

Protocol 20. Factor Xa Protease treatment of fusion proteins containing a Factor Xa Protease recognition sequence

Treatment of fusion proteins containing a Factor Xa Protease recognition sequence consists of three steps: a) Factor Xa Protease Cleavage, b) removal of Factor Xa Protease, and c) cleanup of the digested protein. Protocols for all three steps can be found below.

Protocol 20 a. Factor Xa Protease cleavage

Factor Xa Protease is a site-specific endoprotease that preferentially cleaves the C-terminal peptide bond of the recognition sequence Ile-Glu-Gly-Arg. Factor Xa Protease consists of two polypeptides linked by a disulfide bond. The optimal cleavage conditions must be determined individually for each protein to be cleaved. Accessibility of the cleavage site, the adjacent amino acid sequence, and the degree of protein aggregation all affect the cleavage efficiency. Optimization of Factor Xa Protease concentration, temperature (4° to 37°C), and incubation time (2 to 16 h) is recommended. Bear in mind that excess Factor Xa Protease may result in nonspecific proteolysis at secondary sites. Therefore, optimal enzyme specificity is achieved using the lowest amount of protease necessary to achieve complete cleavage.

Optimization of the cleavage conditions should be performed in small-scale reactions using the following protocol as a starting point. We recommend using 20 mM Tris-Cl, pH 6.5; 50 mM NaCl; 1 mM CaCl₂ as the reaction buffer.

Materials

Fusion protein containing a Factor Xa Protease recognition sequence

Factor Xa Protease

1x reaction buffer (20 mM Tris-Cl, pH 6.5; 50 mM NaCl; 1 mM CaCl₂)

5x SDS-PAGE sample buffer

Buffer compositions are provided in the appendix on pages 111–115.

- 1. Prepare four solutions each containing 10 µg of the protein to be cleaved, in 1x reaction buffer. The solutions should have a protein concentration of at least 0.25 µg/µl.**

Since Factor Xa Protease is sensitive to various buffer constituents we recommend that the protein to be cleaved is prepared in 1x reaction buffer before cleavage. If your individual protein requires other specific buffering conditions, please see the important notes below for the compatibility of some commonly used buffer components with Factor Xa Protease. We also recommend changing the buffer system if you have purified your protein by Ni-NTA affinity chromatography under native conditions, because Factor Xa Protease activity is sensitive to phosphate buffers as well as to high imidazole and NaCl concentrations.

2. **Prepare three serial dilutions of Factor Xa Protease in 1x reaction buffer with concentrations of 1.0, 0.2 and 0.05 Units per μl .**

It is important to mix the dilutions completely before use to avoid localized differences in enzyme concentration. Diluted protease should be used immediately after preparation.

3. **Add 1 μl of each Factor Xa Protease dilution to one solution of the protein to be cleaved, and adjust the reaction volume to 40 μl using 1x reaction buffer. Adjust the volume of the fourth protein solution to 40 μl using 1x reaction buffer. This sample will serve as a negative control to monitor the progress of cleavage.**

Component	Volume/reaction	Final concentration
10 μg fusion protein	variable	0.25 $\mu\text{g}/\mu\text{l}$
Factor Xa Protease dilutions	1 μl	1.0, 0.2, and 0.05 U/40 μl
1x reaction buffer	Adjust to 40 μl	

4. **Incubate the reactions at room temperature (15–25°C). Take an 8 μl aliquot from each reaction after 3, 6, 9 and 16 h. Add 2 μl 5x SDS-PAGE sample buffer to each aliquot and mix thoroughly.**

It is important to mix the aliquots with SDS-PAGE sample buffer immediately to completely quench Factor Xa Protease activity.

5. **Analyze the efficiency of cleavage in each sample by SDS-PAGE.**

Since the cleaved-off peptide may be very small, the bands corresponding to cleaved and uncleaved protein will run very close together. We therefore recommend using a gradient gel that will give good resolution in the size-range of the protein being analyzed.

Important notes for optimization of cleavage

Protease concentration: Excess Factor Xa Protease may result in nonspecific proteolysis at secondary sites. Optimal enzyme specificity is achieved using the lowest amount of protease necessary to achieve complete cleavage.

Concentration of protein to be cleaved: Factor Xa Protease activity is sensitive to the concentration of protein to be cleaved. A minimum of 10 μg protein per 40 μl reaction (0.25 $\mu\text{g}/\mu\text{l}$) is recommended.

Incubation temperature: Factor Xa Protease activity increases with increasing incubation temperature from 4°C to 37°C. However, it should be taken into account that reduced incubation temperatures can minimize the accessibility of secondary cleavage sites.

pH: Factor Xa Protease activity decreases with increasing pH from pH 6.5 to pH 9.0. Therefore, we recommend a pH of 6.5 for the reaction buffer. If your individual protein is sensitive to pH, for example, with relation to protein activity or solubility, increase the pH to 7.5.

Buffer system: Use of phosphate buffers will result in reduced Factor Xa Protease activity compared to Tris-Cl or HEPES buffer systems.

Presence of detergents: Up to 1% Triton X-100, Nonidet P-40 and *n*-octylglucoside has no significant effect on Factor Xa Protease specificity or activity. The presence of SDS will significantly reduce enzyme activity.

Presence of denaturants: Significant loss of protease activity is observed at urea concentrations above 100 mM, and guanidine-HCl concentrations above 10 mM.

Presence of reductants: The presence of reductants in the reaction should be avoided, because the subunits of the heterodimeric protease enzyme are linked via a disulfide bridge. However, in some experiments up to 5 mM β -mercaptoethanol has been used with no significant influence on enzyme activity. The use of DTT is not recommended.

NaCl sensitivity: Factor Xa Protease activity decreases with increasing NaCl concentration. Significant inhibition is observed above 100 mM NaCl.

Imidazole sensitivity: Factor Xa Protease is significantly inhibited at or above 100 mM imidazole.

Anionic polymeric sugars: Some experiments have shown that addition of dextran sulfates (5000–10,000 Da) or heparin (5000 or 15,000 Da) to the reaction mixture results in increased Factor Xa Protease activity. 0.5–1 μ g polymeric sugar can be added for cleavage of 10 μ g recombinant protein in a 40 μ l total reaction volume.

Amino acid sequence: Factor Xa Protease preferentially cleaves at the C-terminal side of the recognition sequence Ile-Glu-Gly-Arg↓X. It has been reported that X is in general unspecific, but Ile and Thr are preferred. Where X is a hydrophobic residue, cleavage efficiency is reduced (Owen et al., 1974, He et al., 1993).

Scaleup

Once the optimal cleavage conditions have been found, the reaction can be scaled up proportionally. Following the above protocol, 1 mg recombinant protein would be digested in a total volume of 4 ml. If there is a need to reduce the total reaction volume, perform small scale experiments in which the reaction volume is varied while the protease:recombinant protein ratio and incubation conditions are kept constant.

Protocol 20 b. Removal of Factor Xa Protease

After protease digestion, Factor Xa Protease can be removed by affinity chromatography using Xa Removal Resin. The Xa Removal Resin binds the protease in the reaction mixture while the cleaved recombinant protein remains in solution. After the resin is pelleted by centrifugation, the cleaved recombinant protein is recovered in the supernatant.

Materials

Xa Removal Resin

1x Reaction buffer (20 mM Tris-Cl, pH 6.5; 50 mM NaCl; 1 mM CaCl₂)

- 1. Calculate the required amount of Xa Removal Resin necessary to capture the Factor Xa Protease present in the cleavage reaction.**

50 μ l bed volume (100 μ l slurry) is sufficient to bind 4 Units Factor Xa Protease enzyme in 1x reaction buffer. Use of slurry volumes of less than 25 μ l is not recommended due to associated handling problems. If your individual recombinant protein requires cleavage buffer other than the recommended 1x reaction buffer, bear in mind that the protease capture step may be sensitive to the use of other buffers. Binding of Factor Xa Protease to the Xa Removal Resin is unaffected by increasing the pH to 7.5, the presence of 20–100 mM Tris-HCl, and up to 1% Triton X-100 or Nonidet P-40. High salt concentrations will reduce binding capacity. For example, increasing NaCl concentration from 50 mM to 500 mM will result in a 20–40% reduction in binding. The recommended 1x reaction buffer (20 mM Tris-HCl, pH 6.5; 50 mM NaCl; 1 mM CaCl₂) supports high-efficiency cleavage and capture.

- 2. Resuspend the Xa Removal Resin completely by gentle inversion and then immediately transfer the required amount of slurry into a centrifuge tube of appropriate size.**

Note: The beads will quickly fall out of suspension. For transfer, use a wide-mouth pipette.

- 3. Centrifuge the beads for 5 min at 1000 x g and discard the supernatant.**

- 4. Resuspend the beads in ten bed-volumes of 1x reaction buffer by gently mixing, centrifuge for 5 min at 1000 x g, and discard the supernatant.**

Equilibration of the beads with 1x reaction buffer is necessary for maximum capture efficiency and prevents contamination of the cleaved recombinant protein with resin storage buffer. Use Xa Removal Resin immediately after equilibration.

- 5. Add the cleavage reaction to the equilibrated resin. Mix gently to resuspend the resin and incubate for 10 min at room temperature. Shake on an orbital shaker or place sealed tube on a roller-table to keep beads in suspension.**

If the cleaved protein is temperature sensitive, binding can be performed at 4°C without any loss of binding efficiency.

6. Centrifuge the reaction at 1000 x g for 5 min to pellet the resin. Collect the supernatant which contains the cleaved protein. Factor Xa Protease remains bound to the resin.

Protocol 20 c. Removal of undigested 6xHis-tagged protein and cleaved 6xHis peptides after Factor Xa Protease cleavage

After digestion of 6xHis-tagged recombinant proteins with Factor Xa Protease, undigested proteins and released 6xHis-tag/Factor Xa Protease recognition site-peptides are present in the cleavage reaction mixture. Both these species can be removed by Ni-NTA affinity chromatography using a batch procedure, leaving pure, de-tagged recombinant protein in the supernatant. The protocol can be carried out either directly after Factor Xa Protease cleavage or subsequent to the removal of the protease with Xa Removal Resin. The protocol consists of two steps: adjusting the pH to 7.5, and binding of 6xHis-tagged contaminants to Ni-NTA resin.

Materials

1M Tris-Cl pH 8

Ni-NTA Agarose

1. Adjust the pH of the cleavage reaction mixture to 7.5.

This can be accomplished by the addition of ~1/100 volume of 1M Tris-Cl, pH 8.0 when the recommended 1x reaction buffer (20 mM Tris-Cl, pH 6.5; 50 mM NaCl; 1 mM CaCl₂) was used for cleavage. It is not necessary to adjust the pH if the protease digestion was performed at pH 7.5. A pH of 7.5 is essential for efficient binding of 6xHis tags to Ni-NTA resin.

2. Calculate the required amount of Ni-NTA Agarose needed to capture the 6xHis-tagged contaminants.

1 ml bed volume (2 ml slurry) is sufficient to bind 5–10 mg 6xHis-tagged protein. For optimal performance, the binding capacity of the Ni-NTA Agarose used for removal should match the total amount of 6xHis-tagged protein that was subjected to Factor Xa Protease cleavage.

3. Resuspend the Ni-NTA Agarose completely by gently inverting the bottle 4–6 times and then immediately transfer the required amount of slurry into a centrifuge tube of appropriate size.

4. Centrifuge the resin for 1 min at 1000 x g and discard the supernatant.

Optional: To prevent contamination of the recombinant protein with Ni-NTA storage buffer, the pelleted beads can be washed with two bed volumes of 20 mM Tris-Cl, pH 7.5, 50 mM NaCl prior to incubation with the reaction mixture.

5. **Transfer the reaction mixture to the equilibrated resin. Mix gently to resuspend the resin and incubate for 10 min at room temperature. Shake on an orbital shaker or place sealed tube on a roller-table to keep beads in suspension.**

If the recombinant protein is temperature sensitive, binding can be performed at 4°C without any loss of binding efficiency.

6. **Centrifuge the suspension at 1000 x g for 1 min to pellet the resin. Collect the supernatant that contains the pure recombinant protein. 6xHis-tagged contaminants remain bound to the resin.**