Yet another Factor Xa test cleavage (Novabiochem Fact. Xa)

To each of 5 50-μL eppendorf tubes was added:

- 6 μL Factor Xa buffer 10X
- 24 μL (≈ 12 μg) FLAG and GST purified protein
- 29 μL DI water
- 1 μL Factor Xa (added after 10 μL of the above mix was removed for gel analysis)

After the 10X buffer, protein solution and water were added, the tubes were flicked to mix and 10 μL were added to 2.5 μL of SDS loading buffer for subsequent gel analysis (as the t = 0 timepoint).

Dilutions to of the 1 μg/μL neb Factor Xa solution were made to give a 0.5, 0.1, 0.05, 0.025 and 0.0125 dilutions were made. 1 μL of Fact. Xa was added to each tube as follows:

- tube a: 0.2 μg
d: 0.025 μg
- tube b: 0.1 μg
e: 0.0125 μg
- tube c: 0.05 μg
f: 0 μg

In ladder

10-μL aliquots were removed after 1, 3, 6 and 20 minutes for Western Blot analysis with anti-GST and anti-FLAG.

Western blots were each loaded with 5 mL of each aliquot (4 mL of protease soln. with 1 mL of 5-fold SDS protein loading buffer.)

(see following page for anti-FLAG and anti-GST gels)

Suggested: 1 μg Factor Xa for 50 μg protein
Factor Xa cleavage at 4°C.

- 11.8 mL rxns set up (before Fact Xa added), then 24 mL removed (added to 24 mL 2x SDS loading buffer) and Fact. Xa added:
  
  12 mL Factor Xa buffer
  48 mL (24 mg) pure protein (fr. p. 39)
  58 mL DI water
  (24 mL of above soln. removed, then)
  1 mL Fact. Xa dilution (0.1 and 0.013 mg)

- The rxn was incubated at 4°C. 24 mL aliquots were removed after 5 hours and after 30 hours. (Aliquots boiled w/ 2x SDS loading buffer. 20 mL of mixture loaded for Coomassie and 8 mL for the Western.)

anti-FLAG

1 2 3 4 5 6 7

in ladder

2 0.1 mg per 20 mg protein, t=0
3 
4 
5 0.013 mg Fact Xa per 20 mg protein, t=0
6 
7 

t= 5 hr


t= 30 hr
Factor Xa cleavage with the addition of ZnCl

4 μM GST-FLAG pure protein
600 μM ZnCl
20 μL (20 μg) protein
3 μL ZnCl soln. (3 μg, 150 eq.)
5 μL 10x Factor Xa buffer
31 μL water
(10 μL of above soln. removed for t=0 point before Factor Xa added)
1 μL Factor Xa (0.5 or 0.25 μg/μL)

lanes 2-4: 0.5 units Novagen Factor Xa
lanes 5-10: 0.25 units Novagen Factor Xa
(rxns run at 24°C)

4 μM pure protein
200 μM ZnCl
20 μL (20 μg) protein
1 μL ZnCl (1 μg, 50 eq.)
5 μL Factor Xa 10x buffer
33 μL water
(10 μL of above soln. removed for t=0)
1 μL Factor Xa (0.5 or 0.25 μg)

lanes 2-4: 0.5 units Factor Xa
lanes 5-7: 0.25 units Factor Xa
(rxns run at 16°C)

Grr! The hope was that adding ZnCl might improve folding and thus decrease secondary site cleavage by organizing the four LIM domains (double Zn fingers: nm binding: that bind a total of 8 Zn).

2° cutting seems to occur faster than at the 1° site to give fragments: >43 KDa, 30 and 34 KDa and <10 KDa.
There are no other IEGR (Factor