

A new firefly luciferase with bimodal spectrum: structural determinants, biological function and biosensing applications of pH-sensitivity in beetle luciferases

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Fireflies emit flashes in the green-yellow region of the spectrum for the purpose of sexual attraction. The bioluminescence color is determined by the luciferases. The *in vitro* bioluminescence color of firefly luciferases can be shifted toward the red by lower pHs and higher temperature; for this reason they are classified as pH-sensitive luciferases: railroadworm and click beetle luciferases which are pH-insensitive. However, the mechanism and structural origin of pH-sensitivity in fireflies remains unknown. Here we report the cloning of a new luciferase from the Brazilian twilight active firefly *Macrolampis* sp₂, which displays an unusual bimodal spectrum. The recombinant luciferase displays a sensitive spectrum with the peak at 569 nm and a shoulder in the red region. Comparison of the bioluminescence spectra of *Macrolampis*, *Photinus* and *Cratomorphus* firefly luciferases shows that the distinct colors are determined by the ratio between green and red emitters under luciferase influence. Comparison of these luciferases and site-directed mutagenesis allowed us to identify important substitutions involved with the pH-sensing mechanism in beetle luciferases, among them the natural substitution E354N which determines the appearance of the shoulder in the red region of *Macrolampis* luciferase bioluminescence spectrum. The potential application of this luciferase in biosensors is discussed. (Financial support: FAPESP).