1 **Super RLuc8; a Novel Engineered *Renilla* Luciferase with a Red-shifted Spectrum and Stable Light**
2 **Emission**

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26 **Abstract**

27 *Renilla* luciferase is a bioluminescent enzyme which is broadly used as a reporter protein in
28 molecular biosensors. In this study, a novel luciferase with desired light emission wavelength and
29 thermostability is reported. The results indicated that the new luciferase, namely super RLuc8, had a
30 red-shifted spectrum and showed stable light emission. Super RLuc8 showed a 10-fold (p-value=
31 0.0084) increase in the thermostability at 37 °C after 20 min incubation, in comparison to the native
32 enzyme. The optimum temperature of the mutant increased from 30 to 37 °C. Molecular dynamics
33 simulation analysis indicated that the increased thermostability was most probably caused by a better
34 structural compactness and more local rigidity in the regions out of the emitter site.

35 **Keywords:** *Renilla* luciferase, enzyme kinetics, emission spectrum, MD Simulation, super RLuc8

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37 **1. Introduction**

38 Luciferases are used as building blocks of diagnostic tools for *in vivo* imaging and gene reporting. Beetle
39 and coelenterazine-based luciferases are two reporter proteins that have been extensively applied over the
40 past several years [1]. Among coelenterazine-based luciferases, *Renilla* luciferase (RLuc) is an applicable
41 diagnostic tool with some advantages over other luciferases. This monomeric enzyme is 311 amino acids
42 long with a total molecular weight of approximately 37 kDa. Structurally, it consists of two domains i.e. a
43 cap-domain and an α/β hydrolase domain which most key residues for enzyme activity such as the
44 presumptive catalytic triad (D120, E144, and H285) and the substrate entrance tunnel being located in the
45 cap-domain [2,3]. During the bioluminescence reaction, RLuc oxidizes the coelenterazine and produces
46 dioxetane. Subsequently, dioxetane loses CO₂ and yields coelenteramide in its excited state. This excited
47 intermediate is then dissipated by the emission of a photon (480nm), which allows the molecule to go
48 back to its ground state; and no cofactors such as ATP are required for the enzymatic reaction. [4, 5]. The
49 bioluminescence characteristics of *Renilla* luciferase make the enzyme attractive for certain applications
50 such as molecular imaging. RLuc-based probes are also being developed in cancer research, targeting and
51 quantitation of specific cells. For instance RLuc-labeled Annexin V has been used for the detection of
52 apoptotic cells [6, 7, 8, and 9].

53 Despite recent applications, the drawbacks of using native RLuc are its rapid inactivation, its blue
54 bioluminescence and its short half-life of light emission which limit its biotechnological applications
55 especially for *in vivo* imaging [10]. Various efforts have been made to improve native RLuc properties

and thus directed evolutions of the native RLuc have been carried out to create new versions of the enzyme with improved properties [11, 12, and 13]. Woo *et al.* reported a variant of RLuc, super RLuc, obtained by substitution of three amino acids (K189V, V267I and M185V) in the RLuc sequence. Compared with the native RLuc, super RLuc has a higher turnover number, increased light output upon expression in *Arabidopsis* and enhanced half-life of photon emission. Nevertheless, super RLuc is a blue light emitting luciferase and represents low thermal stability [14].

An RLuc with eight mutations (A55T, C124A, S130A, K136R, A143M, M185V, M253L, and S287L) in its structure was generated by Loaning *et al.* The enzyme, named as RLuc 8, showed a 4-fold enhancement in light output compared with the native enzyme. In spite of improvement in the thermal stability of RLuc 8, its light emission peaks were observed in the blue region of the visible spectrum; in addition, RLuc 8 has flash-type light emission kinetics. These features make RLuc 8 less favourable for imaging applications. In subsequent studies, different mutants including RLuc8.6-535, RLuc8.6-545 and RLuc8.6-547 were designed and created by substituting certain amino acids in the RLuc8 sequence. Compared with the native RLuc, the manipulated versions of RLuc show red-shifted emission peaks upon reaction with coelenterazine or its analogues; therefore, these mutants seem to be more suitable for imaging in living organisms. Although these variants have red-shifted emission light and a relatively high stability, their flash-type light emission kinetics is a limiting factor for their application in *in vivo* imaging [10].

In the present study, we combined multiple mutations of super RLuc and RLuc8.6,545 to create a new luciferase type with desired light emission wavelength and thermostability. The new mutant, super RLuc8, may provide a new tool for developing and improving RLuc-based biosensors.

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79 **2. Materials and Methods**

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81 **2.1. Materials**

82 The chemical used such as isopropyl- β -D-thiogalactopyranoside (IPTG) were purchased from Bio Basic
83 Inc. (Canada). Primers were purchased from TAG Copenhagen (Denmark). DNA polymerase, restriction
84 enzymes including; *Nde* I and *Xho* I and plasmid extraction kit were purchased from Thermo
85 Scientific™, nickel-nitrilotriacetic acid (NTA) sepharose was purchased from Novagen (Madison, USA).

86 **2.2. Design and Construction of Codon-optimized Super RLuc8**

87 The DNA sequence of the new RLuc was designed by combining RLuc 8.6,545 and K189V, V267I
88 mutations and named super RLuc8. Subsequently, the codon usage of the open reading frame for super
89 RLuc8 was adapted for high yield expression in the *Escherichia coli* (*E. coli*) system using the JCAT tool
90 (<http://www.prodoric.de/JCat>). Consequently, codon-optimized super RLuc8 was chemically synthesized
91 using DNA synthesis technology, ligated into the *Nde* I-*Xho* I site of pET21b (Novagen) expression
92 vector, and used for further experiments.

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94 **2.3. Expression and Purification of Recombinant Protein**

95 For high-level recombinant protein production, super RLuc8-pET21b was transformed into *E. coli* BL21
96 (DE3) competent cells. *E. coli* BL21 (DE3) contains the recombinant pET21b vector under the control of
97 the T7 promoter and may express high levels of the desired protein. The positive transformants were used
98 to inoculate 2 ml LB medium to which ampicillin (100 µg.ml⁻¹) was added and the culture was incubated
99 at 37°C with shaking overnight. Subsequently, the pre-culture medium was used to inoculate fresh LB
100 medium containing ampicillin (100 µg.ml⁻¹), until the optical density at 600 nm (*OD*₆₀₀) reached 0.9. The
101 optimization of recombinant protein expression was performed at different incubation times (2h, 4h, 6h,
102 8h and 16h), with constant isopropylthio-β-Dgalactoside (IPTG) concentration (0.5 mM) at 25°C. Then,
103 the induced cells with recombinant protein were harvested by centrifugation at 4 °C and 3500 rpm for 20
104 min, re-suspended in lysis buffer (50 mM Tris-base, 300 mM NaCl, 10 mM imidazole), and broken by
105 sonication. The lysates were centrifuged at 10,000 rpm for 20 min and the expression level of the
106 recombinant protein was analyzed by SDS-PAGE method. Consequently, protein expression was
107 performed under optimized conditions and after bacterial cell sonication, the lysate was centrifuged and
108 the supernatant of the lysate containing His6-tagged recombinant protein was collected and filtered
109 through a 0.2 µm filter. Purification of the recombinant protein was carried out using Ni-NTA spin
110 column according to manufacturer recommendations. Protein amount was obtained by the Bradford assay
111 [15] using BSA as the standard and the purity of protein fractions was determined by SDS-PAGE
112 analysis.

113

114 **2.4. Bioluminescent Spectra Recording**

115 Bioluminescent spectra were measured from wave lengths of 300 to 700 nm using Cary-Eclipse
116 fluorescence spectrophotometer. This assay was performed by adding 200 µl of each purified luciferase
117 (native RLuc and super RLuc8), diluted with equal volumes of enzyme reaction buffer (Tris-NaCl buffer:
118 100 mM Tris-base, 300 mM NaCl, pH 7.8), to the 200 µl quartz cells; emission spectra were then

recorded from wave lengths of 300 to 700 nm by adding 5 μ L coelenterazine h (74.1 μ M) to the enzyme solution. The experiment was repeated at least three times.

2.5. Kinetic Studies of Super RLuc8 and Native RLuc

Enzyme activity was assayed by measuring the light emission spectra of each luciferase at various coelenterazine h concentrations. To do this, a stock solution of coelenterazine h was prepared by dissolving coelenterazine powder in ethanol/HCl solvent. Various concentrations of coelenterazine h were prepared and enzyme assay was performed by addition of 1 μ L of enzyme, diluted with Tris-NaCl buffer (50 mM, pH 7.8), to 60 μ L of the substrate solution. Measurement of enzyme activity was carried out at 27 °C over 10 s using Berthold luminometer. The experimental data were analyzed using GraphPad prism software and kinetics parameters such as *K_m* and specific activity were calculated according to the Michaelis–Menten equation. Furthermore, optimum temperatures were analyzed in the range of 15 to 50 °C. Finally, luminescence decay was recorded by addition of 1 μ L of enzyme, diluted with Tris-NaCl buffer (50 mM, pH 7.8), to 60 μ L of the substrate solution (25 μ M); the data was recorded over 2 min using Berthold luminometer. The experiment was repeated at least three times.

2.6. Determination of Protein Stability

For thermal inactivation studies, the purified enzymes were incubated at a given temperature (37 °C and 65 °C), aliquots were taken for specified time periods. Then, samples were immediately cooled down to 0 °C and assayed for luciferase activity at room temperature. Moreover, to investigate the thermal stability of the enzymes, samples were heated at 5 °C intervals ranging from 25 to 45 °C for 10 min and quickly withdrawn and chilled on ice; this was followed by luciferase activity assay at room temperature.

2.7. Molecular Dynamic Simulations

The initial coordinates for the enzyme were obtained from homology modelling of super RLuc8 using Swiss-model server (<http://swissmodel.expasy.org>). Furthermore, molecular dynamic (MD) simulation of the native RLuc was based on PDB file 2PSF which is the crystal structure of the mutant RLuc luciferase. The simulation for the information collection was carried out over a 15 ns trajectory at different temperatures (298K, 303K and 310K). The MD simulation was conducted using Gromacs, OPLS-AA/L all-atom force field [16] and periodic boundary conditions. The protein was dissolved in a cubic box containing TIP3P water molecules. Overlapping water molecules were removed, and sodium ions were added for negative charge neutralization. With the aim of limiting steric hindrance, energy

151 minimization was performed over 50000 steps. Finally, the system was equilibrated at constant pressure
152 and temperature using NPT and NVT ensembles respectively.

153 3. Results and discussion

154 3.1. Construction, Expression and Purification of Super RLuc8

155 To obtain super RLuc8, its complete open reading frame (ORF) sequence was designed by substitution of
156 sixteen amino acids (A55T, C124A, S130A, K136R, A143M, M185V, M253L, S287L, A123S, D154K,
157 E155N, D162E, I163L, F261W, K189V and V267I) in the sequence of the native RLuc and generated by
158 synthetic DNA technology and submitted to GenBank (GenBank Accession number KU891681).

159 Super RLuc8 was ligated into an *NdeI-XhoI*-digested pET21b expression vector and transformed into
160 *E.coli* BL21 (DE3) pLys. To optimize the production of recombinant protein, different incubation times
161 for protein expression were tested. Consequently, the highest yield of recombinant protein expression
162 was obtained after six hours incubation and induction with 0.5 mM IPTG (supplementary Fig. 1).

163 Subsequently, the (His₆)-tagged form of super RLuc8 was purified using immobilized-metal (Ni²⁺)
164 affinity chromatography (IMAC). SDS-PAGE analysis revealed that super RLuc8 has an apparent
165 molecular weight of 36 kDa, which is in agreement with previously reported molecular mass for RLuc
166 [10] (Fig. 1). The purified protein content was evaluated by the Bradford assay and results demonstrated
167 that the most concentrated protein fraction had a concentration of 0.14 mg/ml. Also, after the addition of
168 coelenterazine to the obtained purified enzyme, an intense yellow-green light was observed.

170 3.2. Enzymatic Activity and Emission Spectrum of Super RLuc8

171 In order to determine the impact of amino acid substitution on the emitter site of RLuc, kinetic
172 parameters were calculated by measuring the emitted signal with a luminometer. The initial velocity was
173 expressed as relative light units per second (RLU.S⁻¹), and the *K_m* and *V_{max}* values were obtained from
174 the Michaelis–Menten plot (Table. 1). According to Table 1, a significant difference (n = 5, p-value=
175 0.0138) can be observed between the affinity of super RLuc8 and native RLuc to the coelenterazine; and
176 the affinity of the enzyme's emitter site for binding to the substrate is decreased in super RLuc8 as
177 compared to the native enzyme. Moreover, super RLuc8 represents an increase of about 13% in specific
178 activity compared with native RLuc. The half-life of light emission was also measured by following the
179 time decay of luminescence after coelenterazine injection (Fig. 2A). Results showed that the half-life
180 activity of super RLuc8 is much more than that of native RLuc (n = 4, and p-value=0.0126). This
181 property seems to be one of the most important characteristics of the super RLuc8 mutations for most *in*
182 *vivo* imaging applications.

183 In order to determine the impact of amino acid substitution on the emission spectrum of RLuc, the
184 bioluminescence emission spectra of super RLuc8 and native RLuc were recorded from 300 to 700 nm
185 (Fig. 2B). The maximum emission spectra were 485 and 540 nm for native RLuc and super RLuc8,
186 respectively. Interestingly, the results showed a strong and observable 65 nm red-shift in the super RLuc8
187 emission spectrum. Different intermediates with a variety of light emissions from blue to green were
188 proposed for RLuc [4]. Therefore, the red-shifted emission spectrum of super RLuc8 may indicate change
189 in the intermediate(s) of bioluminescence at the emitter site of super RLuc8 as compared to the native
190 enzyme.

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192 **3.3. Thermostability of Super RLuc8**

193 To study the impact of protein manipulation on the stability of RLuc, optimum temperature, thermal
194 stability and thermal inactivation were determined (Fig. 3A and B and supplementary Fig. 2).
195 The optimum temperatures of the purified enzymes were determined by measuring their activity over a
196 temperature range of 15-55 °C (Fig. 3A). The optimal temperatures of super RLuc8 and native RLuc
197 were observed at 37 °C and 30 °C, respectively. The optimal temperature of super RLuc8 is close to the
198 body temperature of small animals such as rodents; thus, this new variant of RLuc may be more suitable
199 for *in vivo* imaging studies.

200 Thermal stability studies were carried out at 25 to 45 °C in Tris-base buffer (pH 7.8). Results indicated
201 that the activity of super RLuc8 remained around 60% after incubation at 45 °C, while native RLuc lost
202 approximately 82% of its initial activity at the same temperature (Fig. 3B). Furthermore, thermal
203 inactivation studies were performed at 37 °C for 60 min and the plot of the remaining activity was
204 obtained for both the native RLuc and super RLuc8 enzymes. According to the thermal inactivation plot,
205 4- and 10-fold ($n = 4$, $p\text{-value}=0.0001$ and $p\text{-value}= 0.0084$) increases in super RLuc8 thermostability
206 are observed at 37 °C after 10 and 20 min incubation, respectively, in comparison to the native enzyme.
207 While the native enzyme completely inactivated after 30 min, the recombinant enzyme retained about
208 30% of its initial activity after the same incubation time (supplementary Fig. 2).

209 The results from the thermal inactivation studies are in line with optimum temperature results and this
210 indicates that mutation brought about an increase in the thermostability of RLuc. Moreover, the results
211 suggest that super RLuc8, compared with native RLuc, can be considered a suitable reporter in typical
212 laboratory animals at body temperatures of 35.9-37.5 °C.

213

214 **3.4. Molecular Dynamics Simulation**

215 In order to simulate mutation impact on the structure of RLuc, comparative modelling and MD
216 simulation of both super RLuc8 and native RLuc were carried out at 303K and 310K with GROMACS
217 4.5.5 using the OPLS-AA/L all-atom force field. The results from the MD simulation were subjected to
218 further structural analyses by Visual Molecular Dynamics (VMD) [17] and Caver 3.0 software [18]. The
219 root mean square fluctuation (RMSF) values of super RLuc8 and native RLuc8 are showed in Figs. 4A
220 and B. Although these values change non-uniformly, RMSF increase at the position of 145 to 165
221 corresponds to the sequence of the main part of the active site. Furthermore, the results of RMSF analysis
222 of super RLuc8 showed that the flexibility of the active site residues at 310K is higher than that of those
223 at 303K and this difference is more obvious in super RLuc8 as compared with the native type (as
224 depicted by boxes in Fig. 4).

225 In order to determine the impact of mutation on the compactness of RLuc, the gyration radius was
226 computed for super RLuc8 and for native RLuc. Super RLuc8 shows a significant decrease in gyration
227 radius compared to the native variant (Fig. 5).

228 The increase of RMSF and the change in the radius of gyration cause a significant change in the
229 conformation and shape of the main tunnel of super RLuc8. Moreover, the orientation of the enzyme
230 emitter site, and, therefore, the solvent accessible surface area of several residues including W153, W156,
231 W261 and F262 at the emitter site increases remarkably in super RLuc8 and these amino acids become
232 more exposed to water (Fig. 5 and supplementary Table). This observation is in accordance with changes
233 in the average number of hydrogen bonds between water molecules and active site residues
234 (supplementary Fig. 3).

235 The relationship between protein structure, stability and the emission spectrum has remained a
236 challenging subject in RLuc. The study results indicate that the mutations have a positive cumulative
237 effect on super RLuc8. Moreover, the MD studies showed non-uniform flexibility in the structure of
238 super RLuc. These data indicate that the rigidity/flexibility of the residues may affect the overall protein
239 structure, stability and activity.

240

241 **3.5. The Relationship between Mutations and Super RLuc8 Activity**

242 While specific activities improved in super RLuc8, its *K_m* value increased significantly ($n = 5$, p -value=
243 0.0138) as compared with this value in native RLuc.

244 The structure of RLuc consists of a main domain and a small cap domain which form a bowl-shaped
245 emitter site [19]. The essential catalytic unit of RLuc has been reported to be the catalytic triad, which
246 consists of D120, E144 and H285. Moreover, this catalytic triad is located at the bottom of a main tunnel,

247 which is solvent accessible and permits substrate influx and product egress during the bioluminescence
248 reaction [20].

249 Conformational flexibility in the vicinity of the enzyme active sites usually has various roles in the
250 enzyme catalysis and it has been reported that the enzyme conformational flexibility may contribute to
251 their catalytic efficiency and the substrate turnover rate [21, 22]. MD simulations indicated that the
252 position of 145 to 165 is the most flexible part of the super RLuc8 structure. This position forms a
253 flexible motif that includes a number of amino acids involved in the emitter site, such as V146, I163,
254 I166. Moreover, in the folded structure, this motif is placed close to the catalytic residues, including
255 D120 and E144 and its flexibility seems to be associated with the conformational changes which are
256 necessary during the catalytic cycle and facilitates emitter site rearrangements during the bioluminescent
257 reaction.

258 Super RLuc8 also showed a decreased substrate binding compared with the wild type. The conformations
259 of super RLuc8 and native RLuc were investigated by MD simulations. W153, W156, F262, and S263
260 are located at the rim of the substrate-binding site. Their side chains adopt in a distinct conformation in
261 super RLuc8 as compared to the native enzyme. In native RLuc, these residues have a proximal distance
262 and the bowl-shaped emitter site shows a buried conformation while in the emitter site of super RLuc8,
263 which is stabilized by new interactions either with the solvent molecules or with adjacent amino acids,
264 these residues are located in a distal position and the emitter site shows an upward conformation. A
265 consequence from the dislocation of emitter site conformation is a significant reduction of *Renilla*
266 luciferase interaction with coelenterazine.

267 In general, results from the MD studies indicated that while the mutations caused the local flexibility in
268 the vicinity of the emitter site of super RLuc8 and improved the enzyme activity, the substrate binding
269 cavity was not in an optimized conformation for binding to coelenterazine and reduced the affinity of the
270 enzyme for the substrate.

271

272 **3.6. The Relationship between Mutations and Super RLuc8 Emission**

273 In coelenterazine-dependent bioluminescence systems, the substrate is oxidized to yield coelenteramide
274 and light. The mechanism of reaction is common for coelenterazine-based luciferases and photoproteins
275 [23] and the maximum emission spectrum strongly depends on the polarity/ hydrophobicity of the
276 environment surrounding the substrate and product(s). It has been reported that the fluorescence
277 emission maximum wavelength of coelenteramide in aequorin varies in various solvents; and by
278 increasing the polarity of the solvent, the fluorescence of coelenteramide shifts to red wavelengths [24-

26]. Furthermore, not only polarity, but the degree of fluorophore motion is crucial for the amount of emission spectral shifts [27].

Super RLuc8 is a red-shifted luciferase, which has its maximum emission spectrum at 540 nm wave length.

Some residues at the emitter site of RLuc such as F260, F262 and S263 have been reported to have an important role in modifying the emission spectrum of the bioluminescence reaction [2]. Moreover, the color of the emission spectrum is dependent on the intermediate(s) of enzyme reaction and changes from blue to green in the phenolate and pyrazine anions, respectively [10].

The MD studies revealed that the cleft of the emitter site in super RLuc8 is more solvent accessible and flexible compared with native RLuc and some amino acids such as W153, W156, W261 and F262 are fully exposed to water in an “open” conformation of the emitter site (Fig. 5B). The cleft, moreover, is stabilized through a network of hydrogen bonds with the solvent. It can be proposed that the multiple interactions of the intermediate(s) with the new polar environment cause the red shift of the bioluminescence spectra in super RLuc8. Moreover, the more superficial localization of the excited intermediate(s) in super RLuc8 may show a higher flexibility in molecule motion, due to decrease in steric hindrance. Therefore, the relaxed state form of the excited intermediate leads to increased loss of energy and thereby shows a red light emission.

Overall, based on the results, it can be discussed that the conformation of the enzyme emitter site that encloses the coelenterazine and the excited coelenteramide was changed by the introduced mutations. Therefore, during the bioluminescence reaction, the excited coelenteramide in super RLuc8 became more water-exposed than the excited form in the native type which gained more motional freedom. These issues lead to the formation of the pyrazine anion instead of the phenolate anion at the emitter site of super RLuc8 and resulted in longer wavelengths emission in it.

302 **3.7. The Relationship between Mutations and Protein Stability**

Super RLuc8 had higher optimum temperature and was more resistant to thermal inactivation as compared with the native enzyme. It has been reported that the radius of gyration and RMSF, which are parameters for describing the compactness and flexibility of protein structures are key features in protein stability [28]. Super RLuc8 showed a significant decrease in the radius of gyration and forms a closed global conformation compared with native RLuc. The closed conformation of super RLuc8 probably caused the increased relative thermostability of this enzyme as compared to the native type. Furthermore, most mutations that are related to RLuc8 mutations, especially the M185V mutation, enhanced the local rigidity of certain parts of the enzyme which are located out of the emitter site (supplementary Fig. 4). In

general, MD simulation indicated that the impact of sixteen mutations on the structure of super RLuc8 is global and these mutations together have provided better structure compactness, more local rigidity in the regions out of the emitter site and better enzyme thermostability.

4. Conclusion

In the present study an engineered novel RLuc, named super RLuc8, has been created by molecular directed evolution. Super RLuc8 has a 65 nm red-shift in the emission spectrum and is more resistance to inactivation compared with the native enzyme. Furthermore, specific activity and light-emission half-life are improved in super RLuc8. These properties make this new variant of luciferase a practical candidate for further studies such as *in vivo* imaging studies.

Furthermore, based on the MD results, we propose that the red-shift in the bioluminescence spectra is due to two phenomena: 1) Increase in the solvent accessibility of some buried residues in the emitter site which leads to an “exposed” form of the tunnel mouth. 2) Enhancement in the degree of motion of the solvent-exposed intermediate(s) which leads to increased loss of energy. Also, the results of this study suggest that the mentioned amino acid substitutions substantially change compactness and overall fluctuations with respect to native RLuc leading to increased thermostability and optimum temperature of super RLuc8. This new mutant of RLuc and the data we have obtained may be used as a foundation for future studies on the structure-activity relationship of RLuc and may also open a new avenue for further stability studies which could possibly result in the employment of this enzyme in novel diagnostic studies.

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Figure legends

Fig. 1. SDS-PAGE analysis of purified protein fractions. Lane M shows protein ladder and lanes 1, 2, 3 and 4 are fractions of purified super RLuc8.

Fig. 2. A) Decay of light emission of native RLuc and super RLuc8. B) Luminescence spectra of the native enzyme and super RLuc8 in Tris-base buffer (pH 7.8).

Fig. 3. A) Effect of temperature on relative enzymatic activity and stability of purified super RLuc8 and native RLuc. B) Thermal stability curves at different temperatures. Standard deviation was shown by error bars.

Fig. 4. The root mean square fluctuation (RMSF) values per residue in last 15 ns of simulations for native RLuc (A) and super RLuc8 (B) at two different temperatures.

Fig. 5. A) The radius of gyration (Rg) over time for native RLuc (blue) and super RLuc8 (green). The variation of Rg indicated the differences on compactness of protein structures on the variants. B) Three-dimensional structure of native RLuc and super RLuc8. (i) New cartoon model of super RLuc8 with some active site residues where they can be seen on the surface of the enzyme. (ii) Structure of native RLuc with the same residues represented in super RLuc8 which are buried in the protein or are relatively near the surface (The different colored parts of catalytic cavity represent different amino acids).

429 **Figures**

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Fig. 1.

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Fig. 2.

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Fig. 3.
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Fig. 4.
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Fig. 5.

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Table 1 Kinetic parameters of the native RLuc and super RLuc8.

	Specific activity (RLU.S ⁻¹ .mg ⁻¹ .10 ¹²)	Km (μM)	Optimum temperature (°C)
Native RLuc	7.5	2.93±0.65	30
Super RLuc8	8.5	9.97±1.6	36