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The smallest natural high-active luciferase: Cloning and characterization of novel 16.5-kDa luciferase from copepod *Metridia longa*

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ABSTRACT

Coelenterazine-dependent copepod luciferases containing natural signal peptide for secretion are a very convenient analytical tool as they enable monitoring of intracellular events with high sensitivity, without destroying cells or tissues. This property is well suited for application in biomedical research and development of cell-based assays for high throughput screening. We report the cloning of cDNA gene encoding a novel secreted non-allelic 16.5-kDa isoform (MLuc7) of *Metridia longa* luciferase, which, in fact, is the smallest natural luciferase of known for today. Despite the small size, isoform contains 10 conservative Cys residues suggesting the presence of up to 5 S—S bonds. This hampers the efficient production of functionally active recombinant luciferase in bacterial expression systems. With the use of the baculovirus expression system, we produced substantial amounts of the proper folded MLuc7 luciferase with a yield of ~3 mg/L of a high purity protein. We demonstrate that MLuc7 produced in insect cells is highly active and extremely thermostable, and is well suited as a secreted reporter when expressed in mammalian cells ensuring higher sensitivity of detection as compared to another Metridia luciferase isoform (MLuc164) which is widely employed in real-time imaging.

1. Introduction

The secreted bioluminescent enzymes are a convenient analytical tool because they enable monitoring of intracellular events with high sensitivity, without destroying cells or tissues [1]. This property is well suited both for application in biomedical research [2,3] and in the development of cell-based assays for high throughput screening [4].

The secreted luciferases are responsible for light emission of a variety of marine crustaceans [5]. The cDNAs encoding luciferases of some ostracods [6,7] and copepods [8–12] have been cloned. These proteins have signal sequences at their N-termini which assure efficient secretion at their expression in mammalian cells. Bioluminescence of the shrimp *Oplophorus gracilirostris* is also determined by secreted luciferase. However, in contrast to ostracod and copepod luciferases which are single polypeptide chain proteins, this luciferase is composed of two, 35-kDa non-catalytic and 19-kDa catalytic, proteins [13]. The copepod luciferases have advantages over other bioluminescent proteins at the use in cell-based assays since their light emission depends only on the substrate, coelenterazine, in contrast to other luciferases which require additional co-factors like ATP, for example, in case of firefly luciferase [14] and they are relatively small (~22–24 kDa) [9–11] as compared to other luciferases (e.g., molecular mass of ostracod *Vargula* luciferase is ~62 kDa [6]).

Despite the fact that nowadays copepod luciferases are widely used as bioluminescent reporters, their bioluminescent properties are poorly understood yet. The main hindrance here is the production of a proper folded protein. In copepod luciferase sequences three parts may be allocated: N-terminal signal peptide of 17 amino acids for secretion, N-terminal variable domain following signal peptide which is unimportant for luciferase activity as its deletion does not destroy bioluminescence function [15], and C-terminal catalytic conservative domain formed by two repeats (Fig. 1). These repeats contain ten highly conservative Cys residues (5 Cys per repeat) supposing the disulfide bonds' presence in proteins. The existence of S—S bonds in these luciferases is also supported by the fact that the dithiothreitol addition results in a complete loss of their activity [10]. The expression of functionally active proteins with disulfide bonds in *Escherichia coli*, the most simple and cost effective host for producing recombinant proteins, is greatly hampered since prokaryotic, as well as eukaryotic organisms, keep their cytoplasm reduced thereby impairing disulfide bond formation. The *E. coli* periplasm is more suitable for expression of disulfide-bond dependent recombinant proteins due to the presence of

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disulfide oxidoreductase and disulfide isomerase. However, this *E. coli* compartment is not always efficient for proper disulfide bond formation in proteins having numerous S—S bonds [16]. Expression in insect cells with the use of baculovirus expression system lacks these shortcomings; the luciferase MLuc164 from *Metridia longa*, for example, expressed in the insect cell system is a monomeric protein with a 3.5-fold greater bioluminescence activity than luciferase expressed and purified from *E. coli* [17].

In the present study, we report for the first time the cloning of cDNA gene encoding a novel secreted non-allelic 16.5-kDa luciferase (MLuc7) of *M. longa*, which, in fact, is the smallest natural luciferase of known for today, its efficient expression in Sf9 insect cells and purification from culture medium, and biochemical characterization of high purity luciferase. We also show that the cloned isoform is well suited as a secreted reporter when expressed in mammalian cells ensuring higher sensitivity of detection as compared to MLuc164 isoform [9] of *M. longa* luciferase.

2. Materials and methods

2.1. Materials

NanoFuel®Coelenterazine was obtained from NanoLight Technology, a division of Prolume Ltd. (Pinetop, USA). A stock coelenterazine solution was prepared by dissolving in methanol, and stored at −20 °C for several days. Concentration was calculated by absorption at 435 nm using the $\varepsilon_{435nm} = 9800\ cm^{-1}\ M^{-1}$ [14].

2.2. Cloning and constructions for expression of MLuc7 in E. coli and insect Sf9 cells

The full-size cDNA encoding MLuc7 isoform was isolated by functional screening from the copepod *M. longa* cDNA library in pTriplEx2 vector as described for MLuc164 cDNA [9]. The nucleotide sequence was deposited in the GenBank with the accession number: KP242023. For expression in *E. coli*, the MLuc7 coding sequence without signal peptide was amplified using specific primers: forward 5′-TACCGCATATGAACCCTACTGTAAACAATGA-3′ with NdeI site (underlined) and reverse 5′-ATGCTCGAGTTTAACGATCTC CAGCAAGAC-3′ with XhoI site, and pTriplEx2-MLuc7 as a template. The synthesized fragment was then cloned to corresponding sites of the pET22b+ vector (Novagen) for direct expression.

For secreted expression in insect Sf9 cells, the Bac-to-Bac Baculovirus expression system (Invitrogen) was used. The MLuc7 coding sequence with native signal peptide was amplified using specific primers: forward 5′-GACGGATCCATGGATATCAAATT-TATTTT-3′ with BamHI site (underlined) and two overlapping reverse primers: first 5′-TGATGATGACCTTGAAAGTACA AGTTCTCACGATCTCCAGCAAGAC-3′ and second 5′-TACTC-GAGTCATTAGTGATGGTGATGGTGATGATGACCTTGAAAG-3′ with XhoI site, using two-step PCR as described for MLuc164 [17]. The oligonucleotide primers were designed to introduce the C-terminal His6-tag followed by a TEV-specific protease site after MLuc7 coding sequence. After digestion, the synthesized fragment encoding MLuc7-His6 was cloned into BamHI/XhoI sites of the pFastBac-1 donor plasmid (Invitrogen). Generation of the recombinant bacmid DNA using DH10Bac *E. coli* cells, transfection of Sf9 cells with recombinant bacmid-MLuc7, obtaining of amplified recombinant MLuc7-baculovirus, and virus titration were done according to manufacturer's manual for the Bac-to-Bac Baculovirus expression system. The MLuc7 bioluminescence activity was used to evaluate the infection efficiency.

2.3. Production and purification of MLuc7

Expression of MLuc7 isoform in *E. coli* cells, its purification from insoluble inclusion bodies and refolding were carried out as reported for MLuc164 and its mutants [15].

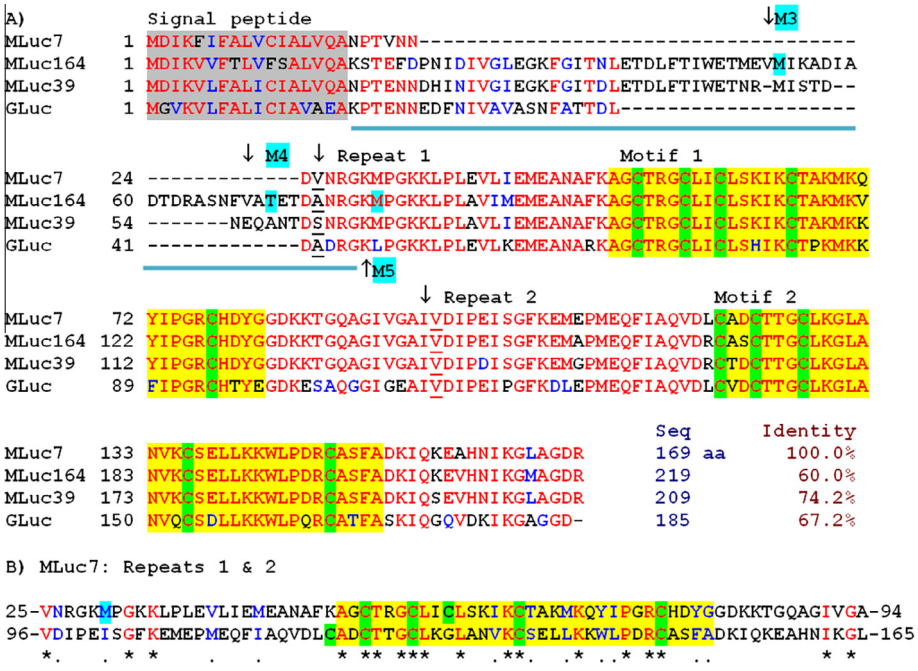

Fig. 1. (A) Sequence alignment of *M. longa* luciferase isoforms (MLuc), and *G. princeps* luciferase (GLuc, GenBank No. AAG54095). The alignment was produced by ClustalW and adjusted manually using nucleotide sequence information. Red letters indicate sequence positions with identical amino acid residues, blue letters represent similar residues, and black letters show nonidentical residues. Gaps are shown by dashes. Two very similar motifs within homologous repeats are marked yellow, conservative Cys residues are in green. Start positions of putative tandem repeats in luciferases are shown by arrows (Repeats 1 and 2). The residues shadowed with cyan indicate the truncation position of highly active ML164M3, ML164M4, and ML164M5 mutants of MLuc164 luciferase [15]. Variable N-terminal part located between signal peptide and the beginning of the shortest ML164M5 mutant of high activity is underlined. (B) Sequence alignment of putative duplicated domains in MLuc7. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

For secreted expression in insect cells, the Sf9 cells (Invitrogen) cultured in suspension at 28 °C without CO_2 using serum-free medium Sf900 II SFM (Life Technologies) were infected with the P2 viral stock (titer ~5×10^7 infectious units per 1 ml) at a multiplicity of infection (MOI) of 2 plaque-forming units and harvested at 72 h post-infection. Cells were pelleted at 2000*g* for 10 min at 4 °C and luciferase was concentrated from culture medium immediately by differential ammonium sulfate precipitation of 40–65% (w/v). Then, the insoluble particles were spun down (6000*g*, 20 min) and dissolved in Ni-binding buffer (0.3 M NaCl, 5 mM imidazole, 50 mM phosphate buffer, pH 7.1). The mixture was passed over a 5-ml HisTrap column (GE Healthcare) and eluted with an imidazole gradient (0–0.5 M) in the same buffer. The luciferase peak was concentrated with Amicon Ultra Centrifugal Filter (EMD Millipore) and the elution buffer was changed for TEV protease cleavage buffer (0.3 M NaCl, 1 mM EDTA, 0.02 mM DTT, 20 mM Tris–HCl, pH 7.5). The His6-tag was digested with TEV protease at ratio 50:1 (w/w) by overnight incubation of the luciferase sample at 4 °C. Then sample was loaded on a Ni-NTA column and the flow-through was collected. The collected sample was concentrated with Amicon Ultra Centrifugal Filter with a replacing buffer (0.3 M NaCl, 1 mM EDTA, 20 mM Tris–HCl, pH 7.6), and passed through a Superdex 75 gel filtration column (GE Healthcare) equilibrated with the same buffer. After gel filtration, luciferase sample was highly pure according to SDS–PAGE. The yield of MLuc7 luciferase was ~3 mg/L of insect cell culture. For storage of the concentrated MLuc7, the sample was transferred into buffer 0.3 M NaCl, 1 mM EDTA, 0.02% NP-40, 20 mM Tris–HCl, pH 7.5. For long storage, the MLuc7 sample was supplemented with 50% glycerol and stored at −20 °C. Under these conditions, MLuc7 luciferase activity is completely retained within 6 months. Protein concentration was determined using a *DC*™ Protein Assay kit (Bio-Rad).

2.4. Bioluminescence assay

Bioluminescence was measured by rapid injection of 5 μl of coelenterazine methanol solution into a luminometer cell containing a protein sample in 0.5 ml of the assay buffer (0.5 M NaCl, 10 mM $MgSO_4$, 0.015% gelatine, 50 mM Tris–HCl, pH 7.5) at room temperature. The temperature of the assay tube was supported by water circulation through a jacketed photometer. To determine thermostability, MLuc7 samples were incubated in a block heater at indicated temperatures and then cooled on ice for 5 min before bioluminescence measurement. The luminometer was supplied with neutral filters to extend detection range.

2.5. Spectral measurements

Bioluminescence spectra were measured with a Cary Eclipse spectrofluorimeter (Agilent Technologies, USA) in the assay buffer with correction for instrument spectral sensitivity. Bioluminescence was initiated by injection of coelenterazine (7.6 μM) in methanol solution (protein/coelenterazine molar ratio was ~$1{:}10^4$).

2.6. Expression of MLuc7 in mammalian cells

The pcDNA3.1(+) (Invitrogen) plasmid harboring a neomycin resistance was used to express MLuc7 in mammalian cells under constitutive cytomegalovirus (CMV) enhancer-promoter. The expression pcDNA3m-MLuc7 plasmid was generated by subcloning the fragment encoding full-size cDNA gene of MLuc7 from the original plasmid pTriplEx2-MLuc7 into the KpnI/XbaI sites of pcDNA3.1(+). The construction of the expression pcDNA3m-MLuc164 plasmid was described elsewhere [15].

The HEK 293 cells were grown in 96-well plates in Dulbecco's modified Eagle Medium supplemented with 10% FCS at 37 °C, 5% CO_2. The transient transfection of HEK 293 cells (~5000 cells/well) with a plasmid was performed with Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. After 7 h, the medium was replaced by fresh medium to monitor secretion time course of MLucs.

The medium aliquots and cells were separately assayed at regular time intervals of 2 h as previously described [15].

3. Results and discussion

Small marine copepod *M. longa* emits bioluminescence as a secretion from epidermal gland into surrounding water in response to various stimuli. Bioluminescence is caused by secreted coelenterazine-dependent luciferases, cDNA genes of which for the two isoforms, MLuc164 and MLuc39, were cloned [9,10]. As a result of additional functional screening of the expression cDNA library [9], we isolated the cDNA gene encoding the third non-allelic luciferase isoform MLuc7 from copepod *M. longa*, the smallest luciferase of known for today; the molecular mass of "mature" luciferase (without signal peptide) is only 16.5 kDa. The sequence differences between MLuc7 and other Metridia isoforms are comparable with those between each of luciferase isoforms of copepod *M. longa* of genus Metridia and luciferase from phylogenetically distant *Gaussia princeps* (GLuc) belonging to other genus of the same family *Metridinidae* (Fig. 1). Such major sequence differences in the cloned Metridia luciferases, especially noticeable at comparison of cDNA genes (Fig. S1), allow assuming that MLuc7, MLuc39, and MLuc164 isoforms are encoded by non-allelic paralogous genes. All these luciferases have variable N-termini (~1/3 of the longest MLuc164), and highly conservative C-terminal parts. The difference between luciferases is mainly determined by gaps in the variable N-terminal sequences (Fig. 1). The truncation of N-terminus of MLuc164 yields deletion mutants (Fig. 1) with even higher bioluminescent activity than that of the wild type enzyme [15]. The sequence of the smallest deletion mutant (ML164M5, ~15 kDa) was even less than the conservative part of the luciferase sequences. Thus it is no wonder, that we cloned a small highly active natural luciferase isoform which sequence practically corresponds to a catalytic part. The comparison of MLuc7 sequence (Fig. 2S) with those of other MLuc isoforms and GLuc shows that MLuc7 sequence practically consists of two duplicated tandem domains (Fig. 1) similar to those identified in GLuc luciferase [18] suggesting that catalytic part of luciferase genes may arise due to duplication. Each repeat includes one of the two motifs of high similarity (32 amino acids) previously identified in MLuc164 [9]. Only within these repeated consensus motifs all conservative Cys residues of copepod luciferases (5 Cys residues per repeat) are found (Fig. 1).

The expression of Metridia isoforms in *E. coli* using the plasmid pET22b+ yields a large amount of protein which is accumulated as inclusion bodies and, consequently, active enzyme obtaining requires renaturation and refolding. The high purity active monomeric isoforms of Metridia luciferase from *E. coli* cells (Fig. S3A) were obtained as described elsewhere [15]. We compared activities of Metridia isoforms produced in *E. coli* and found that the MLuc7 bioluminescence activity exceeds those of MLuc39 and MLuc164 approximately by a factor of 3 and 6, respectively. This agrees with the data obtained for deletion mutants when the removal of N-terminal variable part of MLuc164 resulted in significant increase of bioluminescence activity [15]. It is noteworthy, that the MLuc7 sequence (Fig. 1) practically corresponds to that of ML164M4 mutant which revealed the highest bioluminescent activity [15]. It should be noted that kinetics of MLuc7 biolumines-

cence signal is faster than those of MLuc39 and MLuc164 (data not shown). It is interesting that the kinetics of ML164M4 mutant is also faster than that of full-size MLuc164.

To produce active Metridia isoforms from inclusion bodies, we used oxidative refolding [15]. Although this approach increases the yield of bioluminescence activity, only minor parts of the total proteins attempted to be refolded could be purified as functionally active monomeric luciferases. Our attempts to express MLuc7 in a soluble fraction of *E. coli* using different approaches were disappointing. Intracellular expression of MLuc7 as a fusion protein with thioredoxin Trx-tag in pET32a (Novagen) using RosettaGami2 (DE3) strain to provide an oxidative environment in *E. coli* cytoplasm, expression in periplasm of *E. coli* cells using prokaryotic pelB signal peptide, extracellular expression as a fusion protein with secreted protein YebF [19] resulted in very low protein yields and low recovery of bioluminescence activities (data not shown).

We produced a significant amount of pure and proper folded MLuc7 luciferase (Fig. S3B and C) using Bac-to-Bac Baculovirus expression system and Sf9 insect cells only. The MLuc7 with its own signal peptide for secretion and cleavable His6-Tag at C-terminus for IMAC-purification was expressed at high level (Fig. S3B) (see Section 2), and was purified from serum-free culture media with a yield of high purity protein of ~3 mg/L. The gel electrophoresis under denaturing and reducing conditions revealed one band for the purified protein with the molecular mass corresponding to MLuc7 (Fig. S4B). The semi-native gel electrophoresis (Fig. S4C) and gel filtration (Fig. S4) also showed that MLuc7 luciferase produced from insect cells is a high purity monomeric protein of an appropriate size. This recombinant MLuc7 has ~80-fold greater specific bioluminescence activity than monomeric MLuc7 purified from *E. coli* (Table 1), i.e. the difference of activities is higher than that between similar samples of MLuc164 isoform [17]. Some other bioluminescence properties of these MLuc7 samples (pH and temperature profiles of light intensities, thermostability) also differed (Fig. 2B–D). However, both luciferase samples display practically identical bioluminescent spectra (Fig. 3A). It may be conditioned by incorrect formation of the multiple disulfide bonds in luciferase produced by refolding from inclusion bodies despite the use of monomeric luciferase.

We studied some properties of pure MLuc7 obtained by expression in insect cells and determined optimal conditions for bioluminescent reaction. Similar to MLuc164 obtained from insect cells [17], MLuc7 luciferase has optimum of bioluminescence activity at ~0.5 M NaCl (Fig. 2A) that approximately corresponds to NaCl concentration in sea water where secreted copepod luciferase must effectively function. However MLuc7 is more stable al low and high ionic strength than MLuc164; it retains ~60% of activity without NaCl as compared to ~1% for MLuc164, and displays more than twice higher activity at 1–3 M of NaCl [17] that may be due to a more rigid and stable protein structure of MLuc7. This supposition is supported by its extremely high resistance to

Table 1
Specific activities of MLuc7 luciferase from *M. longa* purified from inclusion bodies of *E. coli* and Sf9 insect cells.

Proteins	Host cells	Specific activity (rlu/mg × 10^{10})	Related specific activity (%)
MLuc7	*E. coli*	1.5	1.2
MLuc7	Sf9 (medium)	121.0	100.0

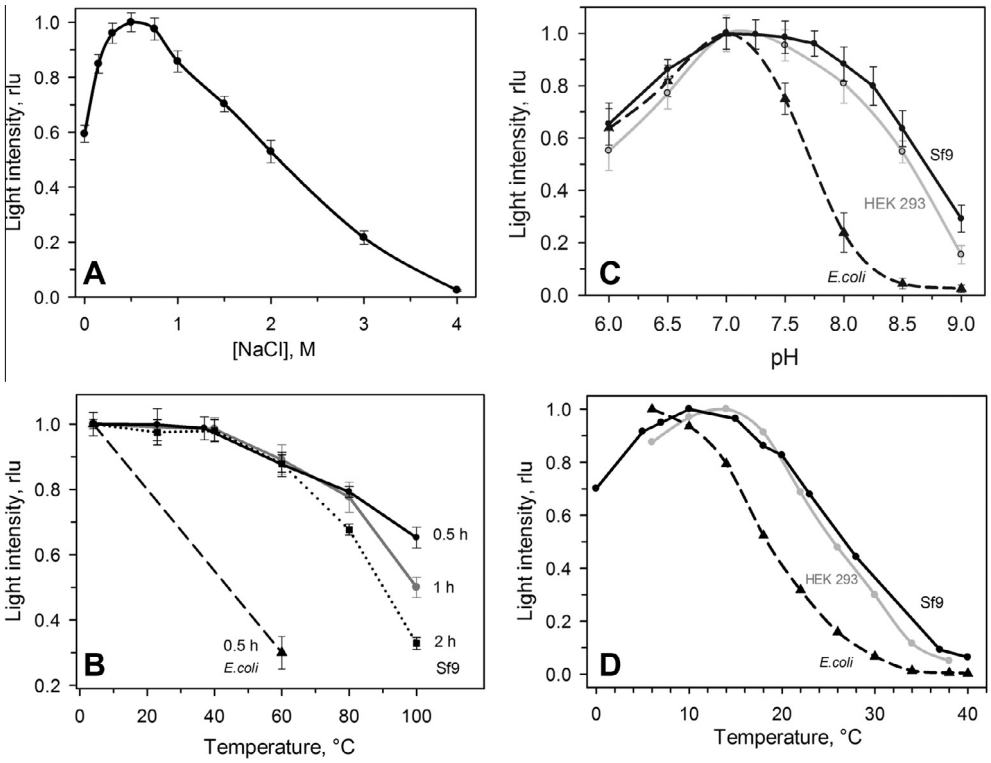

Fig. 2. Bioluminescent properties of recombinant MLuc7 luciferase. (A) Effect of NaCl on bioluminescent activity of MLuc7 produced in insect cells. The buffer was 10 mM $MgCl_2$, 50 mM Tris–HCl, pH 7.5. (B) Thermal inactivation of pure MLuc7 produced in insect cells (●) and *E. coli* (▲) at different temperatures in a storage buffer (0.3 M NaCl, 1 mM EDTA, 0.02% NP-40, 20 mM Tris–HCl, pH 7.5). Protein concentrations were 3.7×10^{-5} and 0.03 mg/ml, respectively. The loss of bioluminescence activity of MLuc7 secreted in medium by HEK 293 cells after 0.5 h of incubation at 60 °C was the same as for MLuc7 produced in insect cells (data not shown). Before measurements, the samples were cooled for 5 min on ice. (C) Effect of pH on bioluminescent activity of pure MLuc7 produced in insect cells (●) and in *E. coli* (▲), and MLuc7 secreted in medium by HEK 293 (○). The buffer was 50 mM Bis-Tris propane of different pH with addition of 0.5 M NaCl. (D) Effect of temperature on light intensity of MLuc7. MLuc7 samples were denoted as in (B). rlu, relative light units. Data are the mean ± SD.

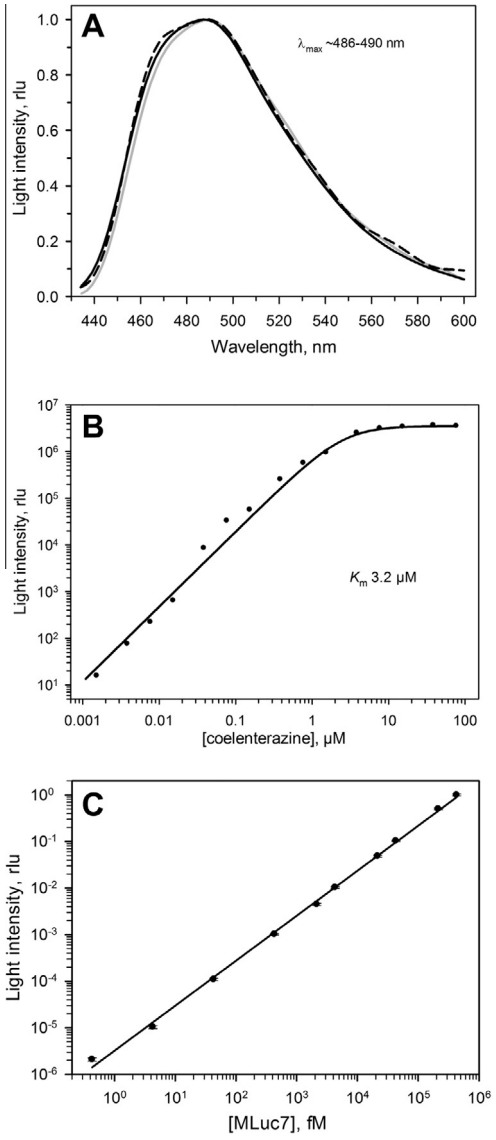

Fig. 3. Bioluminescent properties and kinetics analysis of MLuc7. (A) Normalized bioluminescence spectra of pure MLuc7 from insect (black line) and *E. coli* cells (dashed line), and MLuc7 secreted in medium by HEK 293 cells (grey line). (B) Log–log plot of dependence of light intensity of MLuc7 from insect cells on coelenterazine concentration. The luciferase concentration is 1.1 nM. (C) Log–log plot of dependence of light intensity of MLuc7 from insect cells on its concentration. The lowest and biggest MLuc7 concentrations were 0.43 fM and 0.43 nM, coelenterazine concentration was 7.64 μM, i.e. its concentration always exceeds that of luciferase over the whole range. The points on the plots are average of three measurements. rlu, initial bioluminescence intensity in relative light units.

thermal inactivation; MLuc7 retains complete activity after 2-h incubation at 40 °C and 50% of activity after 1 h of boiling (Fig. 2B).

Fig. 3B shows the concentration dependence of bioluminescence intensity maximum when MLuc7 produced in insect cells is assayed with coelenterazine. Light intensity reaches a constant value at ~10 μM that is twice higher than for MLuc164 produced in *E. coli* [15] but approximately 10 times less than for coelenterazine-dependent luciferase from *Renilla muelleri* [20]. The apparent Michaelis constant determined from dependence of initial light intensity on coelenterazine concentrations amounts to 3.2 μM. It is ~2-fold higher than that for monomeric MLuc164 (K_m = 1.56 - μM) obtained by refolding from inclusion bodies [15], ~3-fold less than for luciferase from *R. muelleri* [20] which is an intracellular protein in contrast to copepod luciferases [14], but practically

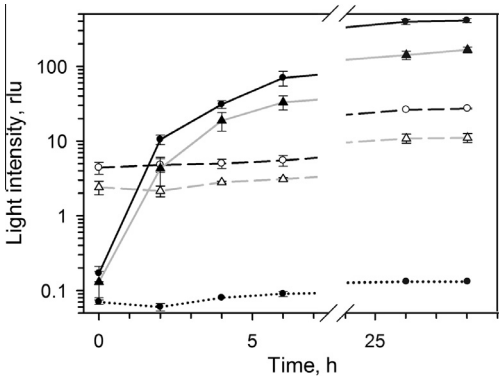

Fig. 4. Time course of MLuc7 (circles) and MLuc164 (triangles) activities in culture media (filled symbols) and cells (open symbols). Dotted line shows light signals from culture medium of cells transfected with pcDNA3. rlu, relative light units. Data are the mean ± SD.

equals K_m (3 μM) determined for ML164M4 mutant [15], which sequence practically corresponds to that of MLuc7.

Fig. 3C represents the dependence of bioluminescence intensity maximum of MLuc7 produced in insect cells on protein concentration. With ordinary luminometer we were able to detect MLuc7 luciferase at concentration of 0.43 fM. In addition, the light intensity was linear over a wide range of luciferase concentrations covering six orders of magnitude. These results evidently show that the novel MLuc7 luciferase displays very promising properties as a bioluminescent reporter to be applied in various assays.

Of note is that when we produced a high active MLuc7 luciferase in insect cells we faced with the problem of a rapid loss of its bioluminescence activity in highly diluted samples. We tested some reagents attempting to stabilize luciferase (Fig. S5). It was found that MLuc7 diluted with the assay buffer up to 2 nM retains its bioluminescence activity during 2 h with 0.02% NP-40 nonionic detergent, and, moreover, NP-40 is more effective for protein stabilization than the commonly used BSA. The addition of glycerol in concentration of 50% along with NP-40 allowed us to preserve MLuc7 bioluminescence activity at −20 °C for more than 6 months. This finding is very valuable since the approach can be applied for MLuc7 stabilization at developing various *in vitro* assays.

The secreted bioluminescent reporters utilizing copepod luciferases from *G. princeps* and *M. longa* (MLuc164) are a powerful tool for the real-time monitoring of intracellular events with high sensitivity. To test novel Metridia luciferase as a secreted reporter, HEK 293 cells were transiently transfected with pcDNA3m-MLuc7, pcDNA3m-MLuc164, and pcDNA3.1 (without insert). Since transfected cells secrete luciferase continuously, for determination of the time course of secretion, the medium was removed and fresh medium was added at the beginning of the experiment (Fig. 4). As anticipated from the *in vitro* experiments, the bioluminescence signals from cells expressing MLuc7 were approximately 3-fold higher than those from cells expressing MLuc164. In addition, we compared some properties of MLuc7 secreted by HEK 293 cells into the culture medium with those determined for pure MLuc7 produced from insect cells. In contrast to MLuc7 produced from *E. coli* cells, both luciferase samples had approximately the same pH and temperature profiles of light intensities and bioluminescence spectra (Figs. 2C and D and 3A). It definitely shows that for proper characterization of bioluminescent and biochemical properties of the secreted copepod luciferases the protein produced in eukaryotic cells should be used.

In summary, in this study we describe for the first time the cloning of cDNA gene encoding a novel secreted luciferase of copepod *M. longa*, the smallest natural luciferase of known for today, and its bioluminescent properties determined for high purity

protein produced in insect cells. We also demonstrate that MLuc7 isoform is a highly active and extremely thermostable protein. In addition, we show that novel luciferase is well suited as a secreted reporter when expressed in mammalian cells ensuring higher sensitivity of detection as compared to MLuc164 Metridia isoform.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.12.082.

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