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 10× 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 ~~t~~of the 1µg/µL neb Factor Xa solution ~~was ma~~ 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		tube	
a:	0.2 µg	d:	0.025 µg
b:	0.1 µg	e:	0.0125 µg
c:	0.05 µg	f:	0 µg

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

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

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

suggested: 1 µg Factor Xa for 50 µg protein

ln		
1	ladder	
2	a,	t=0
3	a,	t=1hr
4	a,	t=3hr
5	a,	t=6hr
6	a,	t=20hr
7	b,	t=0
8	b,	t=1hr
9	b,	t=3hr
10	b,	t=6hr
11	b,	t=20hr
12	c,	t=0
13	c,	t=1hr
14	c,	t=6hr
15	c,	t=20hr

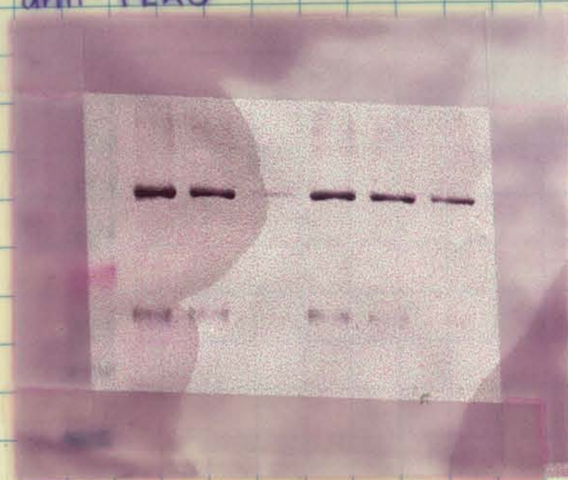
Factor Xa cleavage at 4° C.

- 118 µL rxns set up (before Fact Xa added), then 24 µL removed (added to 24 µL 2× SDS loading buffer) and Fact. Xa added:

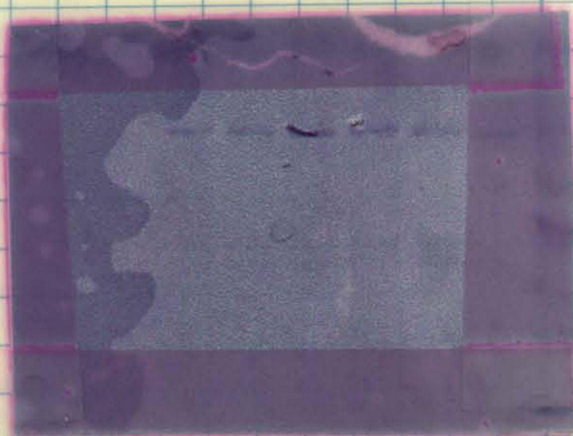
  12 µL Factor Xa buffer
  48 µL (24 µg) pure protein (fr. p. 39)
  58 µL DI water
  (24 µL of above soln. removed, then)
  1 µL Fact. Xa dilution (0.1 and 0.013 µg)

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

anti-FLAG



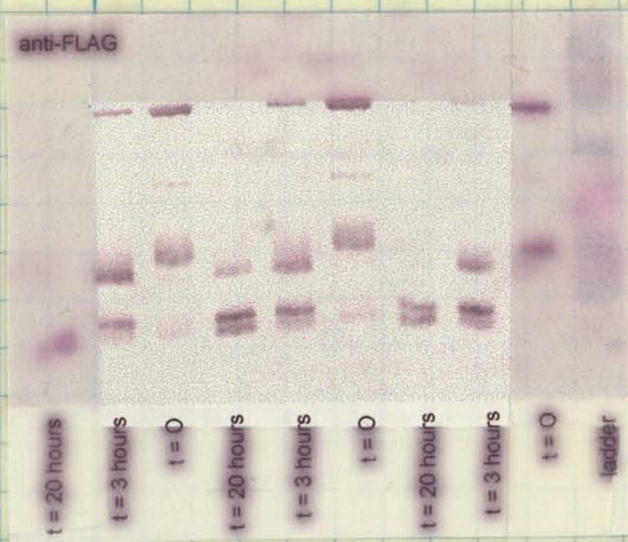
1 2 3 4 5 6 7



7 6 5 4 3 2 1

ln 1 ladder
2 0.1 µg per 20 µg protein, t=0
3 " t=5 hr
4 " t=30 hr
5 0.013 µg Fact Xa per 20 µg protein, t=0
6 " t=5 hr
7 " t=30 hr

Factor Xa cleavage with the addition of ZnCl

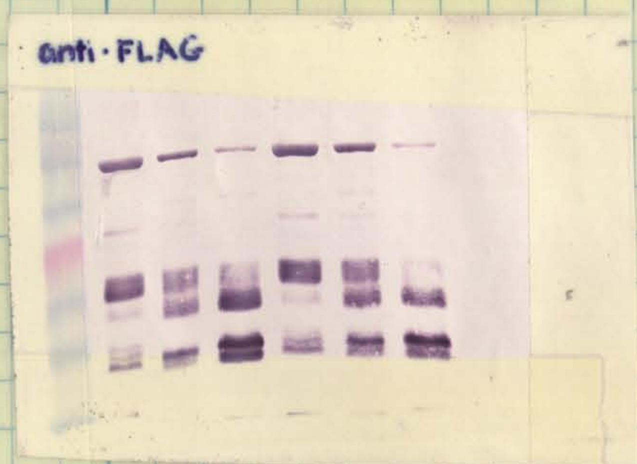


10 9 8 7 6 5 4 3 2 1
(5 µL protein soln. and 5 µL of 2 x SDS loading buffer per lane.)

4 µM GST + FLAG pure protein
600 µM ZnCl

20 µL (20 µg) protein
3 µL ZnCl soln. (3 µg, 150 eq.)
5 µL 10 x 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~~ units)

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



1 2 3 4 5 6 7
(same time course as above gel)

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 U)

lanes 2-4 0.5 units Factor Xa
lanes 5-7 0.25 units Factor Xa
(rxn 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