Supplementary material

Identification of potential mutational hotspots in serratiopeptidase to address its poor pH tolerance issue

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Table S1. (a) Surface accessibility percentage for asparagine (ASN) residues in serratiopeptidase using Hotspot Wizard.

Position	Amino Acid	Secondary structure	Accessible surface area	Mutability & Mutability score	Relative flexibility	B-factor (Angstrom)
191	ASN (N)	Loop (L)	57.4%	Moderate (4)	High	65.54
20	ASN (N)	Hydrogen bond turn (T)	98.6%	Moderate (5)	High	52.07
25	ASN (N)	Hydrogen bond turn (T)	52.3%	Moderate (4)	High	44.78
412	ASN (N)	Hydrogen bond turn (T)	88.0%	High (6)	High	29.34
289	ASN (N)	Loop (L)	60.1%	High (6)	Moderate	18.72
262	ASN (N)	Hydrogen bond turn (T)	73.2%	Moderate (5)	Moderate	17.87
264	ASN (N)	Loop (L)	58.8%	Moderate (4)	Moderate	15.87
300	ASN (N)	Loop (L)	61.0%	Moderate (5)	Moderate	15.77
346	ASN (N)	Loop (L)	60.4%	Moderate (5)	Low	11.14

Table S1. (b) Surface accessibility percentage for glutamine (GLN) residues in serratiopeptidase using Hotspot Wizard.

Position	Amino Acid	Secondary structure	Accessible surface area	Mutability & Mutability score	Relative flexibility	B-factor (Angstrom)
144	GLN (Q)	Hydrogen bond turn (T)	68.8%	High (6)	High	52.52
146	GLN (Q)	Extended strand (E)	54.7%	High (7)	High	49.25
23	GLN (Q)	Extended strand (E)	59.4%	Moderate (5)	High	44.09
396	GLN (Q)	Loop (L)	55.9%	High (6)	Moderate	19.18

Table S2. (a) Missense mutation prediction of serratiopeptidase mutants (N to D) using DynaMut server.

Position	NMA	mCSM	SDM	DUET	Prediction outcome
N20D	0.107	0.000	-0.070	0.248	0.224
N25D	-0.119	0.079	-0.230	0.286	-0.059
N191D	0.274	0.149	0.310	0.395	0.705
N412D	0.098	0.033	-0.640	0.123	0.098
N262D	-0.086	0.191	-1.010	0.187	0.205
N264D	0.024	0.295	-0.630	0.455	0.162
N289D	0.035	-0.61	-0.60	-0.314	-0.047
N300D	-0.167	-0.365	0.180	0.009	0.038
N346D	0.068	-0.714	0.020	-0.390	0.254

Table S2. (b) Missense mutation prediction of serratiopeptidase mutants (Q to E) using DynaMut server.

Position	NMA	mCSM	SDM	DUET	Prediction outcome
Q23E	**-0.102**	**0.002**	**0.480**	**0.559**	**-0.316**
Q144E	0.121	0.027	0.540	0.420	0.529
Q146E	0.098	-0.030	0.540	0.540	0.556
Q396E	0.311	0.017	0.520	0.359	0.455

Table S3. (a) Amino-acid substitution stability of serratiopeptidase mutants (N to D) using CUPSAT program.

Position	Solvent accessibility	Overall stability	Torsion favoured (%)	Predicted delta G (kcal/mol)
N20D	114.75 %	Unstable	Favorable	-0.57
N191D	67.62%	Stable	Unfavorable	2.15
N262D	83.33 %	Unstable	Favorable	-0.13
N264D	65.57 %	Stable	Favorable	0.26
N300D	69.67 %	Stable	Favorable	0.65
N346D	70.36 %	Stable	Favorable	0.28
N412D	101.09 %	Unstable	Favorable	-1.68

Table S3. (b) Amino-acid substitution stability of serratiopeptidase mutants (Q to E) using CUPSAT program.

Position	Solvent accessibility	Overall stability	Torsion favoured (%)	Predicted delta G (kcal/mol)
Q144E	75.59 %	Stable	Unfavorable	0.85
Q146E	59.91 %	Unstable	Unfavorable	-0.31
Q396E	63.83 %	Stable	Unfavorable	0.67

Table S4. (a) Protein stability prediction of serratiopeptidase mutants (N to D) using I-Mutant 2.0 prediction server.

Mutant type	Stability	T	pH	RI	RSA
N20D	Increase	40	1.5	7	112.7
N191D	Increase	40	1.5	4	69.0
N262D	Increase	40	1.5	6	84.0
N264D	Increase	40	1.5	0	65.6
N300D	Increase	40	1.5	3	71.0
N346D	Increase	40	1.5	0	69.7
N412D	Increase	40	1.5	6	101.8

Table S4. (b) Protein stability prediction of serratiopeptidase mutants (Q to E) using I-Mutant 2.0 prediction server.

Mutant type	Stability	T	pH	RI	RSA
Q144E	Increase	40	1.5	6	75.0
Q146E	Increase	40	1.5	5	60.5
Q396E	Decrease	40	1.5	4	64.9

Table S5. (a) Physicochemical characterization of serratiopeptidase native and mutant forms (N to D) using ExPASy ProtParam tool.

Position	Molecular weight	PI	Total no of atoms	Instability index
Wild	50275	4.54	6875	22.54
N20D	50276.48	4.51	6874	23.04
N191D	50276.48	4.51	6874	23.04
N262D	50276.48	4.51	6874	23.21
N264D	50276.48	4.51	6874	22.40
N300D	50276.48	4.51	6874	22.52
N346D	50276.48	4.51	6874	22.36
N412D	50276.48	4.51	6874	22.77

Table S5. (b) Physicochemical characterization of serratiopeptidase native and mutant forms (Q to E) using ExPASy ProtParam tool.

Position	Molecular weight	PI	Total no of atoms	Instability index
Wild	50275	4.54	6875	22.54
Q144E	50276	4.52	6874	22.54

Table S6. (a) Protein model generation of mutants (N to D) in contrast to Wild-Type serratiopeptidase using SWISS-MODEL.

Position	Mol probity score	Clash score	Ramachandran favoured	Rotamer outlier	C-beta deviation	Bad bonds (3644)	Bad angles (4955)
Wild	0.94	0.44	97.65 %	1.92 %	2	0	35
N20D	0.93	0.44	97.44%	1.92 %	2	0	36
N191D	0.83	0.00	97.44%	1.64%	3	0	35
N262D	0.98	0.44	97.44%	1.92 %	2	0	38
N264D	0.98	0.44	97.44%	1.92 %	2	0	38
N300D	0.98	0.44	97.44%	1.92 %	2	0	36
N346D	0.98	0.44	97.44%	1.92 %	2	0	38
N412D	0.98	0.44	97.44%	1.92 %	3	0	35

Table S6. (b) Protein model generation of mutants (Q to E) in contrast to Wild-Type serratiopeptidase using SWISS-MODEL.

Position	Mol probity score	Clash score	Ramachandran favoured	Rotamer outlier	C-beta deviation	Bad bonds (3644)	Bad angles (4955)
Wild	0.94	0.44	97.65 %	1.92 %	2	0	35
Q144E	0.98	0.44	97.44 %	1.92 %	3	1	35

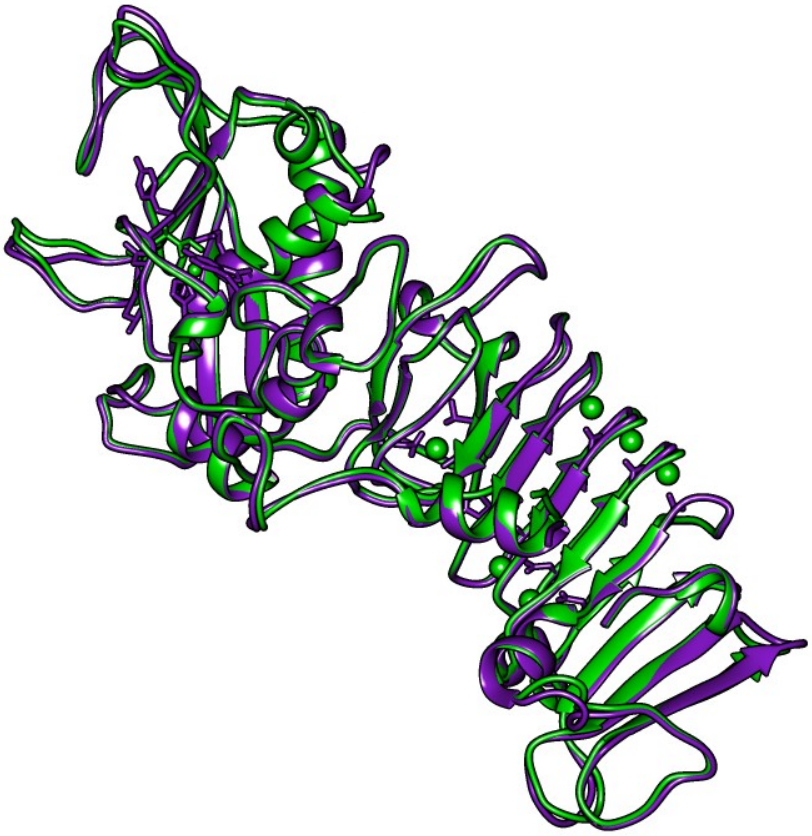

Fig. S1. Comparison of modeled structure N412D mutant of serratiopeptidase (PDB ID: 1SAT) by superimposition: SWISS-MODEL (purple) and AlphaFold (green) models.

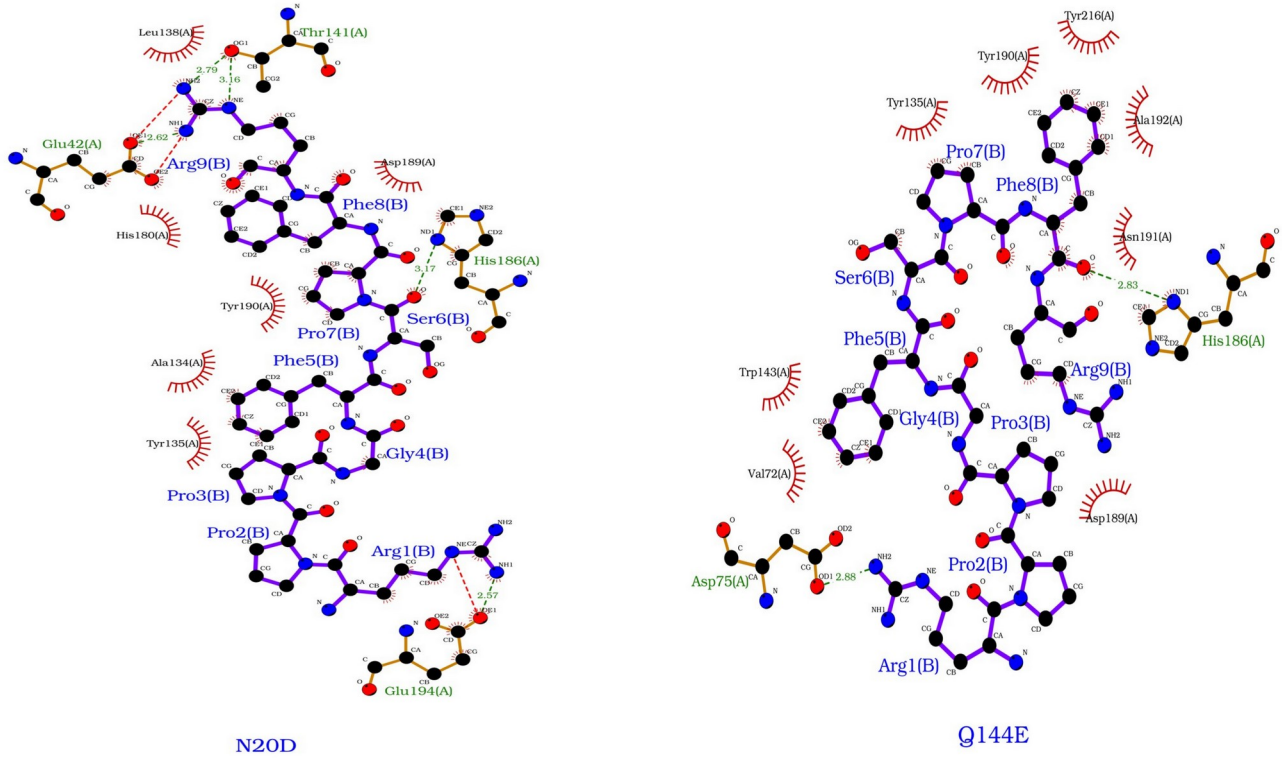

Fig. S2. Intramolecular H-bond demonstration of serratiopeptidase mutants (N20D and Q144E) in complex with bradykinin (substrate) using ligplot.

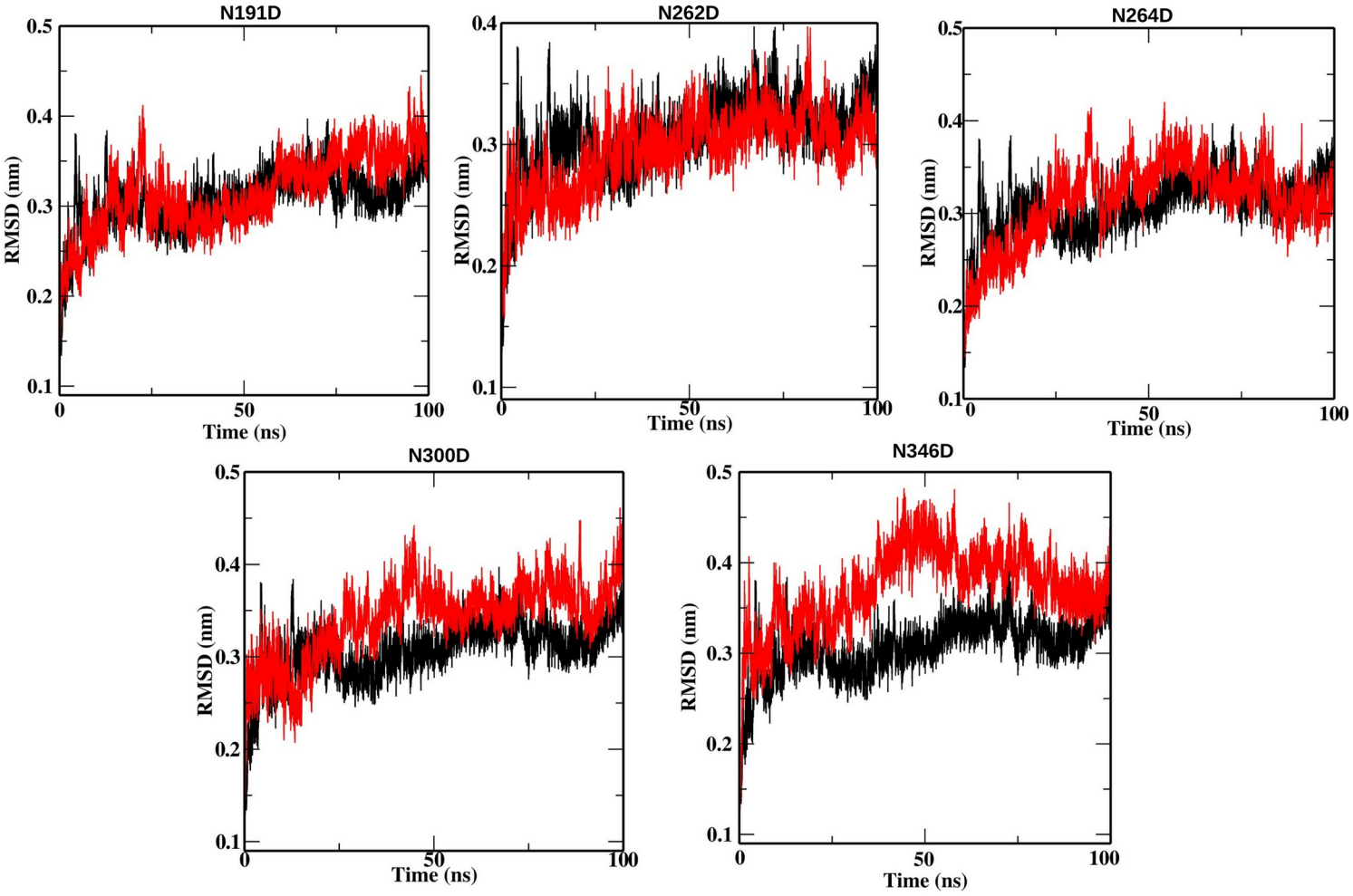

Fig. S3. RMSD plots analysis of all the mutants (red) with respect to Wild-Type serratiopeptidase (black).

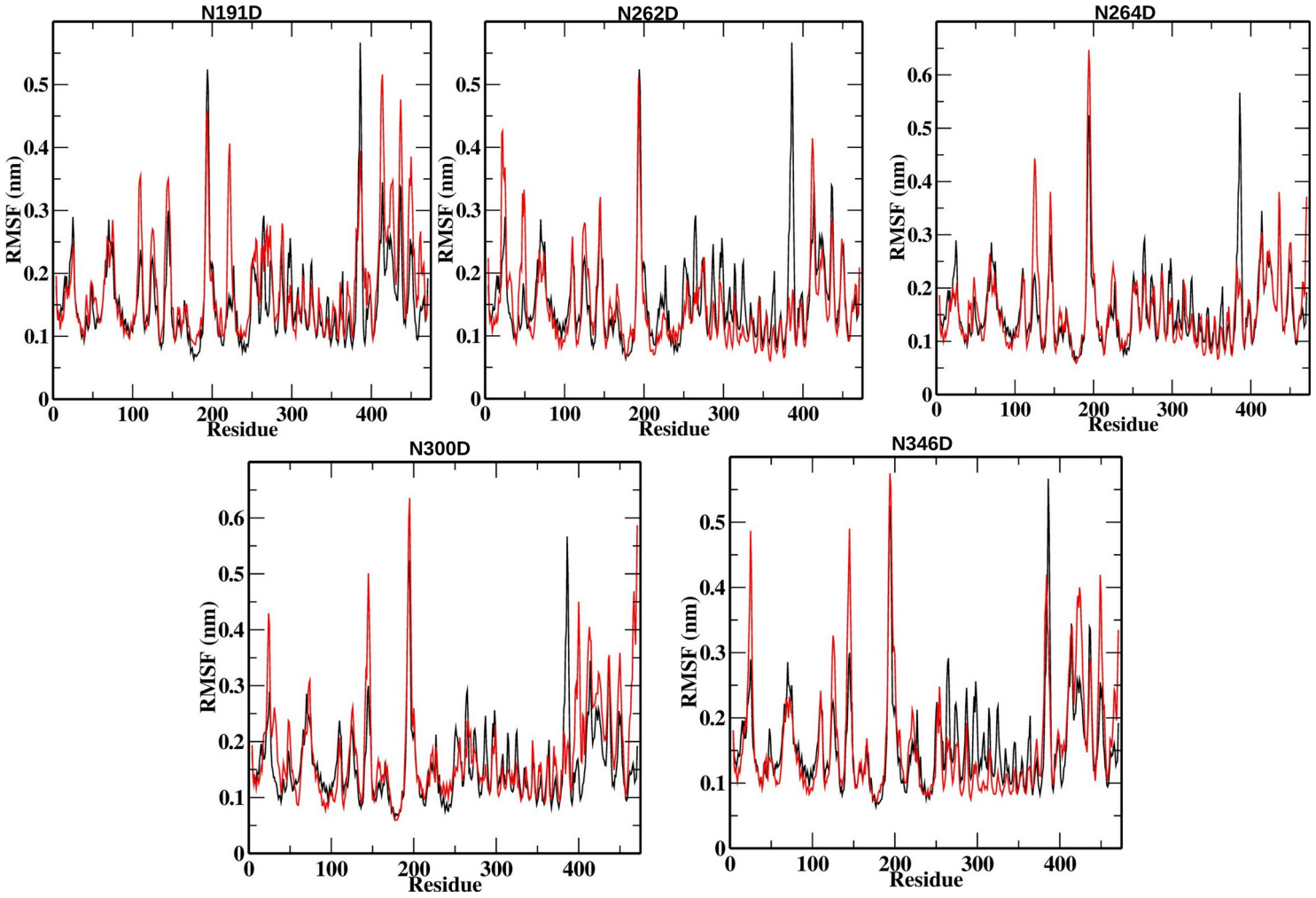

Fig. S4. RMSF plots analysis of all the mutant forms (red) in contrast to Wild-Type serratiopeptidase (black).

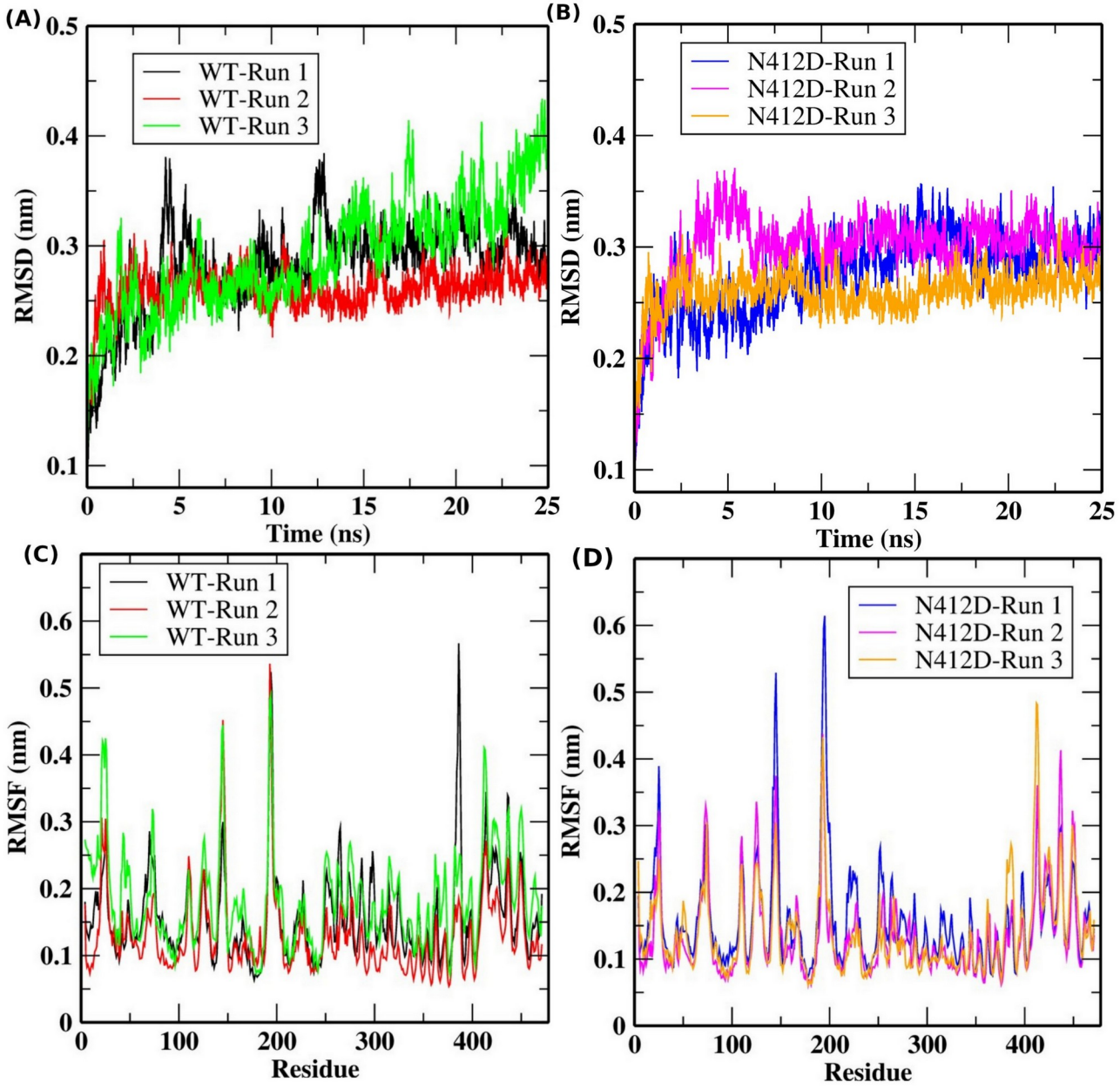

Fig. S5. Graphical representation of MD trajectories in triplicate to check the robustness of simulated systems.

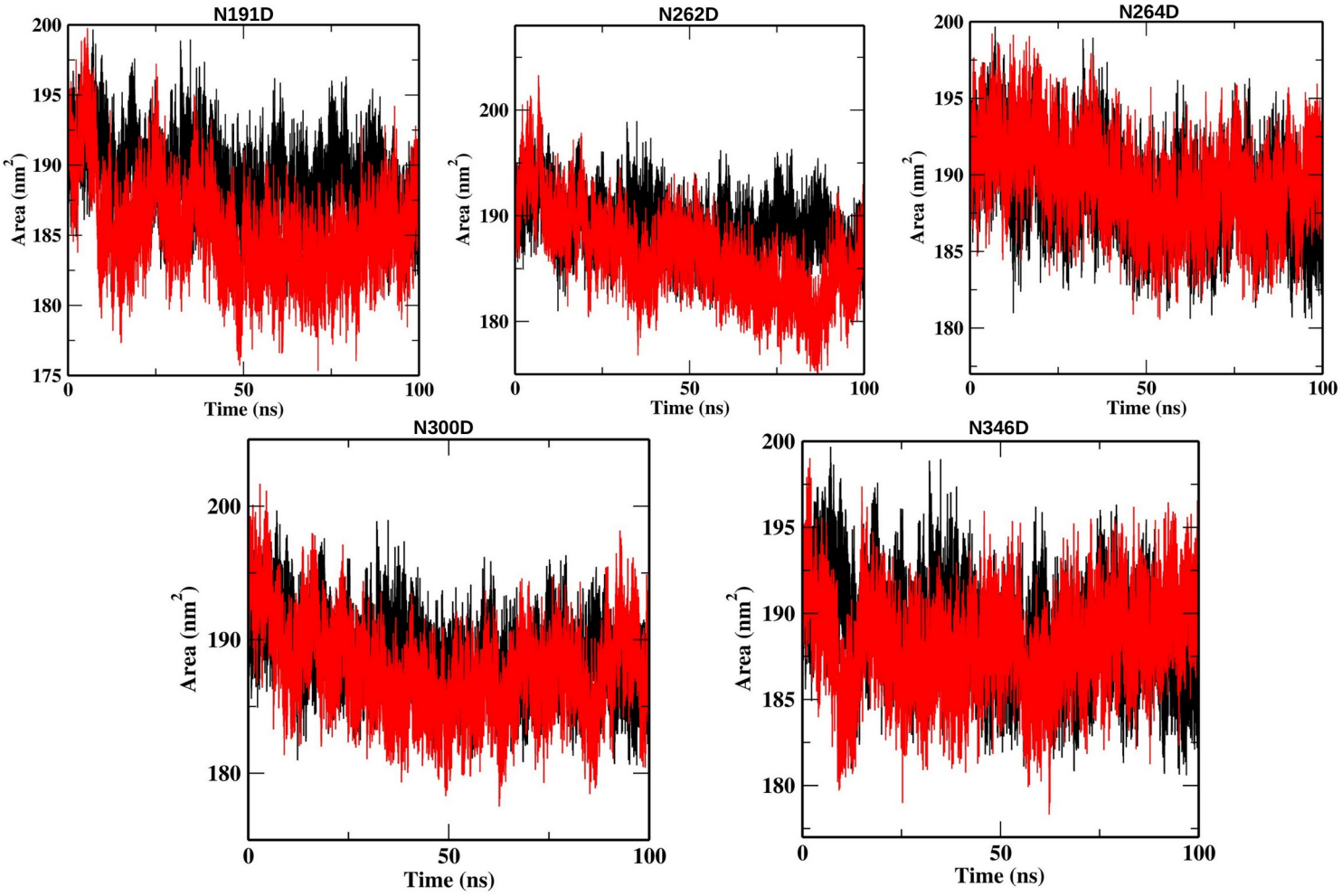

Fig. S6. SASA analysis plots for the generated mutant forms (red) in comparison to Wild-Type serratiopeptidase (black).

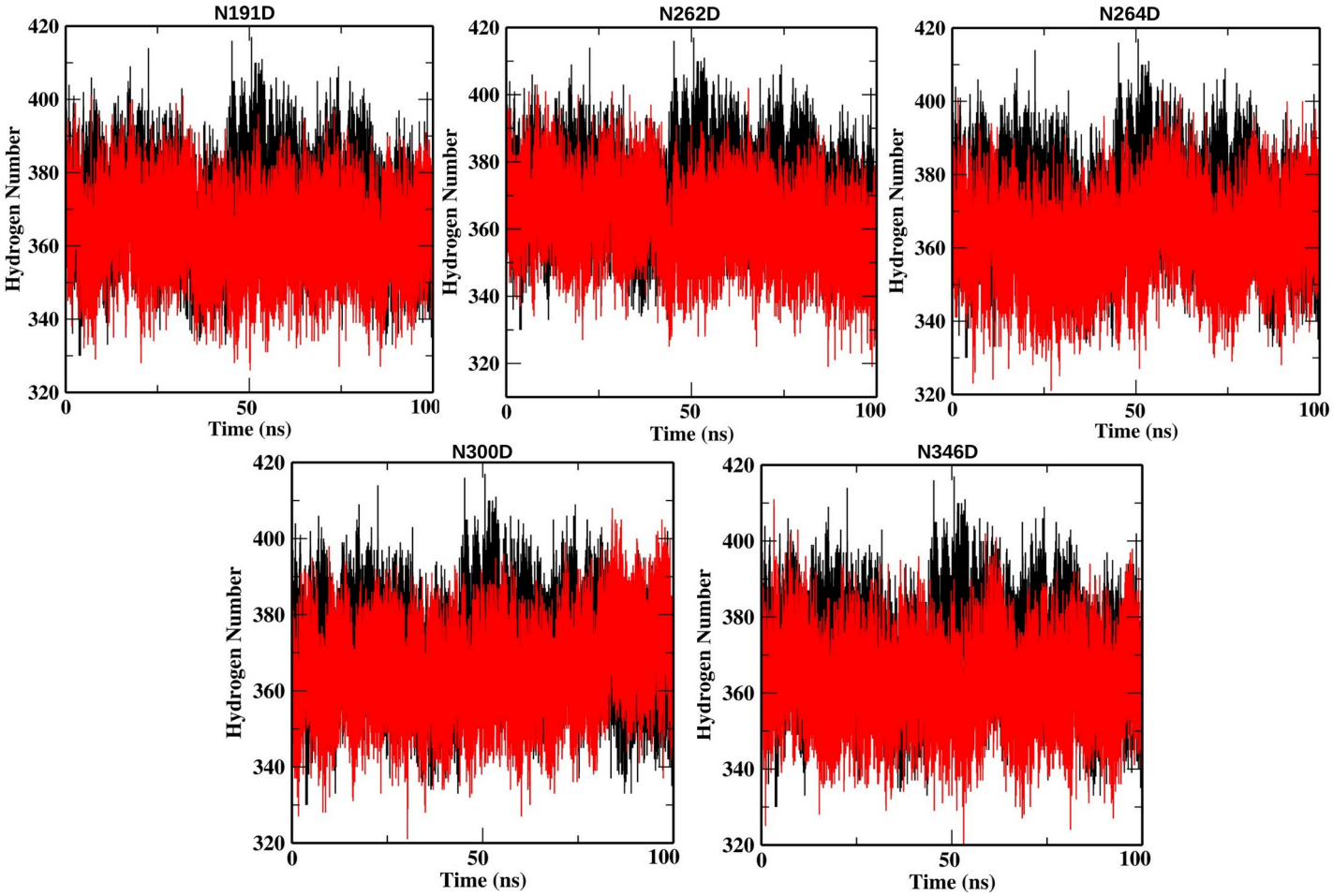

Fig. S7. H-bond analysis of the mutants (red) in contrast to Wild-Type serratiopeptidase (black).

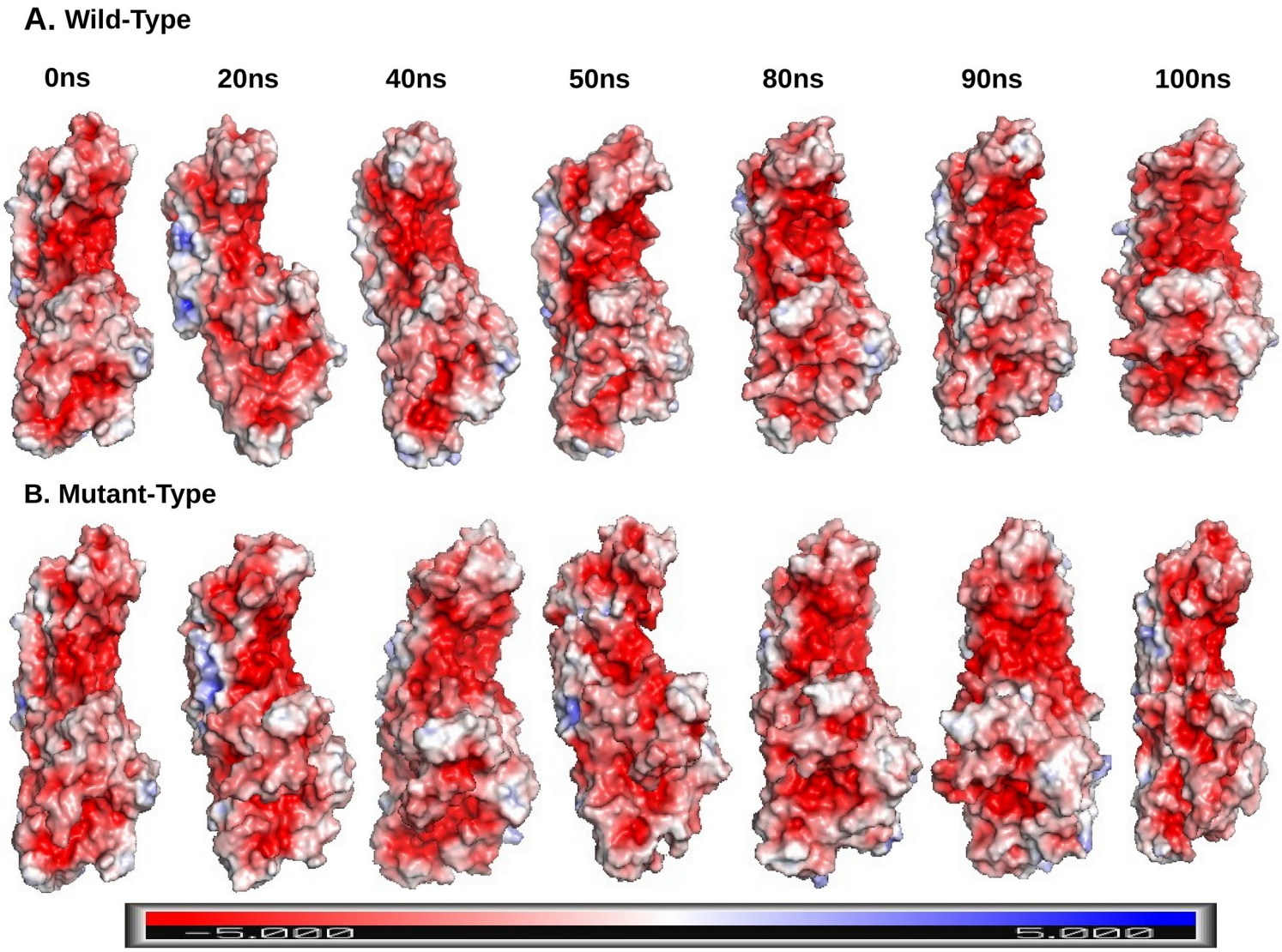

Fig. S8. Electrostatic potential analysis of N412D mutant of serratiopeptidase at different time-steps using ABPS program.