0273-2289/00/88/0127/$12.50

Recombinant Firefly Luciferase in *Escherichia coli*

Properties and Immobilization

Irina Lundovskikh, Ekaterina Dementieva,* and Natalya Ugarova

Chemistry Faculty, Lomonosov Moscow State University, Moscow, 119899, Russia

Abstract

The authentic recombinant luciferase, the luciferase with the structure similar to that of the native protein, was obtained using random mutagenesis, and its properties were studied in comparison with several fusion proteins. Thermoinactivation curves of the recombinant luciferases within the 10–50°C temperature interval showed that thermoinactivation involves reversible and irreversible steps. Immobilization of the recombinant *Luciola mingrelica* and *Photinus pyralis* firefly luciferases on BrCN-activated sepharose was carried out. Immobilization resulted in the preparation of enzymes with high catalytic activity. Physicochemical properties and analytical characteristics of the immobilized recombinant and native luciferases were studied. The catalytic properties of the immobilized recombinant *L. mingrelica* luciferase were close to those of the native luciferase but the former enzyme appeared to be significantly more stable. The immobilized recombinant luciferases can be used for ATP assay within 0.01–10000 n*M* range.

Index Entries: Recombinant firefly luciferase; bioluminescence; immobilization; ATP assay.

Introduction

Firefly luciferase catalyzes oxidation of luciferin (LH_2) in the presence of ATP and Mg^{2+} ions followed by emission of visible light. The unique mechanism and high quantum yield of light emission and the absolute specificity toward ATP and luciferin are of great interest to investigators. The firefly luciferin–luciferase system is widely used in many biochemical assays *(1)*. Cloning of luciferase genes from different firefly species and the possibility

*Author to whom all correspondence and reprint requests should be addressed.

of synthesizing the recombinant protein in preparative amounts opened new perspectives in its practical application.

Previously we have shown that the catalytic properties of the recombinant *Luciola mingrelica* luciferase synthesized by *Escherichia coli* cells carrying the plasmid-superproducer pJGR *(2)* are similar to those of the native luciferase, but the recombinant enzyme is less stable due to the presence of the Cys-residue among the 11 additional amino acids at the N-terminus *(3)*. All our previous studies of the *L. mingrelica* recombinant luciferase were carried out with the fusion recombinant proteins. We have now obtained an authentic recombinant luciferase, the luciferase with a structure similar to that of the native protein, and examined its properties. In this work we also carried out immobilization of the recombinant *Photinus pyralis* and *L. mingrelica* luciferases on BrCN-activated sepharose and studied catalytic properties and analytical characteristics of the recombinant and native luciferases in immobilized forms.

Materials and Methods

Strains, Media, and Plasmids

Escherichia coli cells, strain LE 392, were used as a host for the recombinant plasmids. The cells were grown in Luria–Bertani (LB) broth containing 0.1 mg/mL ampicillin. The overexpression vector pJGR carrying *L. mingrelica* luciferase gene *(2)* was used for expression of luciferase and random mutagenesis. The pME61 plasmid *(4)* with the thermoinducible promoter was used for the expression of the *P. pyralis* firefly luciferase.

Materials

Native *L. mingrelica* luciferase was isolated from freeze-dried firefly lanterns and purified as previously described *(5)*. Bacto tryptone and yeast extract were purchased from Difco. BrCN-activated sepharose 4B and chromatography carriers were from Pharmacia, Sweden; ATP was from Reanal, Hungary. Crystallized *P. pyralis* firefly luciferase, dithiotreitol (DTT), and D(+)-trehalose were purchased from Sigma. Luciferin was synthesized as previously described *(6)*.

Random Mutagenesis and Screening of Mutants

An aqueous solution of the pJGR plasmid (0.5 mL) was irradiated with ultraviolet rays for 5 min. Colonies of *E. coli* cells transformed with the mutated plasmid were tested for luciferase activity and thermal stability at 50°C in vivo and in vitro. For the in vivo tests, luciferase activity in the living cells was measured. In the in vitro tests, the cells were first destroyed with lysozyme and then luciferase activity in cell lysates was determined. The lysates were incubated at 50°C, and aliquots were taken and assayed for luciferase activity. One colony with the mutated plasmid with the highest thermal stability was selected. The plasmid from this colony (pLR plasmid) was purified using a Qiagen (Germany) plasmid kit and sequenced.

Preparation and Purification of Soluble Recombinant Luciferases

The recombinant luciferases were synthesized in *E. coli* cells carrying pJGR or pLR plasmids. The cells were grown to A_{590} = 3.0–3.5 at room temperature to avoid thermoinactivation of luciferase and destroyed with lysozyme in 0.1 *M* Na-phosphate buffer, 2 m*M* EDTA, 1 m*M* dithiotreitol (DTT), 8% saccharose, 0.5% Triton X-100, pH 8.0. Cell walls and DNA were precipitated with protamine sulfate (2 mg/mL) and centrifuged (50,000*g*, 20 min). Luciferase was precipitated from the supernatant with ammonium sulfate (35–75% saturation), diluted with 0.1*M* Na-phosphate buffer, 2 m*M* EDTA, 1 m*M* DTT, 0.1 *M* NaCl, pH 7.8, and purified to homogeneity using the previously described procedure *(2)*.

Assay of Luciferase Activity

Luciferase activity was determined by measuring the maximum intensity of light emitted during the enzyme reaction at saturating concentration of the substrates. The reaction mixture containing 0.05 *M* Tris-acetate buffer, pH 7.8, 2 m*M* EDTA, 10 m*M* $MgSO_4$ (0.4 mL), 4 m*M* ATP solution in the same buffer (0.3 mL), and a luciferase solution or cell lysate, or suspension of immobilized luciferase (10 μL) were placed into a polystyrene cell 10 mm in diameter. Then 1 m*M* luciferin (LH_2) solution (0.3 mL) was quickly added and the bioluminescence intensity was recorded. Luciferase activity was expressed in arbitrary units: 1 arb.un. = 1×10^9 quanta/s.

Immobilization of Luciferases

Native *L. mingrelica* luciferase was isolated from freeze-dried firefly lanterns. The lanterns (1.5 g) were triturated and 0.1 *M* Na-phosphate buffer, 2 m*M* EDTA, 0.4 *M* NaCl, pH 8.0 (30 mL) were added to the powder. The suspension was kept at 4°C for 2 h and centrifuged (50,000*g*, 20 min) and the supernatant was used for immobilization.

The crystallized native *P. pyralis* firefly luciferase was diluted with 0.1 *M* Na-phosphate buffer, 2 m*M* EDTA, 0.1 *M* NaCl, pH 8.0 and used for immobilization.

The recombinant luciferases were isolated from *E. coli* cells. Cell lysates were treated with ammonium sulfate (35–75% saturation) and centrifuged. The precipitate was diluted in a minimal amount of 0.1 *M* Na-phosphate buffer, 2 m*M* EDTA, 0.1 *M* NaCl, pH 8.0, and used for immobilization.

A weighted portion of dry BrCN-activated sepharose (200 mg of the carrier per 1 mL of a luciferase solution) was washed with 1 m*M* HCl and water, added to an enzyme solution, and incubated at 4°C for 20–24 h with constant stirring. The immobilized enzyme was washed for the separation of the unbound enzyme, suspended in 0.05 *M* Tris-acetate buffer, 2 m*M* EDTA, 0.1 *M* $MgSO_4$, 6.5 m*M* DTT, pH 7.6, and stored at 4°C. The effectiveness of immobilization (%) was calculated as a portion of the activity of the obtained immobilized enzyme with respect to the initial enzyme activity.

Instruments

Luciferase activity was measured on an LKB 1250 luminometer, Sweden. Bioluminescence spectra were recorded on a Perkin Elmer LS-50B fluorimeter, UK.

Results and Discussion

The Authentic Recombinant L. mingrelica Luciferase

The plasmid pLR was obtained from the plasmid-superproducer pJGR using random mutagenesis. The plasmid pJGR encodes a fusion of the 11 additional amino acid residues of *V. fischeri* Lux C of bacterial luciferase with the *L. mingrelica* firefly luciferase *(2)*. The pJGR-encoded recombinant luciferase was shown to be less stable than the native enzyme *(3)*. UV irradiation of the pJGR plasmid resulted in the deletion of the 29 bp, including the ATG codon from a portion of the bacterial luciferase (Fig. 1). In this case the protein synthesis starts from the first ATG codon of the firefly luciferase. The sequence analysis of the whole luciferase gene showed that all the rest of the nucleotides encoding 548 amino acids of the *L. mingrelica* firefly luciferase remained unchanged. Thus, the pLR plasmid encodes authentic *L. mingrelica* luciferase under autoinducible promoter of bacterial luciferase. The overexpression of the enzyme in *E. coli* cells does not need any inductor and allows one to obtain up to 50 mg of the active luciferase per 1 L of cell culture (10% of all cell proteins). The absence of the additional Cys residue, capable of oxidation, at the *N*-terminus results in the increase in luciferase stability.

The pLR- and pJGR-encoded recombinant luciferases were both purified to homogeneity. Physicochemical properties (K_{mATP}, K_{mLH_2}, bioluminescence spectra) of the native and recombinant luciferases were similar (Table 1). The specific activity of the homogeneous authentic recombinant luciferase was 20% higher than that of the native one, which is explained by the higher stability of the authentic enzyme.

Thermoinactivation of the Recombinant L. mingrelica Luciferases

Thermoinactivation of the recombinant firefly luciferases in the temperature range 10–50°C does not obey first-order kinetics (Fig. 2A) and depends on the initial concentration of the enzyme (at least with the [0.05–5] × 10^{-7} *M* range [Fig. 2B]) indicating a complex kinetics of changing of luciferase activity under the action of temperature. In addition, reactivation of the luciferase activity was observed under certain conditions, for example, when thermoinactivated at a high temperature enzyme was cooled to 4°C. Therefore, thermoinactivation was studied in more detail. Thermoinactivation curves were obtained by two methods: 1) inactivation at 30–50°C and immediate determination of enzymatic activity at the same temperature and 2) inactivation at 30–50°C and determination of the activity after keeping the enzyme at 4°C. The first method gives the sum of the

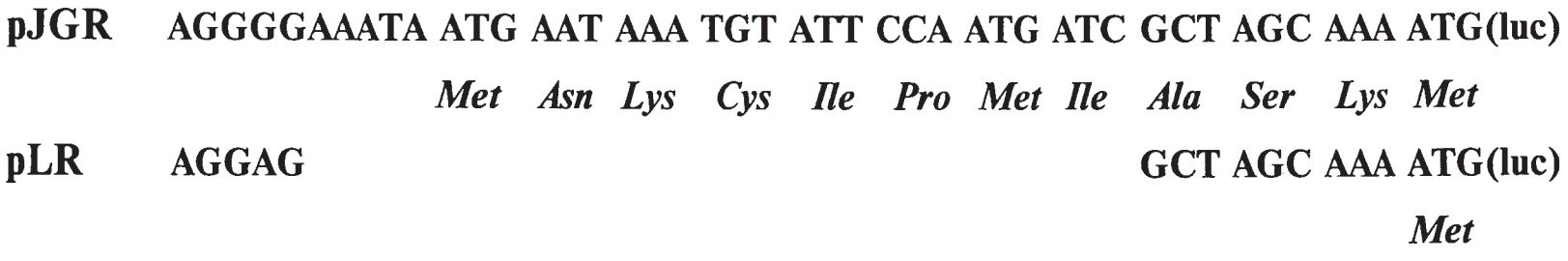

Fig. 1. Alignment of the *N*-terminal amino acids for the pJGR- and pLR-encoded *L. mingrelica* luciferases.

Table 1
Physicochemical Properties of the Native and Recombinant *L. mingrelica* Firefly Luciferases

Enzyme	Native firefly luciferase	pJGR-Encoded recombinant luciferase	pLR-Encoded recombinant luciferase
Bioluminescence maximum, nm	570 ± 5	570 ± 5	570 ± 5
Specific activity, arb.un./mg	(1–1.3) × 10^9	1 × 10^9	(1.2–1.5) × 10^9
K_{mLH_2}, μ*M*	20 ± 4	28 ± 3	20 ± 2
K_{mATP}, m*M*	0.15 ± 0.03	0.16 ± 0.03	0.18 ± 0.02

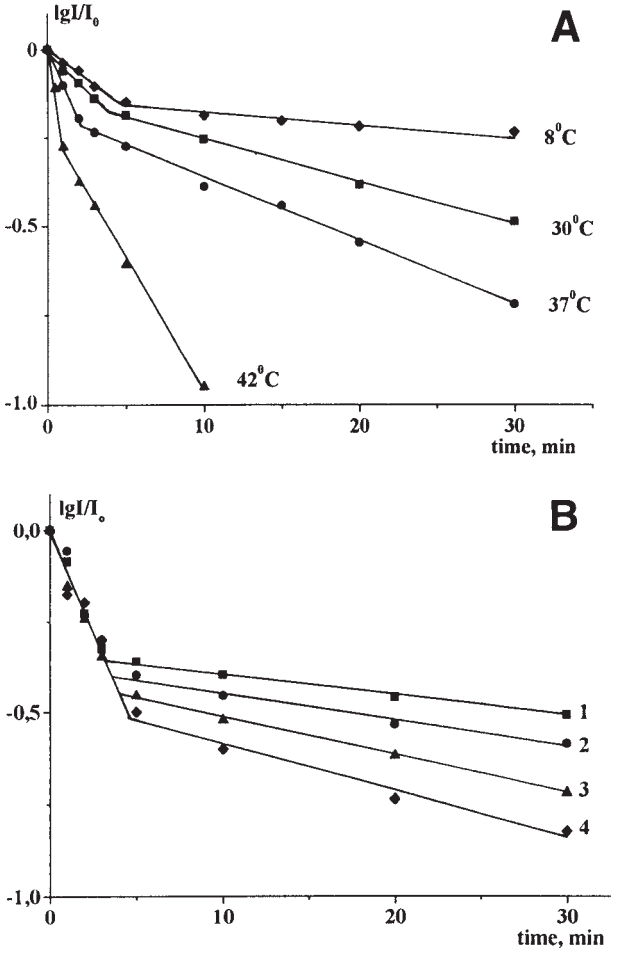

Fig. 2. Sum of the reversible and irreversible thermoinactivation of the pLR recombinant luciferase: **(A)** 340 n*M* enzyme, different temperatures; **(B)** 30°C, enzyme concentration, n*M*: 340 (1); 90 (2); 30 (3); 10 (4).

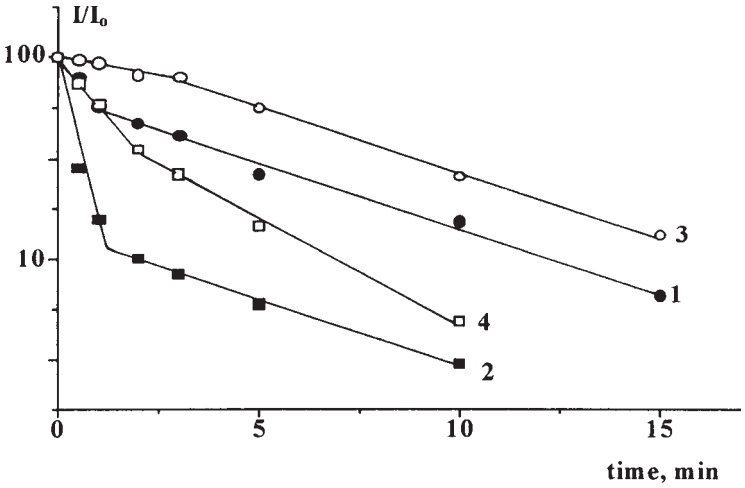

Fig. 3. Thermoinactivation of the pLR (1,3) and pJGR (2,4) recombinant luciferases at 42°C. 1,2—sum of the reversible and irreversible inactivation; 3,4—the irreversible inactivation, 340 n*M* enzyme.

reversible and irreversible inactivation and the second one—the irreversible inactivation only. To avoid the dilution effect on the luciferase activity, in all thermoinactivation experiments the concentration of luciferase was practically the same during enzyme incubation and activity assay. The data obtained indicate at least two-step kinetics of luciferase thermoinactvation (Fig. 3). The luciferase inactivated at 42°C is able to regain spontaneously a part of its initial activity when the samples are transferred to 4°C. Reactivation occurs quickly (5 min at 4°C) in the absence of any cellular factors. Reactivation yield depended on the portion of the inactivated luciferase, reactivating samples exhibited 80–100% of initial activity after thermoinactivation to 50% and only 20–30% of its initial activity after thermoinactivation to 90%. Thus, thermoinactivation of luciferase involves the reversible step followed by the irreversible one. The data obtained indicate that the pJGR-encoded luciferase is less stable than the authentic pLR recombinant luciferase at 10–50°C mainly with respect to the reversible inactivation.

Immobilization of the Native and Recombinant Luciferases

Immobilization of luciferases on different carriers *(7–10)* allows one to increase the stability of enzymes and expand the field of their analytical application. For the native luciferases, BrCN-activated agarose is the most suitable carrier for which the activity of the immobilized enzyme can reach 20% of the starting one *(10)*.

We immobilized the native and recombinant luciferases on BrCN-activated sepharose. The results of the immobilization are given in Table 2. Immobilization allowed us to obtain very active enzyme preparation with the recovery of the luciferase activity up to 30%. To prepare an active

Table 2
Immobilization of the Native and Recombinant *L. mingrelica* and *P. pyralis* Luciferases

	L. mingrelica luciferase		*P. pyralis* luciferase	
	Native (firefly lanterns extract)	Recombinant (*E. coli* cell lysate)	Native (homogeneous enzyme)	Recombinant (*E. coli* cell lysate)
Specific activity of the solution before immobilization, arb.un./mg of protein	(4.0–8.0) 10^8	2.0×10^8	1×10^9	6.7×10^6
Specific activity of the immobilized luciferase, arb.un./mg of the carrier	1×10^6	1×10^6	1.3×10^4	2.2×10^4
Effectiveness of immobilization, %	~10	20–30	~2	~10
Background signal, arb.un.	10	0.2	0	0.1
Immediately after preparation of the reaction mixture				
after 5 h	1–2	0	0	0
Retention of 100% activity, days[a]				
At 20°C	4–5	7	—	5
At 4°C	8–10	14		8

[a]Experimental conditions: 0.1*M* Tris-acetate buffer, pH 7.6, 2 m*M* EDTA, 10 m*M* $MgSO_4$, 6.5 m*M* DTT, 1 mg/mL BSA, 1 mg/mL sepharose with the immobilized enzyme.

reagent, immobilization should be carried out either from firefly extract (native luciferase) or from partially purified lysates of recombinant *E. coli* cells. Homogeneous luciferases, both *P. pyralis* and *L. mingrelica* ones, inactivate during incubation with BrCN-sepharose; as a result, the specific activity of the immobilized enzyme is 100 times lower than that of the enzyme prepared from the extract or lysate. A stabilizing effect of trehalose on the *P. pyralis* luciferase has been previously described *(11)*. In our experiments, the addition of trehalose (200 mg/mL) increased the stability and the activity of the recombinant luciferase in solution by 1.5–2 times. A similar effect was observed in the presence of 1 mg/mL lecithin. Trehalose, as well as lipids, may keep the enzyme in a catalytically active form in solution.

Catalytic Properties and Stability of the Immobilized Luciferases

The specific activity of the immobilized recombinant *L. mingrelica* luciferase (with respect to the proteins bound to the carrier) was by an order

Table 3
Catalytic Properties of the Soluble and Immobilized on BrCN-Activated Sepharose Luciferases

Enzymes	Specific activity, arb.un./mg of protein	K_{mLH_2}, μ*M*	K_{mATP}, m*M*
Luciola mingrelica luciferase			
Native soluble	1×10^9	20 ± 4	0.15 ± 0.03
Native immobilized	4×10^5	12 ± 1	0.19 ± 0.02
Recombinant soluble	1×10^9	24 ± 5	0.16 ± 0.03
Recombinant immobilized	4×10^6	10.5 ± 1.0	0.17 ± 0.01
Photinus pyralis luciferase			
Native soluble	1×10^9	20[a]	0.1[a]
Native immobilized	1.6×10^5	—	—
Recombinant soluble	1×10^9	24[a]	0.1[a]
Recombinant immobilized	3×10^5	4.2 ± 0.8	0.04 ± 0.01

[a]Literature data *(9)*.

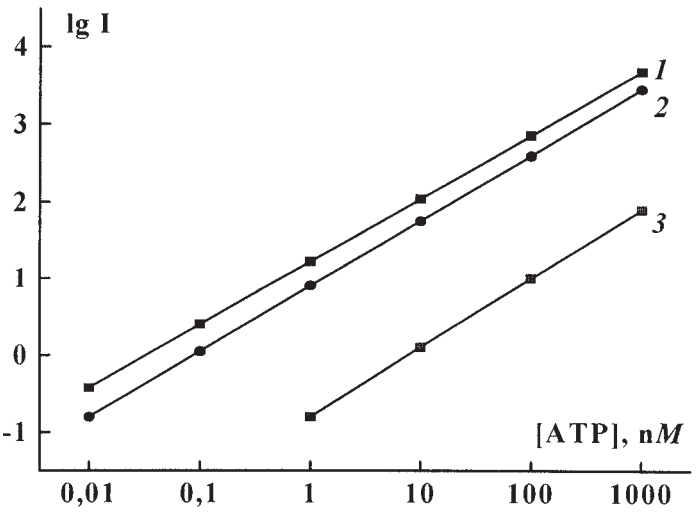

Fig. 4. Bioluminescent determination of ATP based on the immobilized native *L. mingrelica* (1), recombinant *L. mingrelica* (2), and recombinant *P. pyralis* (3) firefly luciferases.

of magnitude higher than that of the immobilized native luciferase (Table 3) since the immobilization of the recombinant luciferase was carried out from the partially purified lysate.

Immobilization of the luciferases did not change the bioluminescence spectra; maximum of bioluminescence was observed at 570 nm for the *L. mingrelica* and at 565 nm for the *P. pyralis* luciferases. Immobilization resulted in a decrease in the Michaelis constants both for LH_2 and ATP for *P. pyralis* luciferase and approx two times decrease in K_m for LH_2 for

L. mingrelica luciferase (Table 3). Such effect can be explained by a change in the luciferase conformation at immobilization.

Immobilization of the luciferases on BrCN-sepharose led to a substantial stabilization of the enzymes both at 20°C and 4°C, the immobilized recombinant luciferase being even more stable than the immobilized native luciferase (Table 2). An even larger stabilization effect was observed in the presence of trehalose (200 mg/mL).

Immobilization affected also the time-course of the bioluminescent signal. At the concentration of ATP 10^{-7} mol/L and lower a bioluminescent signal stable for several minutes was observed for the immobilized luciferases.

A high activity and stability of the immobilized *L. mingrelica* and *P. pyralis* firefly luciferases made possible to use them for the assay of ATP within the 0.01–100.00 n*M* interval (Fig. 4). Because of the absence of background signal for the immobilized recombinant luciferase the sensitivity of the assay was increased at least by two orders of magnitude as compared with the soluble enzyme.

References

1. Ugarova, N. N., Brovko, L. Yu., and Kutuzova, G. D. (1993), *Biochemistry* (*Moscow*) **58,** 976–992.
2. Devine, J. H., Kutuzova, G. D., Green, V. A., Ugarova, N. N., and Baldwin, T. O. (1994), *Biochim. Biophys. Acta* **1173**, 121–132.
3. Dementieva, E. I., Kutuzova, G. D., Zheleznova, E. E., Lundovskikh I. A., and Ugarova, N. N. (1996), *Biochemistry* (*Moscow*) **61**, 115–119.
4. Kutuzova, G. D., Skripkin, E. A., Belogurova, N. G., Skrjabin, G. A., Ugarova, N. N., and Varfolomeev, S. D. (1990), *Dokl. Akad. Nauk SSSR* **314**, 757–761.
5. Dementieva, E. I., Kutuzova, G. D., and Ugarova, N. N. (1989), *Moscow Universitet Chem. Bull.* (Engl. trans.) **44**, 69–73.
6. Talebarovskaya, I. K., Katkova, V. A., Rizhova, V. V., Schogolev, A. A., and Berezin, I. V. (1983), Method of D-luciferin synthesis. USSR patent no. 1192324.
7. Lee, J., Jablonski, I., and DeLuca M. (1977), *Anal. Biochem.* **80**, 496–501.
8. Blum, L. J., Coulet, P. R., and Gauteron, D. C. (1985), *Biotech. Bioeng.* **27**, 232–237.
9. Roda, A., Grigolo, B., Girotti, S., Ghini, S., Carrea, G., and Bovara, R. (1991), in *Bioluminescence and Chemiluminescence*. (Stanley, P. E. and Kricka, L. J., eds.), Wiley, Chichester, pp. 487–489.
10. Ugarova, N. N., Brovko, L. Yu., and Kost N. N. (1982) *Enz. Microb. Technol.* **4**, 224–228.
11. Wang, C. and Andrade, J. (1994), in *Bioluminescence and Chemiluminescence.* (Campbell, A. K., Kricka, L. J., and Stanley, P. E., eds.), Wiley, Chichester, pp. 423–426.