Supplementary Information

Insights to the assembly of functionally active leptospiral ClpP1P2 protease complex along with its ATPase chaperone ClpX

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Running Title: Caseinolytic serine proteases of *Leptospira*

MATERIALS AND METHODS

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA of *L. interrogans* was isolated from the log-phase of the growth curve using the TRIzol (Invitrogen) method. The obtained total RNA was treated with DNase (New England BioLabs [NEB]) and converted to cDNA as described before in our laboratory[1]. The oligonucleotides used for qRT-PCR were designed using the OligoPerfect primer design program (Invitrogen) from the available *L. interrogans* serovar Copenhageni genomic sequence. The PCR (CFX96 Real-Time, Bio-rad) system was programmed for 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by melt curve analyses of the PCR products. The primers used in RT-PCR are listed in Table S1. Transcripts of target genes were quantified using $2^{-\Delta\Delta C_T}$ method[2] and normalized with the 16S *rRNA* gene transcripts of *Leptospira*.

Estimation of rClpX ATPase activity. The known phosphate standards (0, 12.5, 25, 31.25, 37.5, and 50 μM) were prepared from a working concentration of 500 μM phosphate provided in the ATPase activity assay kit (Cat No. MAK113). The absorbance values obtained of these standards were used to plot the phosphate curve (620 nm wavelength). The concentration of free phosphate generated in the ATPase activity of rClpX was determined using the standard phosphate curve. The ATPase activity of rClpX was calculated as described in the protocol of the assay kit: ATPase activity of rClpX = $(S_a \times R_v)/(S_v \times T)$, where S_a = concentration of free phosphate (μM) generated by rClpX ; R_v = reaction volume (μL); S_v = sample volume (μL) added to well; T = reaction time (min).

Table S1. Oligonucleotides used for the quantification of gene transcripts of *Leptospira*

Primer	Sequence (5'-3')
qclpP1_F	ATGGAGCGTAATCCCGTATGTG
qclpP1_R	AAACAACAATTGAGCTGTAATGACA
qclpP2_F	ATGCCAGAAACAGAGAAAAAATCG
qclpP2_R	CCGGGACTATTGATATAAAAAGTGAT
16S rRNA_F	TTATTGCTCGGAGATGAGCC
16S rRNA_R	TTCAGGGTTCCCCCCATT

Table S2. Dynamic light scattering (DLS) data of rClpP isoforms of *Leptospira*

Sample Name	Polydispersity (nm)	Pd Index	Count rate (cps)	Estimated Molecular Weight (kDa)* (Mean ± SD) *[Major peak]
rClpP1_1 h or 24 h incubation	18.1	0.498	3,44,000	657.6±198.9
rClpP2_1 h or 24 h incubation	7.3	0.219	1,82,000	474.1±172.4
rClpP isoforms mixture_1 h incubation	21.3	0.570	2,66,900	658.1±182.5
rClpP isoforms mixture_24 h incubation	9.4	0.257	2,01,200	591.0±179.8

Pd –Polydispersity; cps – count per second

LEGENDS TO SUPPLEMENTARY FIGURES

Figure S1. Differential transcription analysis of *Leptospira clpP1* and *clpP2* under *in vitro* condition. Transcript analysis of *clpP1* and *clpP2* genes by qRT-PCR of cDNA synthesized from *Leptospira interrogans* serovar Copenhageni grown in EMJH (Ellinghausen-McCullough-Johnson-Harris) medium. The gene transcripts were calculated based on the threshold cycle (C_T) values by the use of $2^{-\Delta\Delta C_T}$ method and normalized with 16S *rRNA* transcripts values. Error bars represent the standard deviations (SDs) from two independent qRT-PCR analyses.

Figure S2. Oligomerization of *Leptospira* pure rClpP isoforms and their mixture. Leptospiral pure rClpP isoforms (1.0 µg each) and its mixture (0.5 µg of each of the pure rClpP isoforms) were incubated for 1 h and 48 h at 4°C before analysing the samples on native-PAGE. The samples were resolved on 7.5% native-gel and visualized by Coomassie staining.

Figure S3. DLS analysis of *Leptospira* pure rClpP isoforms. (A and B) Correlation coefficient and mass distribution graphs of *Leptospira* pure rClpP isoforms after 24 h incubation (4°C).

Figure S4. DLS analysis of *Leptospira* rClpP isoforms mixture. (A) Correlation coefficient and mass distribution graphs of *Leptospira* rClpP isoforms mixture after 1 h incubation (4°C) (B) Correlation coefficient and mass distribution graphs of *Leptospira* rClpP isoforms mixture after 24 h incubation (4°C).

Figure S5. Oligomerization of pure rClpP serine mutants. Native 4-20% gradient gel image demonstrate oligomerization of pure rClpP serine mutants identical to wild-type pure ClpP isoforms of *Leptospira*.

Figure S6. Estimation of rClpX ATPase activity. (A) Phosphate standard curve plotted using known standard phosphate concentrations provided in the ATPase assay kit. (B) Absorbance readings of rClpX ATPase activity assay at 620 nm wavelength. Experiments were performed twice independently in duplicates; error bars represent the SDs from the two independent experiments.

REFERENCES

1. Ghosh, K. K.; Prakash, A.; Balamurugan, V.; Kumar, M., Catecholamine-Modulated Novel Surface-Exposed Adhesin LIC20035 of Leptospira spp. Binds Host Extracellular Matrix Components and Is Recognized by the Host during Infection. *Appl Environ Microbiol* **2018,** *84* (6).
2. Schmittgen, T. D.; Livak, K. J., Analyzing real-time PCR data by the comparative CT method. *Nature Protocols* **2008,** *3*, 1101.

Figure S1.

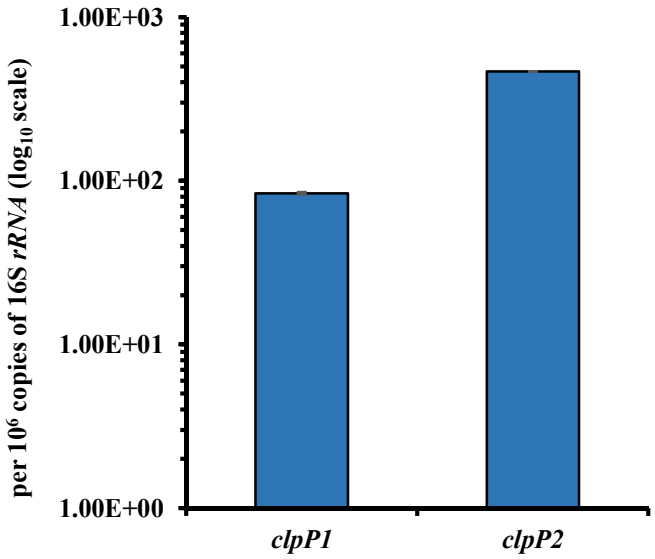

1.00E+03
1.00E+02
1.00E+01
1.00E+00
per 10^6 copies of 16S rRNA (log10 scale)
clpP1
clpP2

Figure S2.

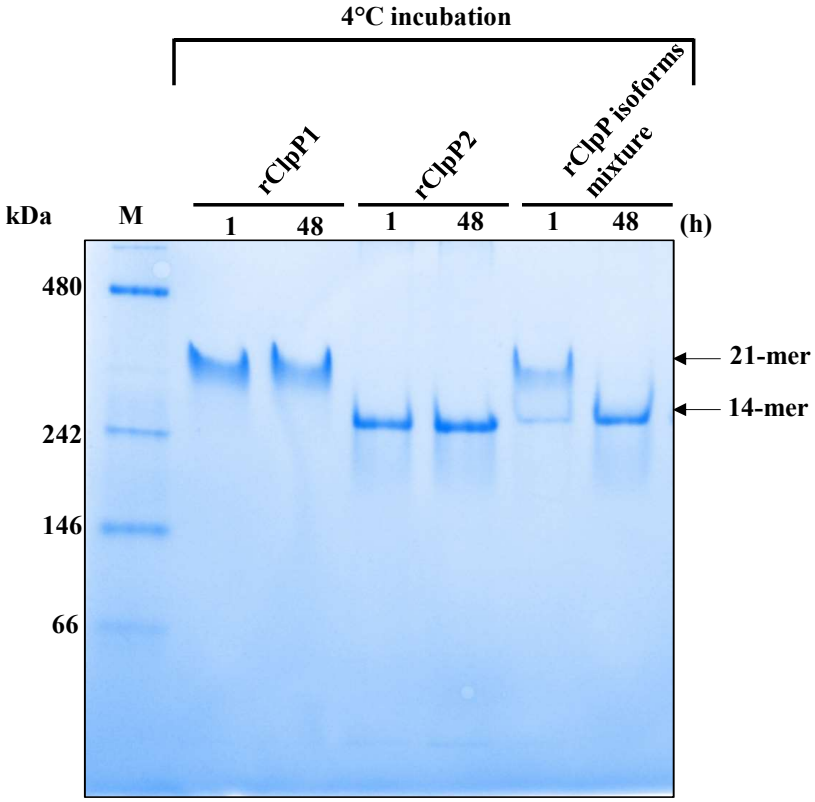
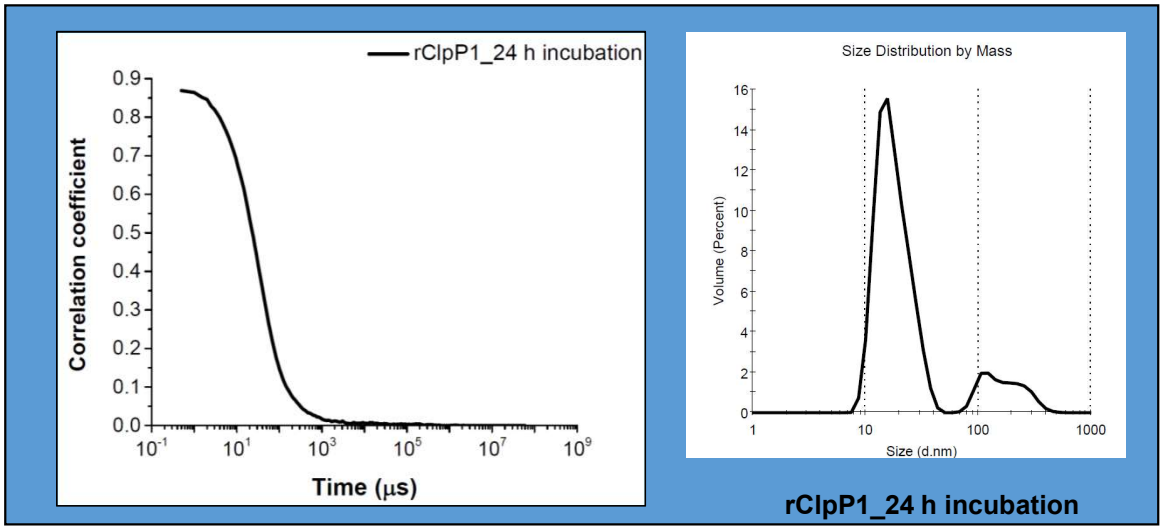

4°C incubation
rClpP1
rClpP2
rClpP isoforms mixture
kDa
M
1
48
1
48
1
48
(h)
480
242
146
66
21-mer
14-mer

Figure S3.

A


rClpP1_24 h incubation
Correlation coefficient
Time (μs)
Size Distribution by Mass
Volume (Percent)
Size (d.nm)
rClpP1_24 h incubation

B

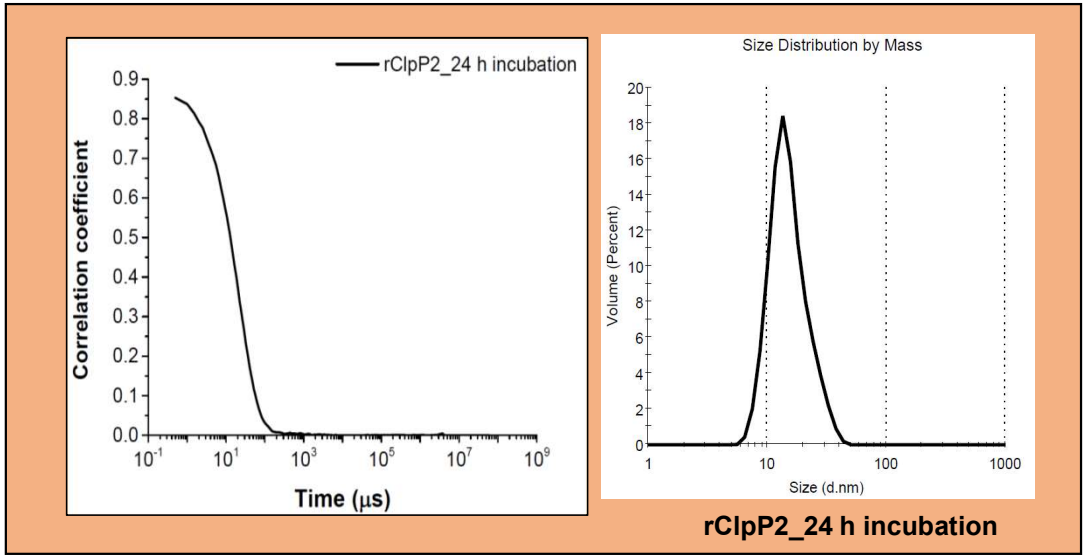
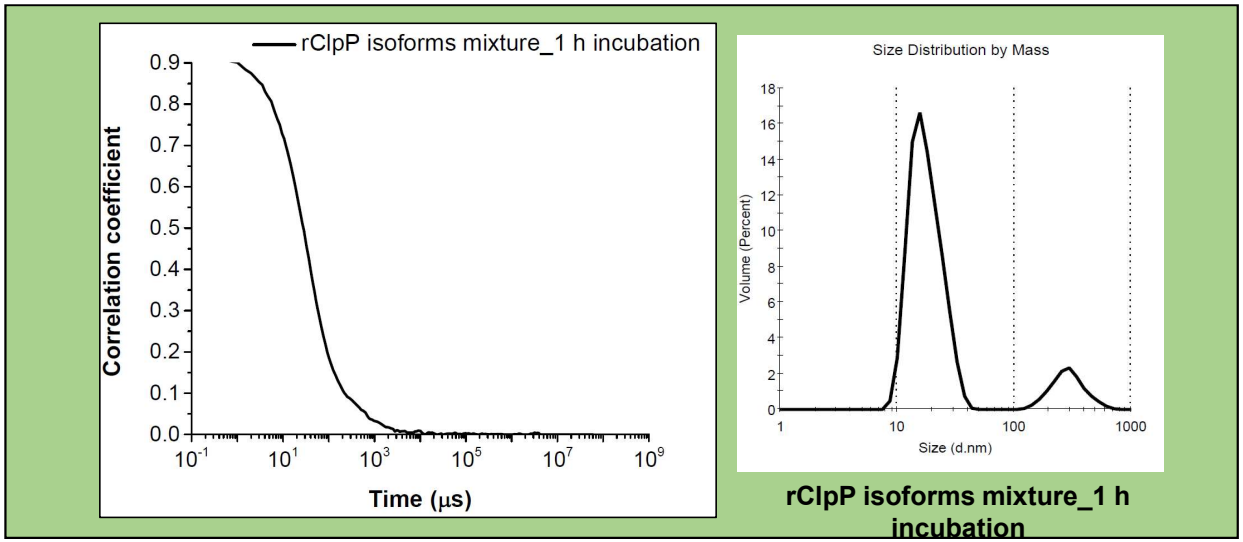

rClpP2_24 h incubation
Correlation coefficient
Time (μs)
Size Distribution by Mass
Volume (Percent)
Size (d.nm)
rClpP2_24 h incubation

Figure S4.

A


rClpP isoforms mixture_1 h incubation
Correlation coefficient
Time (μs)
Size Distribution by Mass
Volume (Percent)
Size (d.nm)
rClpP isoforms mixture_1 h incubation

B

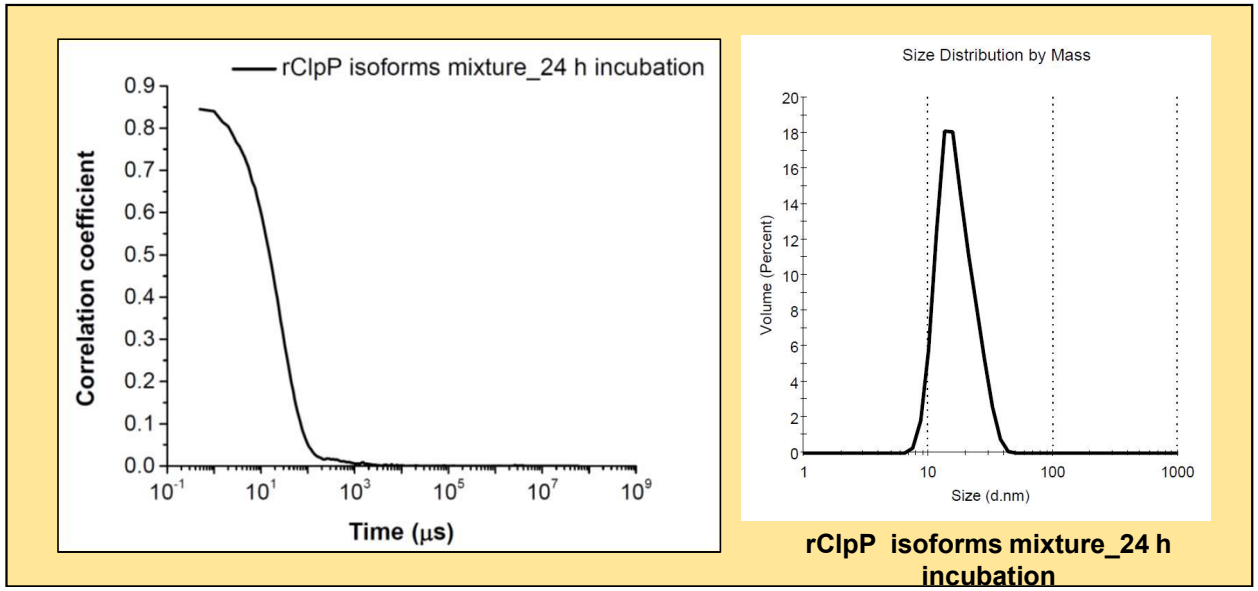

rClpP isoforms mixture_24 h incubation
Correlation coefficient
Time (μs)
Size Distribution by Mass
Volume (Percent)
Size (d.nm)
rClpP isoforms mixture_24 h incubation

Figure S5.

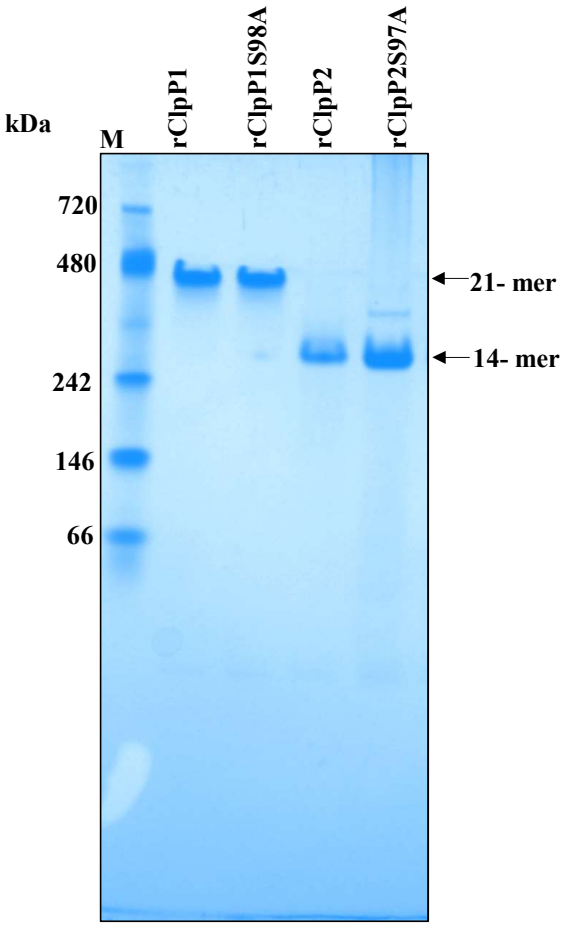
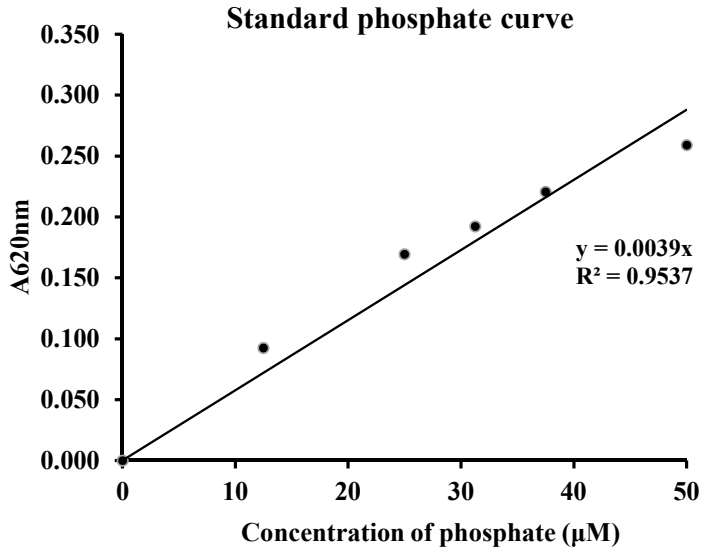
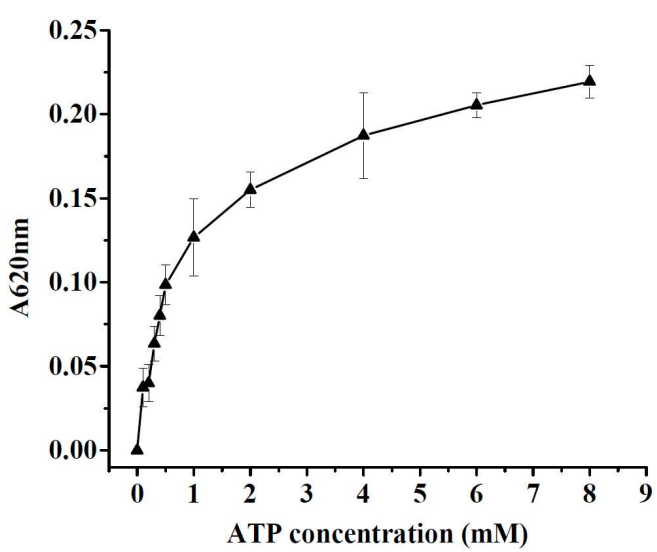

kDa
M
rClpP1
rClpP1S98A
rClpP2
rClpP2S97A
720
480
242
146
66
21- mer
14- mer

Figure S6.

A


Standard phosphate curve
A620nm
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0
10
20
30
40
50
Concentration of phosphate (μM)
y = 0.0039x
R² = 0.9537

B


A620nm
0.25
0.20
0.15
0.10
0.05
0.00
0
1
2
3
4
5
6
7
8
9
ATP concentration (mM)