

REFOLDING OF THE RECOMBINANT LUCIFERASES OF *METRIDIA LONGA*

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INTRODUCTION

Metridia longa is a marine copepod from which a blue bioluminescence originates as a secretion from epidermal glands in response to various stimuli. Its bioluminescence is conditioned by secreted coelenterazine-dependent luciferases. Using functional screening the three cDNAs encoding different luciferases were cloned from the expression cDNA library of *Metridia*. One of them, MLuc164, was successfully applied as a secreted reporter enzyme in mammalian cells.¹ Two luciferases, MLuc164 and MLuc39, without signal peptides for secretion were expressed in *E. coli*. In *E. coli* cells, most of the synthesized protein is accumulated in insoluble inclusion bodies. Here we report the results of testing various approaches for solubilization and refolding of these luciferases. Despite high identity of luciferases, MLuc39 yielded more active monomeric proteins.

MATERIALS AND METHODS

Reagents. Coelenterazine was from PJK GmbH (Kleinblittersdorf, Germany). Dithiothreitol (DTT) and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were from Aldrich and Serva respectively. Oxidized (GSSG) and reduced (GSH) glutathione were from Sigma. All other reagents were of highest quality commercial grade.

Bacterial expression. *E. coli* cells BL21 CodonPlus (DE3)-RIPL (Stratagene) transformed with the plasmids pET22-MLuc164 and pET22-MLuc39 were cultivated with vigorous shaking at 37°C in LB medium containing 200 µg/mL ampicillin. When the culture reached an OD (600 nm) of 0.8-1.0 the luciferase synthesis was induced with 100 mg/L IPTG. After induction, the cultivation was continued for 3 h. Cells were harvested by centrifugation at 4°C.

Isolation and purification. The cell paste was resuspended in 20 mM Tris-HCl, pH 7.0 (1:5, w/v), disrupted by sonication (20 sec × 6) at 0°C and centrifuged. The pellet was sequentially washed with 0.9% NaCl, 0.5% Tween-20 (× 3), and 20 mM Tris-HCl pH 7.0. All the washing procedures were followed by centrifugation at 4°C. The final pellet was extracted either with 6 M urea, 20 mM Tris-HCl pH 8.8 or 6 M guanidine HCl. The 6 M urea extracts of the luciferases were purified on DEAE Sepharose Fast Flow (Pharmacia) column preliminary equilibrated with 20 mM Tris-HCl pH 8.8. The proteins were eluted with linear salt gradient: 0–0.5 M NaCl in the same buffer, then dialyzed overnight at 4°C against 0.1 M NaCl, 20 mM Tris-

HCl pH 8.8. Luciferases were concentrated and subjected to gel filtration on a column of Bio-Gel P100 equilibrated with 0.5 M NaCl, 50 mM Tris-HCl pH 8.8.

Refolding experiments. The inclusion bodies were dissolved in 6 M guanidine HCl with or without DTT and then diluted 100 times into refolding solution: No. 1, 20 mM Tris-HCl pH 8.8; No. 2, 0.1 M NaCl, 20 mM Tris-HCl pH 8.8; No. 3, 2 M NaCl, 20 mM Tris-HCl pH 8.8; No. 4, 1% Tween-20, 20 mM Tris-HCl pH 8.8; No. 5, 0.1% Tween-20, 20 mM Tris-HCl pH 8.8; No. 6, 5 mM GSSG, 0.5 mM GSH, 20 mM Tris-HCl pH 8.8; No. 7, 0.1 M NaCl, 20 mM Tris-HCl pH 7.0; No. 8, 0.1 M NaCl, 20 mM Tris-HCl pH 6.0; No. 9, 0.1 M NaCl, 5 mM GSSG, 0.5 mM GSH, 20 mM Tris-HCl pH 8.8; No. 10, 0.1 M NaCl, 5 mM GSSG, 0.5 mM GSH, 20 mM Tris-HCl pH 7.0; No. 11, 0.1 M NaCl, 5 mM GSSG, 0.5 mM GSH, 20 mM Tris-HCl pH 6.0 (Table 1). Samples were concentrated and analyzed by native PAGE.

Table 1. Distribution of bioluminescent activity and protein between fractions at gel filtration on Bio-Gel P100

	polymer		monomer	
	activity %	protein %	activity %	protein %
MLuc164	3.9	63.0	96.1	37.0
MLuc39	2.5	43.8	97.5	56.3

Native PAGE. Samples were run on a 12.5% polyacrylamide gel. Protein solutions were mixed 2:1 with 50 mM Tris-HCl pH 7.0 buffer including 0.1% bromophenol blue, 10% glycerol, and loaded directly onto gel without boiling. A low range prestained molecular weight marker (Bio-Rad) was used as size standards.

Quantitation of SH-groups. The SH-groups were determined with DTNB (Ellman's reagent).² The luciferase was treated with 20 molar DTNB excess in 20 mM Tris-HCl pH 8.0 and incubated at room temperature for 10 min prior to measurement of absorbance at 412 nm. The concentration of 5-thio-2-nitrobenzoate (TNB) was determined using an extinction coefficient of $14.140 \text{ M}^{-1} \text{ cm}^{-1}$ at pH 8.0.

Bioluminescence assay. The luciferase activity was measured with a BLM 8801 luminometer (SKTB "Nauka", Russia) by injection of 5 μL of 10^{-5} M coelenterazine in methanol into cuvette containing 5 μL of luciferase sample and 500 μL of buffer (50 mM Tris-HCl pH 7.5, 0.1 M NaCl, 10 mM MgSO_4 , 0.01% gelatin).

RESULTS AND DISCUSSION

Both MLucs, expressed in high yields, accumulated in insoluble inclusion bodies, and both luciferases, after purification on DEAE Sepharose, had high purity by SDS-PAGE (Fig. 1), but gel filtration of these revealed two peaks that respectively corresponded to proteins with the molecular masses over 100 kDa (polymeric fraction) and 27.4 kDa for MLuc39 or 30.4 kDa for MLuc164 (monomeric fraction).

Most of a protein is in a polymeric fraction, and the corresponding bioluminescent activity is much lower than that of monomeric fraction (Table 1).

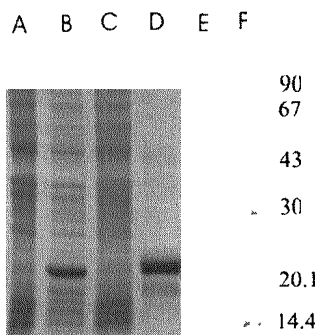


Fig. 1. SDS-PAGE of MLuc39 samples.
Lanes: A, B – *E. coli* cells before and after induction; C – cells lysate; D – inclusion bodies in 6 M urea; E – after DEAE Sepharose; F – molecular weight markers

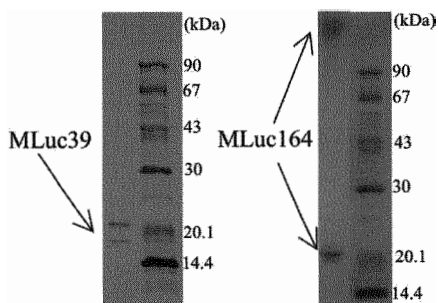


Fig. 2. Native PAGE analysis of MLuc39 and MLuc164 after refolding at No. 9 and No. 6 conditions

Table 2. Bioluminescent activity of MLuc164 and MLuc39 depending on refolding conditions, with maximum of bioluminescent activity marked in bold

refolding solution	MLuc164		MLuc39		
	RLU		RLU		
	- DTT	+ DTT	- DTT	+ DTT	
No. 1	550	148	No. 2	288	360
No. 2	200	973*	No. 7	61	4
No. 3	290	48	No. 8	18	0.3
No. 4	2012	381	No. 9	640	480
No. 5	1000	677	No. 10	0.64	0.016
No. 6	3250	719	No. 11	0.48	0.032

It is well known that, for the majority of the proteins, a removal of the denaturant leads rather to aggregation than to the correct protein folding. Although in recent years various strategies for overcoming aggregation have been developed, the folding conditions for each protein are unique and their finding is still empirical.³

MLuc164 and MLuc39 are single chain polypeptides with molecular masses 22 kDa and 21 kDa respectively. They display high degree of identity (82 %), and both contain 10 cysteine residues. A lot of cysteine residues suggest these luciferases to have some disulfide bonds, which might be responsible for bioluminescent activity

or the stability of a protein conformation. The quantity of disulphide bonds in monomeric luciferases (after gel filtration on Bio-Gel P100) was estimated by comparing the number of free SH-groups in the unfolded proteins produced with 6 M guanidine HCl with or without DTT. Both luciferases contain no less than four disulphide bonds. However at the surface of monomeric molecules, free SH-groups are lacking. This shows that two SH-groups might be buried into molecule core and, therefore, they will be inaccessible for modification with DTNB. The DTT adding to luciferases turns those into monomeric forms. It might be evidence that *Metridia* luciferases aggregate through intermolecular disulfide bond formation. It should be also noted that both luciferases lose bioluminescent activity after DTT addition.

All in all, eleven different conditions were tested to find the correct conditions for refolding of luciferases (Table 2). Refolding accuracy was monitored by bioluminescent activity, free SH-groups quantity and native PAGE data. For Mluc164 proper refolding condition was not found. Under all tested refolding conditions MLuc164 is a mixture of polymeric and monomeric forms (Fig. 2). That's why the number of free SH-groups in the samples was not determined. However, the largest bioluminescent activity was found at refolding under alkaline condition with GSSG/GSH (Table 1, No. 6). In contrast to MLuc164, MLuc39 is a monomer under all examined refolding conditions (Fig. 2). Nevertheless, the most of bioluminescent activity was found at refolding under alkaline conditions with GSSG/GSH adding (Table 1, No. 9). At that free SH-groups at the molecule surface are not determined, though at another refolding conditions the ones were found. It is interesting that the number of free SH-groups correlates with bioluminescent activity; the increase of the number of free SH-groups is accompanied by the decrease of bioluminescent activity. Although both *Metridia* luciferases display a high degree of identity it was surprisingly to find that at tested refolding conditions MLuc39 reveals the better yield of the active monomeric protein than MLuc164.

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