in-line with the fraction collector. The auxiliary column was matched to the protease used for cleavage. The column used was either GSTrap FF, Ni^{2+}-charged HiTrap Chelating HP, or HiTrap Benzamidine FF (high sub) with specific affinity for PreScission Protease (GST-fused enzyme), recombinant polyhistidine tagged factor Xa, or thrombin, respectively. Each column was pre-equilibrated with the appropriate cleavage buffer specific for the enzyme used.

The auxiliary column detains any cleaved material upon flow start-up, which minimizes loss of cleaved product and allows for rapid baseline recalibration before peak elution. The column also acts as a filter to remove the proteolytic enzyme from the released target protein.

Elution of the released target protein occurred immediately upon flow start-up (flow rate of ~1 ml/min). Following elution and the return of absorbance to the baseline, the GST-affinity peak was eluted with elution buffer (optimized buffer for each enzyme) containing 10 mM reduced glutathione applied in a one step gradient (100% elution buffer). This step elutes the GST moiety, any uncleaved GST-fusion protein, endogenous *E. coli* proteins that have affinity for glutathione, and PreScission Protease, if it was used for cleavage.

Eluted protein fractions were analyzed with SDS-PAGE according to standard procedures (5) and protein quantitation was determined using Coomassie™ Protein Assay Reagent (Pierce).

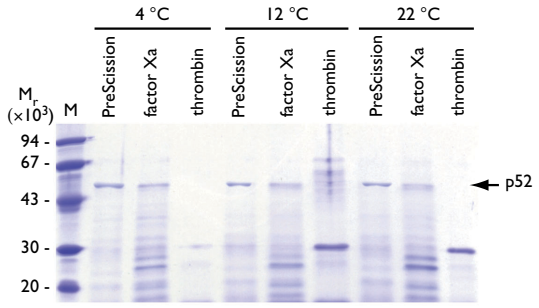

Fig 1. SDS gel electrophoresis of eluates following on-column cleavage of GST-p52 fusion protein at different temperatures. The incubation time was 12 h. Proteases and incubation temperatures are shown above the lanes. The arrow indicates the location of the released p52 protein. M = molecular weight markers.

Temperature-dependence of proteolytic cleavage

The effect of temperature on cleavage of GST-p52 fusion protein was tested using a 12 h digestion period (Fig 1). Each enzyme was tested at 4 °C, 12 °C, and 22 °C, the optimum temperatures for PreScission Protease, factor Xa, and thrombin, respectively.

PreScission Protease shows stable proteolytic processing at 4 °C with the highest enriched fraction migrating to the appropriate theoretical molecular weight of the cleaved p52 fusion protein. At 12 °C and 22 °C, an increase in degraded p52 protein was observed relative to cleavage reactions at 4 °C.

Factor Xa cleavage significantly increases degradation of the p52 protein with increasing temperature. When the temperature was elevated to 12 °C and 22 °C, the band corresponding to p52 protein decreased in intensity and shifted to a lower molecular weight breakdown product of ~M_r 26 000.

Thrombin cleavage is temperature dependent with poor cleavage occurring at 4 °C. The majority of cleaved p52 protein was in the form of a breakdown product migrating at ~M_r 32 000. In addition, aggregation and precipitation of p52 occurred upon elution from the column.

At elevated incubation temperatures for thrombin, cleavage activity was increased. Aggregation and proteolytic breakdown of p52 protein occurred and the formation of a stable p52 degradation product

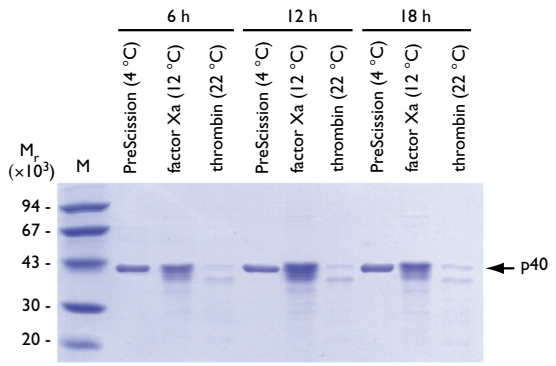

Fig 2. SDS gel electrophoresis of eluates following on-column cleavage of GST-p40 fusion protein for different incubation times at optimum temperatures. Proteases, optimum incubation temperatures, and incubation times are shown above the lanes. The arrow indicates the location of the released p40 protein. M = molecular weight markers.

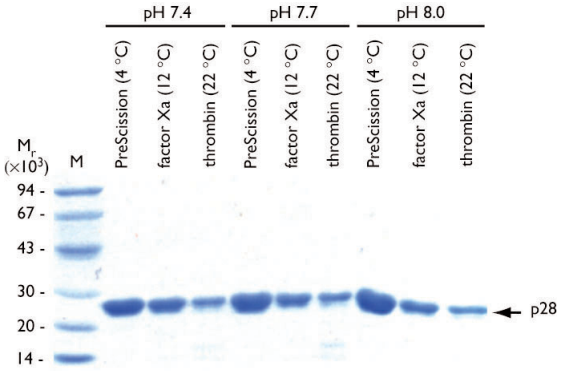

Fig 3. SDS gel electrophoresis of eluates following on-column cleavage of GST-p28 fusion protein at different pH values at optimum temperatures. The incubation time was 12 h. Proteases and pH values are shown above the lanes. The buffers used at each pH value were phosphate buffered saline, pH 7.4; HEPES, pH 7.7, and Tris-HCl, pH 8.0. The arrow indicates the location of the released p28 protein. M = molecular weight markers.

with ~M_r 32 000 was observed. Upon sample loading on the SDS polyacrylamide gel, cleaved p52 protein aggregated and precipitated, necessitating resolubilization prior to electrophoresis.

Time-dependent proteolytic cleavage

The effect of incubation time on cleavage of GST-p40 was examined using each protease at its optimum temperature (Fig 2). Samples were incubated for 6, 12 and 18 h.

Longer incubation times did not affect results when using PreScission Protease at its optimum temperature.

Factor Xa displayed a slight increase in p40 protein degradation with longer incubations. More p40 breakdown products were observed after each successive incubation period.

Thrombin cleavage resulted in immediate spurious proteolytic attack on the p40 protein. The primary breakdown product yields (~M_r 37 000) appeared after 6 h of incubation. After 18 h, the yield of the breakdown product is approximately equal to that of intact p40 protein. In addition, observed levels of p40 protein were significantly decreased following thrombin cleavage because of protein aggregation and precipitation upon elution from the column.

pH-dependent proteolytic cleavage

The effect of pH on cleavage of GST-p28 was tested at three pH values using a 12 h digestion (Fig 3). Each enzyme was tested at its optimum temperature at pH 7.4 (phosphate buffered saline), 7.7 (HEPES), and 8.0 (Tris-HCl).

pH did not affect purity or concentration of the final p28 protein during cleavage with PreScission Protease.

For factor Xa or thrombin, pH did not considerably affect the final p28 protein in terms of homogeneity. The final yield of p28 is, however, generally reduced compared with normalized protein levels due to aggregation and precipitation of the p28 protein after on-column cleavage.

Salt-dependent proteolytic cleavage

Salt sensitivity of the cleavage reactions was tested at two commonly used salt concentrations; 30 mM $(NH_4)_2SO_4$ and 300 mM NaCl. Digestions were performed at optimum temperatures for the proteases for 6 h with GST-p71 as substrate (Fig 4).

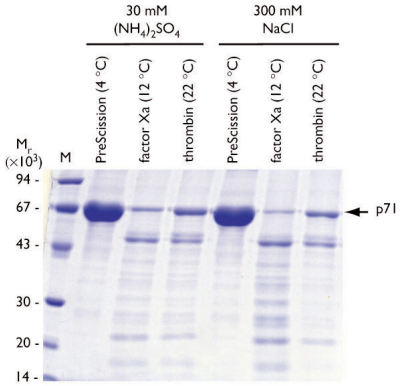

Fig 4. SDS gel electrophoresis of eluates following on-column cleavage of GST-p71 fusion protein at different salt concentrations at optimum temperatures. The incubation time was 6 h. Proteases and salt concentrations are shown above the lanes. The arrow indicates the location of the released p71 protein. M = molecular weight markers.

Under these conditions, PreScission Protease did not affect the homogeneity of the final p71 protein product in the presence of the selected salts.

Factor Xa cleavage reduced the level of soluble cleaved p71 protein relative to PreScission Protease, due to cleaved p71 protein aggregation and precipitation following column elution. A stable p71 breakdown product of ~M_r 53 000 was observed.

Thrombin cleavage also reduced the level of soluble cleaved p71 protein relative to PreScission Protease. The presence of 30 mM $(NH_4)_2SO_4$ appears to be more effective in terms of soluble p71 protein yield. As in the case of the factor Xa-cleaved product, a stable p71 breakdown product of ~M_r 53 000 was observed.

Additive-dependent proteolytic cleavage

The on-column cleavage strategy was used to examine the effect of additives on cleavage of GST-p105 fusion protein (Fig 5). The additives used were 2 mM DTT, 2% (v/v) glycerol, or 0.1% (v/v) Triton™ X-100. Cleavage reactions were incubated at the optimum time and temperature for each enzyme (12 h, 4 °C for PreScission Protease; 6 h, 12 °C for factor Xa; 3 h, 22 °C for thrombin).

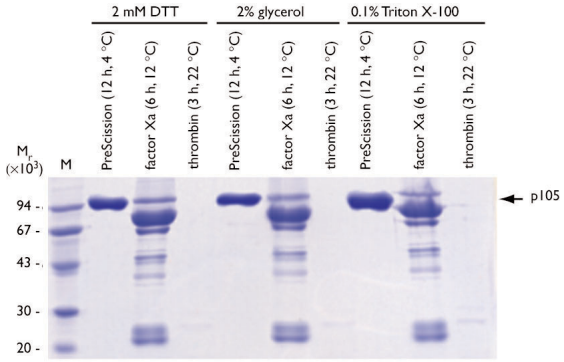

Fig 5. SDS gel electrophoresis of eluates following on-column cleavage of GST-p105 fusion protein in the presence of various additives. Cleavage reactions were incubated at the optimum time and temperature for each enzyme as indicated above each lane. The arrow indicates the location of the released p105 protein. M = molecular weight markers.

Negligible differences among the additives were observed in the final p105 protein product following cleavage with PreScission Protease. In the presence of 0.1% (v/v) Triton X-100, the PreScission Protease-cleaved p105 protein displayed a diffuse migration pattern on the gel.

Factor Xa cleavage in the presence of the additives resulted in low yields of p105 protein. A common degradation pattern was observed, with a predominant degradation product of M_r ~75 000.

Thrombin cleavage reactions displayed poor solubility following on-column cleavage and elution. Soluble, cleaved p105 protein was not detected following thrombin cleavage in the presence of the additives. In the presence of 0.1% Triton-X-100, a minor soluble p105 protein breakdown product migrating at M_r ~75 000 was observed. Additional breakdown products migrating at M_r ~30 000 were also detected. The M_r ~75 000 degradation product is similar in size to the product observed in the Factor-Xa experiments. The remaining cleaved p105 protein material was precipitated on-column or immediately following elution in all experiments with thrombin.

Conclusion

The GST Gene Fusion System is a popular *E. coli* expression system for the expression of large quantities of recombinant proteins. The protein of interest is produced as a fusion to GST, making subsequent proteolysis necessary to obtain a pure "native-like" protein of interest. Three protease sites (PreScission protease; factor Xa; and thrombin) were tested on a variety of target proteins to elucidate a trend in proteolytic behavior. Although protease digests have to be optimized for each protein of interest, some general conclusions can be made.

Performing the digest at optimum temperature is crucial for efficient cleavage. PreScission protease was superior to the other enzymes tested, primarily for its activity at 4 °C, making this protease a good choice when working with temperature-sensitive proteins.

Thrombin performed the poorest in these sets of experiments, demonstrating that non-specific cleavage is more likely to take place with this enzyme. Thrombin also possesses a relatively high temperature optimum (22 °C), which can adversely affect protein stability.

With factor Xa, problems concerning specific cleavage were frequently observed.

Salt concentrations and pH values tested in this study did not make a significant difference in the proteolytic

behavior of these proteases, indicating a relatively high tolerance for variation in these parameters.

The cleavage conditions are more dependent on the protein of interest and should be optimized for each target protein separately taking into consideration protein instability, cleavage efficiency, aggregation, and purity of the final cleaved protein product.

Acknowledgements
The authors would like to thank Anna Heijbel and Marianne Carlsson for valuable discussions concerning experimental techniques.

References

1. Cordingley M. G. *et al., J. Biol. Chem.* **265**, 9062–9065 (1990).
2. Walker P. A. *et al., BioTechnology* **12**, 601–605 (1994).
3. Ausubel, F. M. *et al.* (eds.), *Short Protocols in Molecular Biology*, John Wiley & Sons, New York, (1992).
4. Knaust, R. *et al., Life Science News* **6**, 12–13 (2000).
5. Fling, S. P, and Gregerson, D. S., *Anal. Biochem.* **155**, 83–88 (1986).

ORDERING INFORMATION

GSTrap FF	1 × 5 ml	17-5131-01
GSTrap FF	2 × 1 ml	17-5130-02
GSTrap FF	5 × 1 ml	17-5130-01
HiTrap Benzamidine FF (high sub)	2 × 1 ml	17-5143-02
HiTrap Benzamidine FF (high sub)	5 × 1 ml	17-5143-01
HiTrap Chelating HP	5 × 1 ml	17-0408-01
PreScission Protease	500 units	27-0843-01
Thrombin	500 units	27-0846-01
Factor Xa*	400 units	27-0849-01
pGEX-2T	25 µg	27-4801-01
pGEX-5X-1	25 µg	27-4584-01
pGEX-6P-1	25 µg	27-4597-01

*Native, non-tagged enzyme.

◊ See licensing information.