Plant Leucine Aminopeptidases Moonlight as Molecular Chaperones

Melissa A. Scranton, Ashley Yee, Sang-Youl Park, and Linda L. Walling

SUPPLEMENTARY TABLES

Supplementary Table 1. Primers used for cloning of *AtLAP* cDNAs.

Locus	Gene	Primer name	Primer sequence[A]
At2g24200	*AtLAP1*	LAP1-F	5'-AGCATATGATGGCTCACACTCYCGGT-3'
		LAP1-R	5'-ATGCGGCCGCTCACGAAGATGAATTCTTC-3'
At4g30920	*AtLAP2*	LAP2-F	5'-GCATATGGC- TCATACAATCTCACACGC-3'
		LAP2-R	5'-GCTCGAGTTAAGAAGAAGAATGGTTCTGT-3'

[A] Restriction enzyme sites for cloning of *AtLAP* cDNAs were incorporated into the forward (F) and reverse (R) primers. These sites are underlined and correspond to NdeI (LAP1-F, LAP2-F), NotI (LAP1-R), and XhoI (LAP2-R) sites.

Supplementary Table 2. Summary of Chaperone Assays for Plant LAPs.

	CS Protection at 1:3	Lowest conc (μM) of NdeI Protection	% Luc Recovering at 1:3
LAP-A	40-60%	1	14-20%
LAP-N	40-60%	0.2	17%
AtLAP1	0%	1	16%
AtLAP2	N.D.	0.2	18-19%

Supplementary Table 3. Summary of Chaperone Assays for Tomato LAP-A and LAP-N mutants.

		CS Protection at 1:3	Lowest conc (μM) of NdeI Protection	% Luc Recovering at 1:3	Site Disruption	Hexamer Disruption
LAP-A Mutants	D347N	0%	1	17-18%	Zinc ion binding	no
	D347R	100%	0.2	~30%	Zinc ion binding	yes
	E429R	0%	0.4	~30%	Zinc ion binding	yes
	E429V	40-60%	1	17-18%	Zinc ion binding	no
	K354E	100%	0.2	40%	Catalysis	yes
	K354R	40-60%	1	17-18%	Catalysis	no
	R431A	40-60%	1	~22%	Catalysis	no
LAP-N Mutants	K357E	0%	0.2	13%	Catalysis	no
	K357R	N.D.	0.2	0%	Catalysis	no

Supplemental Table 4. Percent Wild-type His_6-LAP-N enzyme activity on Leu-AMC substrate

His_6-LAP-N enzyme	% Wild-type His_6-LAP-N enzyme activity
Wild-Type	100
K357E	0.35 ± 0.03
K357R	0.93 ± 0.07
K357L	0.7 ± 0.09
K357C	0.26 ± 0.11
K357M	1.47 ± 0.04
K357G	0.84 ± 0.07
K357T	0.28 ± 0.03
K357P	0.34 ± 0.13

Purified His_6-LAPs (2160 ng) were assayed for activity towards Leu-AMC (1.58 μM) in assay buffer (50 mM Tris-HCl, pH 8, 0.5 mM $MnCl_2$) in a total volume of 162 μl. Activity assays proceeded for 30 min at 37°C. Rate of hydrolysis was determined and activity expressed as percent of wild-type His_6-LAP-N activity which was 0.24 ± 0.01 μmol mg^{-1} min^{-1} of protein (± SD).

SUPPLEMENTAL LEGENDS AND FIGURES

SUPPLEMENTARY FIGURE LEGENDS

FIGURE S1. **LAP-A protects NdeI from thermal inactivation in the absence of glycerol.** His_6-LAP-A was isolated and stored in the absence of glycerol. Restriction enzyme NdeI (1 U) was incubated in 1X restriction enzyme buffer 4 with or without His_6-LAP-A (0-2 μM) for 90 min at 43°C. After thermal deactivation, 140 ng of plasmid DNA was added and digested for 90 min at 37°C. Control lanes show plasmid DNA only with supercoiled (SC) monomer and multimers and DNA after digestion with unheated NdeI. NdeI released a 4.6-kb and 0.2 kb fragment (not shown).

FIGURE S2. ***Pisum sativum* Hsp18.1 protects NdeI from thermal inactivation**. Restriction enzyme NdeI (1 U) was incubated alone or with 0.2-2 μM His_6-LAP-A or PsHSP18.1 (Panel *A*) or protein A (Panel *B*). NdeI was heat denatured at 43°C and plasmid DNA was digested as described in Fig. 1. Control lanes show plasmid DNA only and DNA after digestion with unheated NdeI.

FIGURE S3. **PsHsp18.1 protects citrate synthase from thermal aggregation.** Citrate synthase (300 nM; CS) was incubated in 50 mM HEPES-KOH (pH 7.5), 5% glycerol, and 0 (□) or 900 nM His_6-LAP-A (■), PsHSP18.1 (●), or protein A (x) at 43°C for 60 min. Aggregation of CS was determined by measuring light scattering at 360 nm. Neither PsHSP18.1 (○) nor protein A (Δ) aggregated on their own. Data shown is representative of two independent experiments.

FIGURE S4. ***Pisum sativum* Hsp18.1 aids in refolding of firefly luciferase.** Luc (1 μM) was heated at 42°C for 11 min with 1 μM PsHsp 18.1 (●, Panel *A*), 3 μM His_6-LAP-A (■, Panel *B*), 3 μM protein A (x, Panel *B*), or no chaperone (□, Panels *A*-*B*) and then allowed to refold in rabbit reticulocyte lysate (RRL) in refolding buffer supplemented with 2 mM ATP. Percent activity corresponds to the relative luminescence compared to unheated luciferase. Measurements were taken for three technical replicates. Data is representative of at least 2 independent experiments.

FIGURE S5. **LAP1 does not protect citrate synthase from thermal aggregation**. Citrate synthase (300 nM; CS) was incubated in 50 mM HEPES-KOH (pH 7.5), 5% glycerol, and 0 (▲) or 900 (□) nM His_6-LAP1 at 43°C for 60 min. Aggregation of CS was determined by measuring light scattering at 360 nm. LAP1 (Δ) did not aggregate on its own. Data shown is representative of two independent experiments.

FIGURE S6. **Oligomeric structure of LAP-A mutants.** *A,* Purified His_6-LAP-As (10 μg) were fractionated on a native polyacrylamide gel (9% w/v). Protein bands were visualized by staining with Coomassie Brilliant Blue R-250. Masses of each multimer were determined as in Panel *B*. *B,* Purified wild-type and mutant His_6-LAP-As (10 μg) were fractionated on a set of four native polyacrylamide gels (7.5-12% w/v). Molecular mass standards were chicken egg albumin (45 kDa); bovine serum albumin monomer (66 kDa) and dimer (132 kDa); and tomato His_6-LAP-A hexamer (357 kDa). LAP oligomer species masses were determined by their relative mobility and retardation coefficient as previously described (36).

FIGURE S7. **LAP-A active site point mutants protect a NdeI from thermal inactivation**. Restriction enzyme NdeI (1 U) was incubated alone or with 0.2-2 μM His_6-LAP-A, K354E, R431A, E429V, E429R, K354R, D347N, or D347R. NdeI was heat denatured and plasmid DNA was digested as described in Fig. 1. Control lanes show plasmid DNA only and DNA after digestion with unheated NdeI.

FIGURE S8. **Oligomeric structure of LAP-N mutants.** Purified His_6-LAP-Ns (10 µg) were fractionated on a native polyacrylamide gel (9% w/v). Protein bands were visualized by staining with Coomassie Brilliant Blue R-250.

FIGURE S9. **LAP-N mutants protect NdeI from thermal inactivation**. Restriction enzyme NdeI (1 U) was incubated alone or with 0.2-2 µM His_6-LAP-N, K357E, or K357R. NdeI was heat denatured at 43°C and plasmid DNA was digested as described in Fig. 1. Control lanes show plasmid DNA only and DNA after digestion with unheated NdeI.

FIGURE S10. **LAP-N mutants do not protect CS from thermal aggregation** Citrate synthase (300 nM; CS) was incubated in 50 mM HEPES-KOH (pH 7.5), 5% glycerol, and 0 (□) or 900 nM His_6-LAP-N (■), K357E (♦), or K357R (▲) at 43°C for 60 min. Aggregation of CS was determined by measuring light scattering at 360 nm. K357E (◊) had a small amount of aggregation on its own while K357R (Δ) highly aggregated its own. Data shown is representative of two independent experiments.

FIGURE S11. **LAP-N mutants have lower chaperone activity based on the Luc refolding assay.** Luc (1 µM) was heated for 11 min at 42°C with 3 µM His_6-LAP-N (■), K357E (♦), or K357R (▲), or no chaperone (□) and then allowed to refold in rabbit reticulocyte lysate (RRL) in refolding buffer supplemented with 2 mM ATP. Percent activity corresponds to the relative luminescence compared to unheated luciferase. Measurements were taken for three technical replicates. Data is representative of at least two independent experiments.

Figure S1

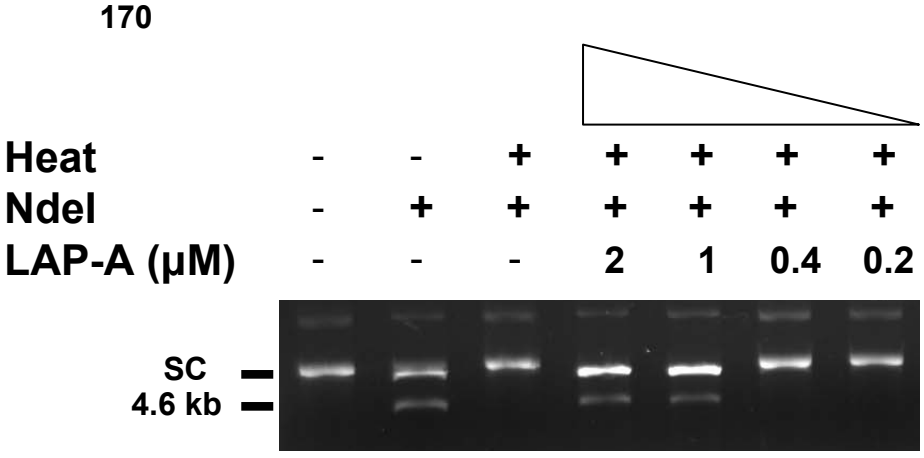


Figure S2

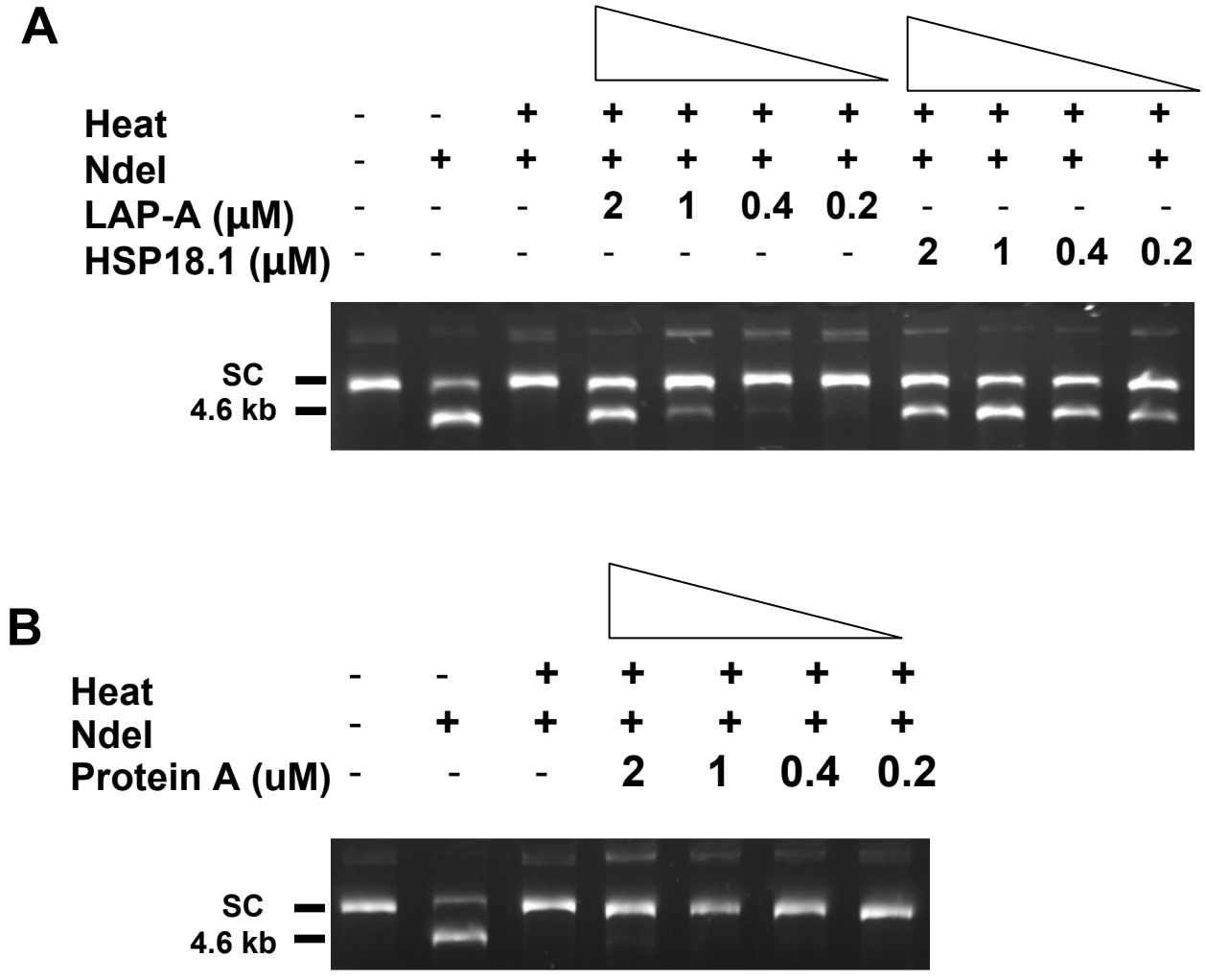

A
Heat - - + + + + + + + + +
NdeI - + + + + + + + + + +
LAP-A (µM) - - - 2 1 0.4 0.2 - - - -
HSP18.1 (µM) - - - - - - - 2 1 0.4 0.2
SC
4.6 kb
B
Heat - - + + + + +
NdeI - + + + + + +
Protein A (uM) - - - 2 1 0.4 0.2
SC
4.6 kb

Figure S3

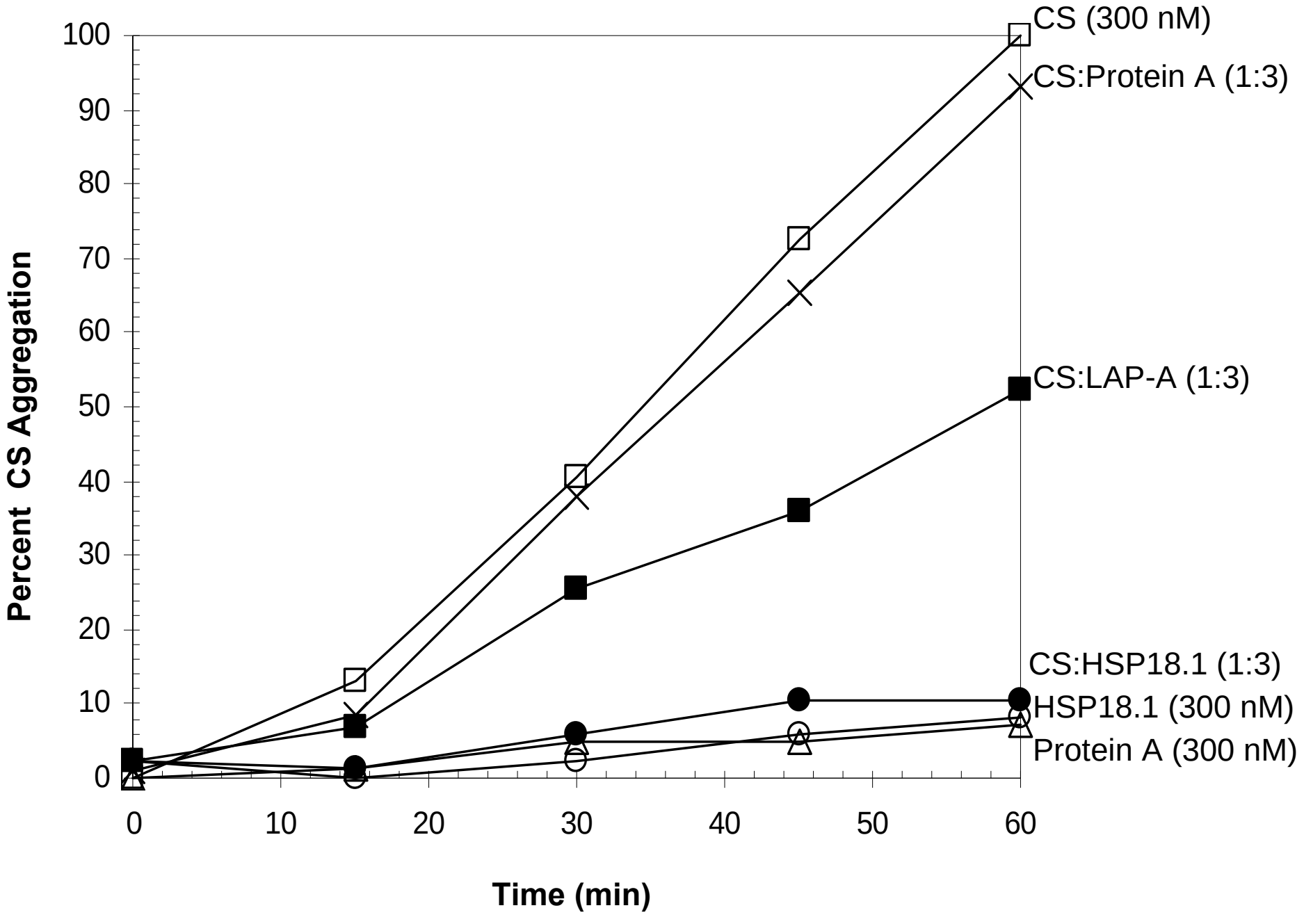

CS (300 nM)
CS:Protein A (1:3)
CS:LAP-A (1:3)
CS:HSP18.1 (1:3)
HSP18.1 (300 nM)
Protein A (300 nM)
Percent CS Aggregation
Time (min)
0
10
20
30
40
50
60
70
80
90
100

Figure S4

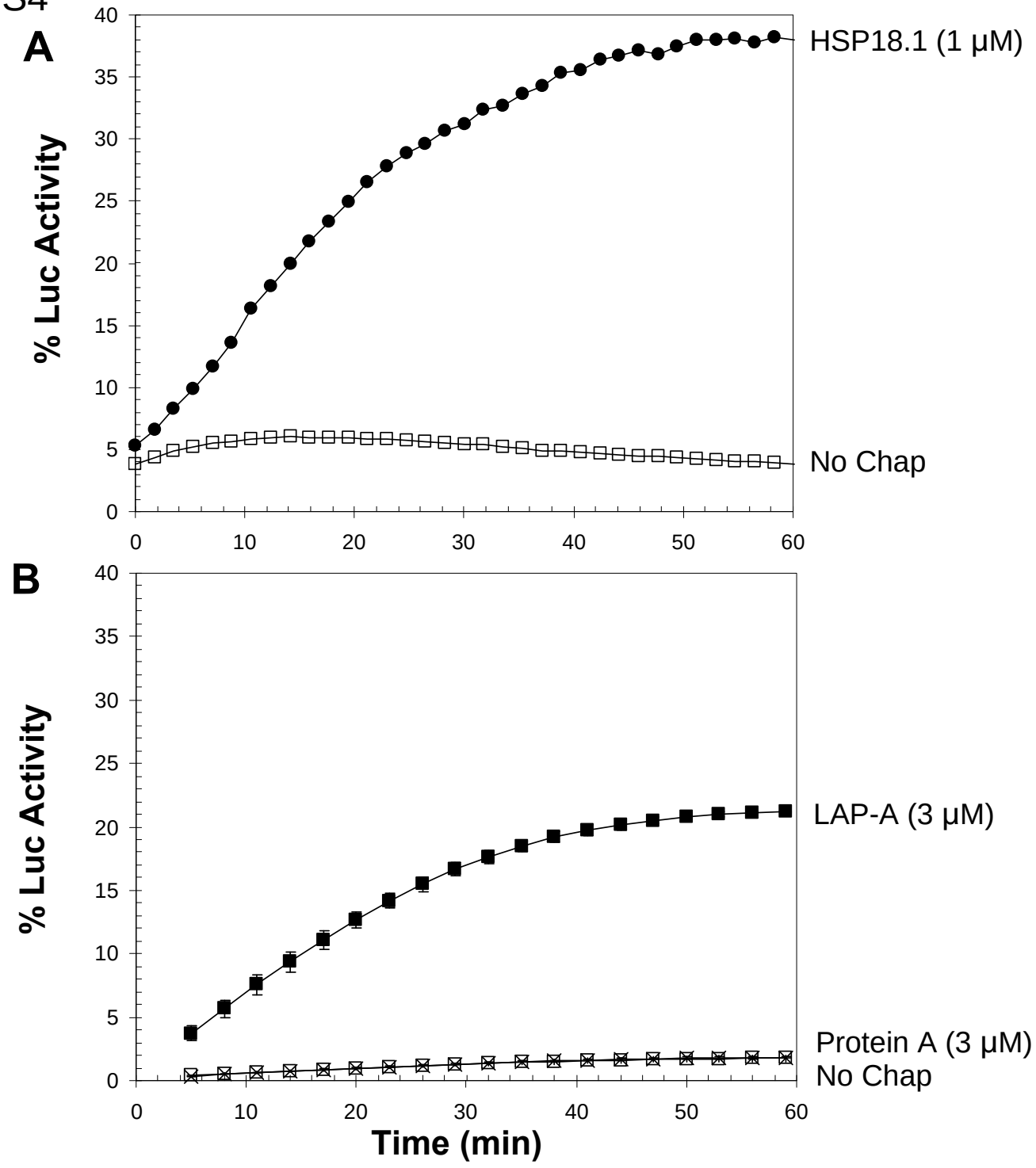

A
% Luc Activity
HSP18.1 (1 µM)
No Chap
B
% Luc Activity
LAP-A (3 µM)
Protein A (3 µM)
No Chap
Time (min)

Figure S5

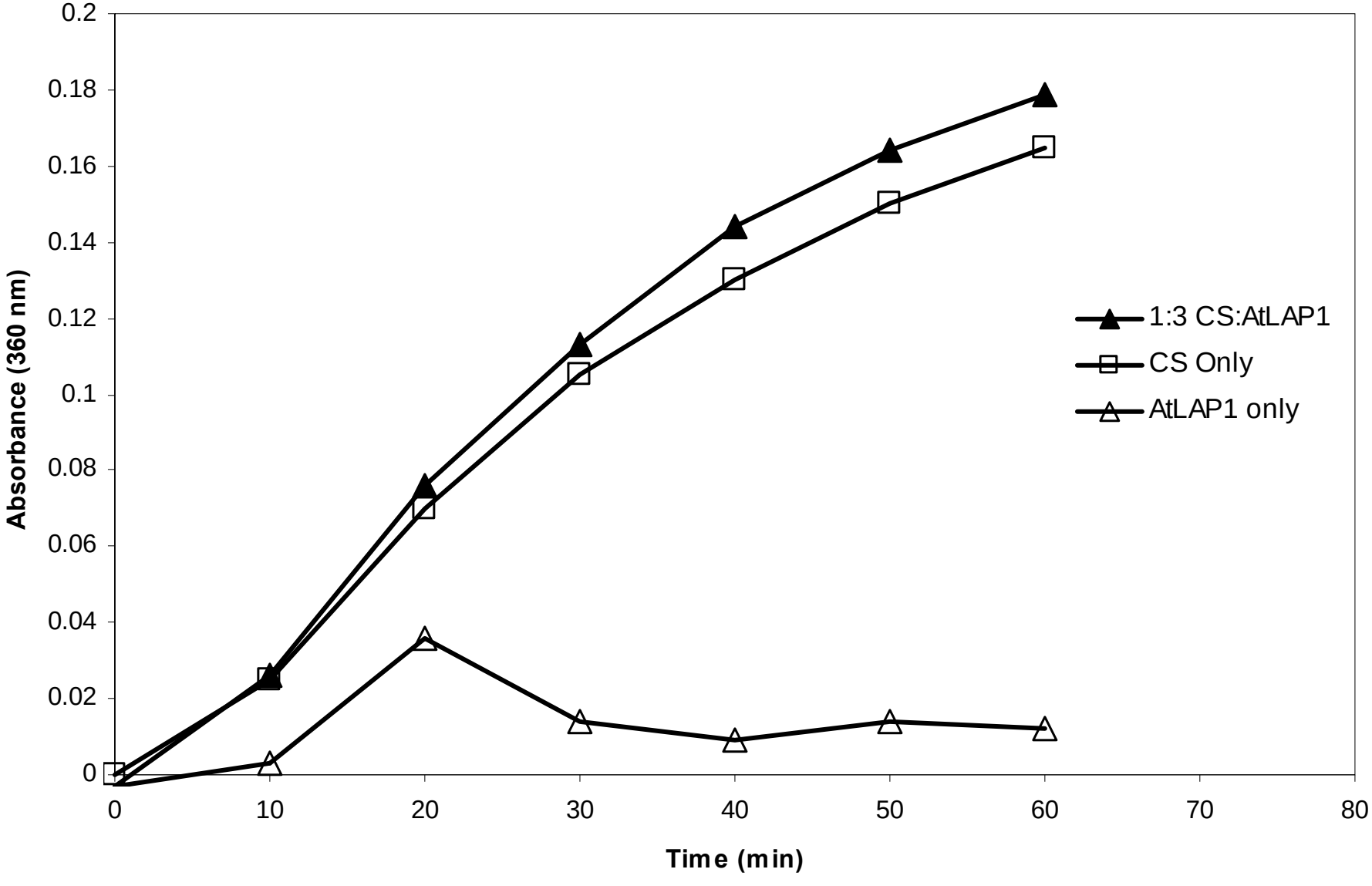

0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Absorbance (360 nm)
0
10
20
30
40
50
60
70
80
Time (min)
1:3 CS:AtLAP1
CS Only
AtLAP1 only

Figure S6

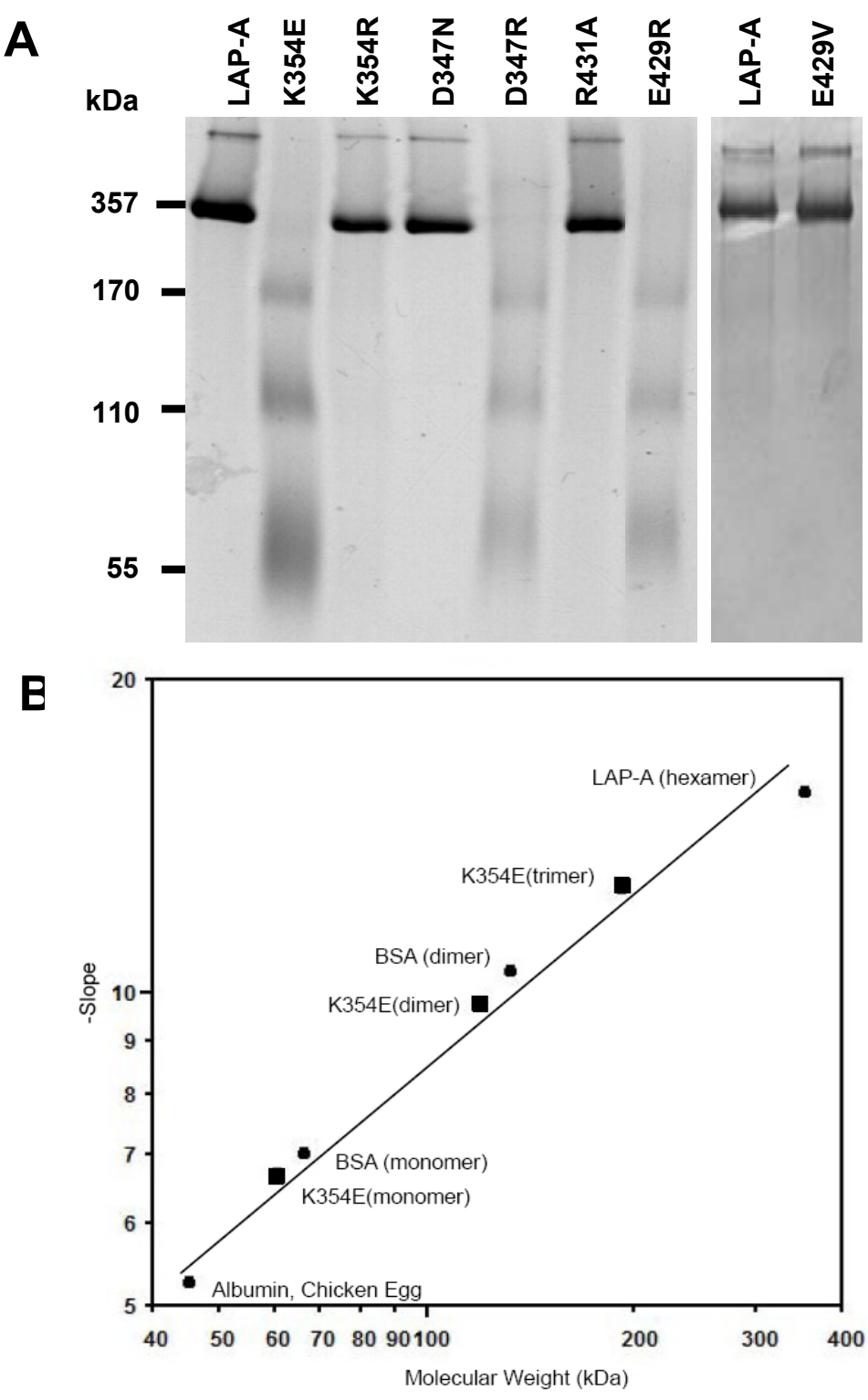

A
kDa
LAP-A
K354E
K354R
D347N
D347R
R431A
E429R
LAP-A
E429V
357
170
110
55
B
-Slope
20
10
9
8
7
6
5
LAP-A (hexamer)
K354E(trimer)
BSA (dimer)
K354E(dimer)
BSA (monomer)
K354E(monomer)
Albumin, Chicken Egg
40
50
60
70
80
90
100
200
300
400
Molecular Weight (kDa)

Figure S7

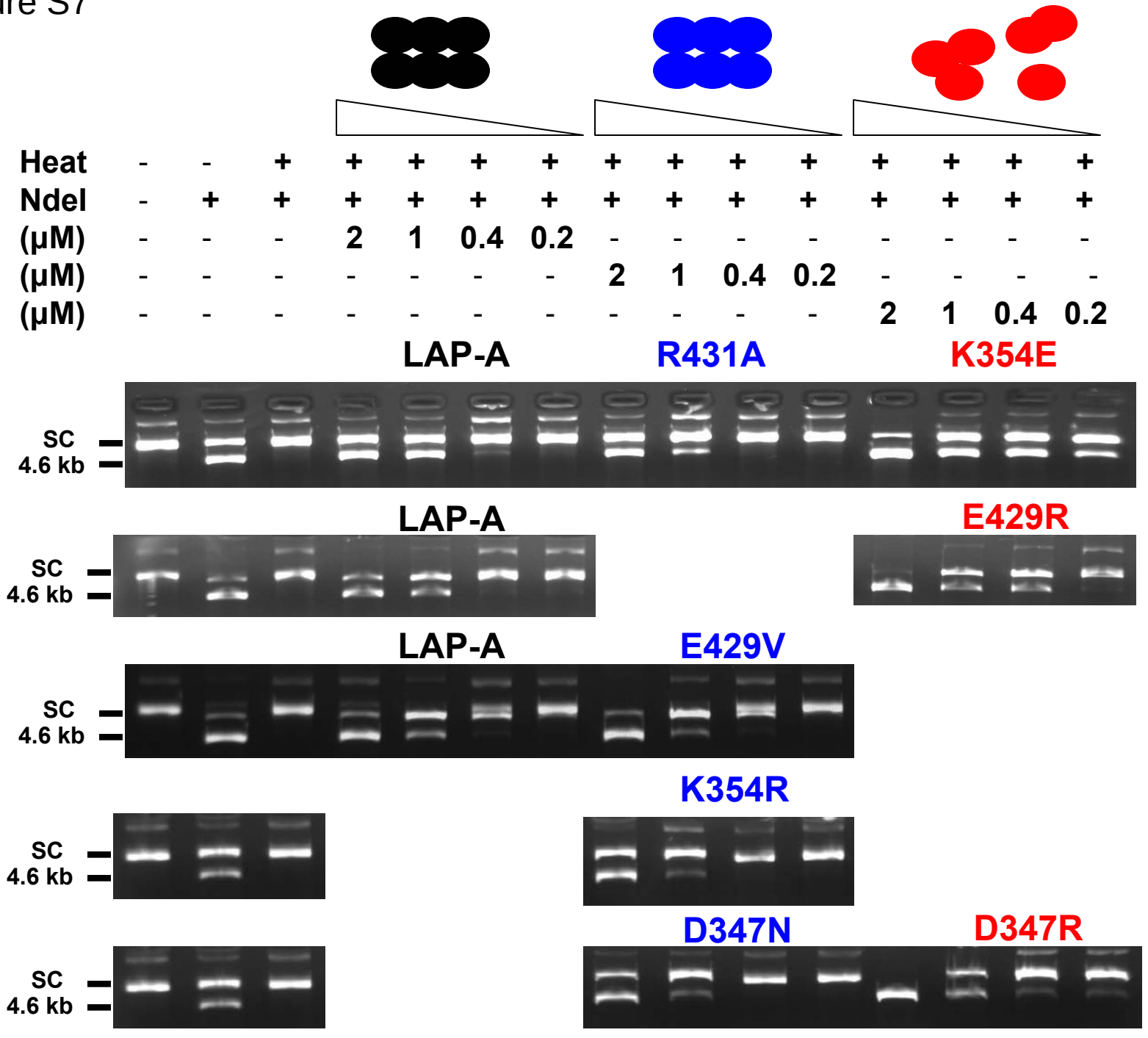

Heat - - + + + + + + + + + + + + + +
NdeI - + + + + + + + + + + + + + + +
(μM) - - - 2 1 0.4 0.2 - - - - - - - -
(μM) - - - - - - - 2 1 0.4 0.2 - - - -
(μM) - - - - - - - - - - - 2 1 0.4 0.2
LAP-A
R431A
K354E
SC
4.6 kb
LAP-A
E429R
SC
4.6 kb
LAP-A
E429V
SC
4.6 kb
K354R
SC
4.6 kb
D347N
D347R
SC
4.6 kb

Figure S8

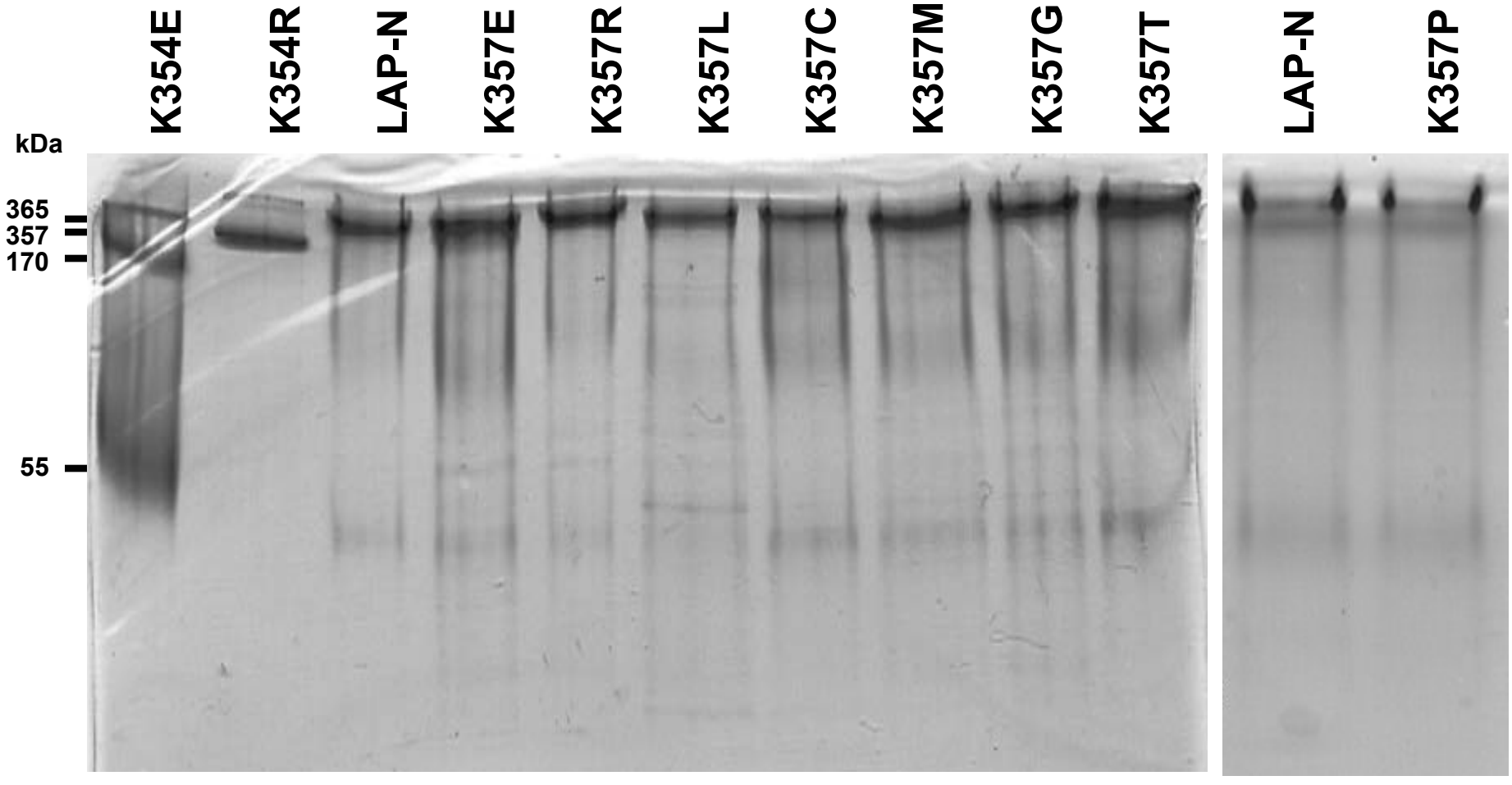

K354E
K354R
LAP-N
K357E
K357R
K357L
K357C
K357M
K357G
K357T
LAP-N
K357P
kDa
365
357
170
55

Figure S9

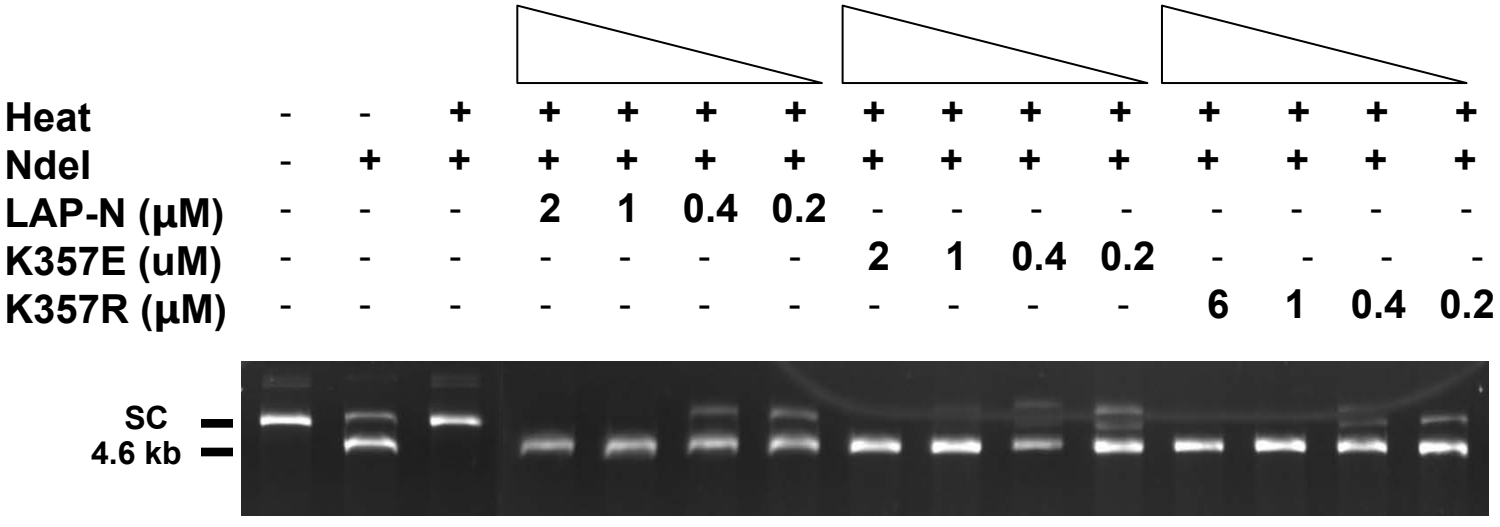

Heat - - + + + + + + + + + + + + + +
NdeI - + + + + + + + + + + + + + + +
LAP-N (µM) - - - 2 1 0.4 0.2 - - - - - - - -
K357E (uM) - - - - - - - 2 1 0.4 0.2 - - - -
K357R (µM) - - - - - - - - - - - 6 1 0.4 0.2
SC
4.6 kb

Figure S10

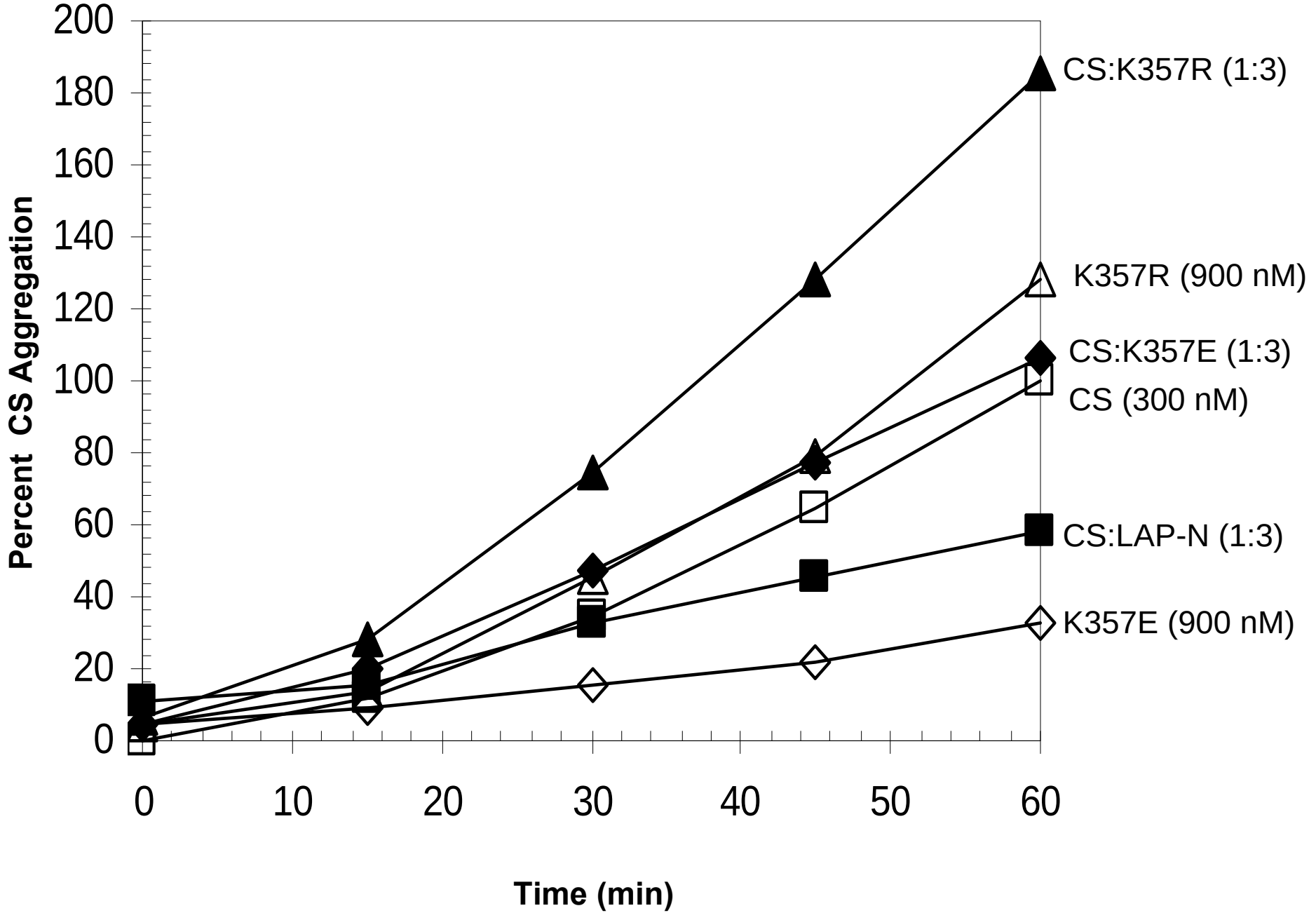
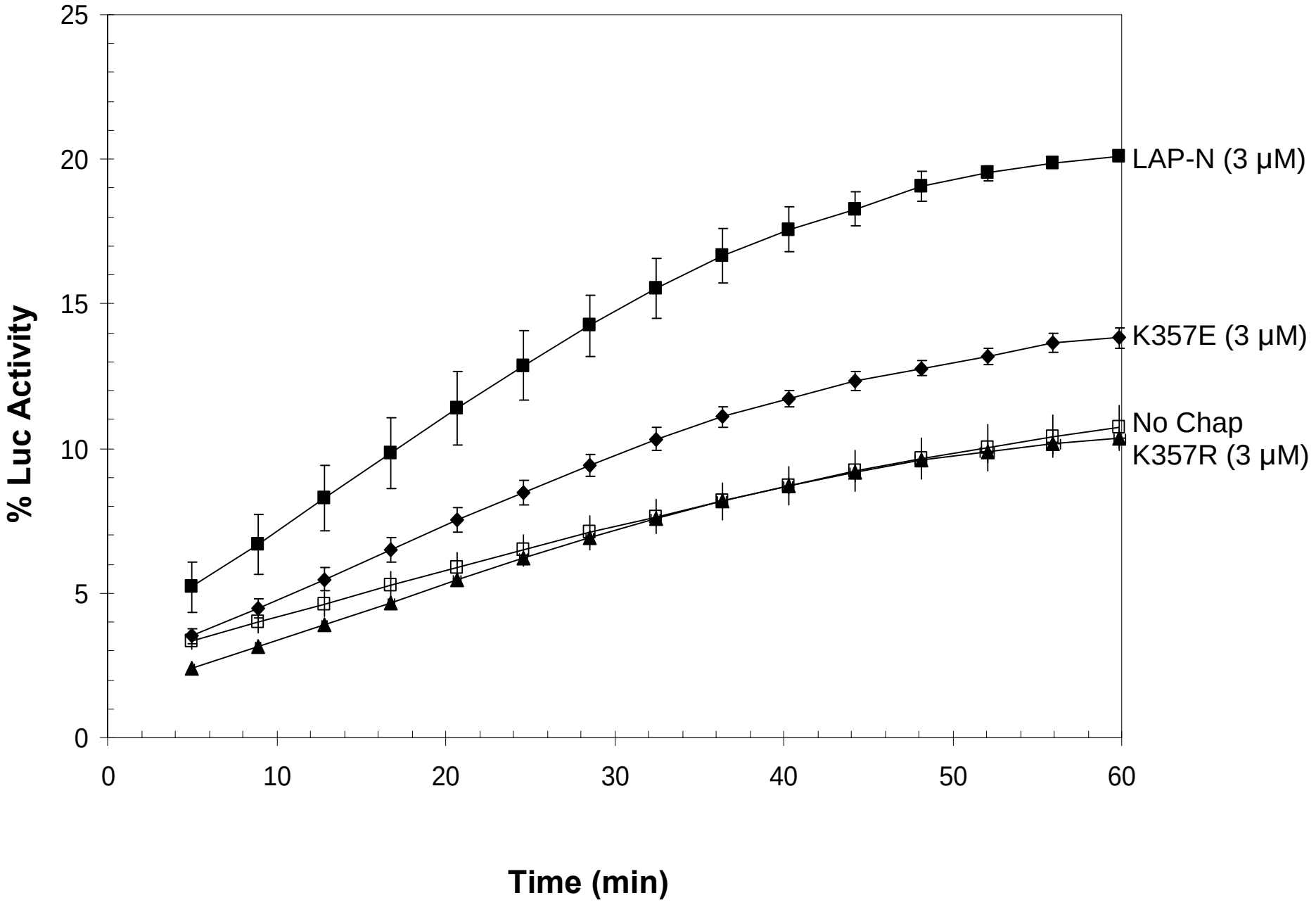

Percent CS Aggregation
200
180
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (min)
CS:K357R (1:3)
K357R (900 nM)
CS:K357E (1:3)
CS (300 nM)
CS:LAP-N (1:3)
K357E (900 nM)

Figure S11


25
20
15
10
5
0
% Luc Activity
0
10
20
30
40
50
60
Time (min)
LAP-N (3 μM)
K357E (3 μM)
No Chap
K357R (3 μM)