ISSN 0027-1314, Moscow University Chemistry Bulletin, 2008, Vol. 63, No. 2, pp. 67–70. © Allerton Press, Inc., 2008.
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Stabilization of ATP Reagents Containing Firefly *L. mingrelica* Luciferase by Polyols

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Received November 23, 2007

Abstract—ATP reagents containing 2–20% of ethylene glycol, glycerol, or PEG-8000 have been prepared. Polyols inhibit the activity of the ATP reagents by 20–40%, and addition of 0.75 mM Triton X-100 prevents this inhibition. The optimal polyol concentration is 10%. The kinetics of thermoinactivation of the ATP reagents in solution at 37, 20, and 4°C has been studied. At all temperatures, the stability of ATP reagents increases two- to fivefold in the presence of polyols. A solution of ATP reagents kept at 4°C for 180 h retains 100% activity. The activity half-life is 15 days.

DOI: 10.3103/S0027131408020041

Firefly luciferase catalyzes the oxidation of the substrate (luciferin) by air oxygen in the presence of adenosine 5'-triphosphate (ATP) and magnesium ions. This reaction is accompanied by visible emission, bioluminescence [1]. Interest in this bioluminescent process is caused not only by study of the mechanisms of transformation of biochemical energy to light energy but also by its practical significance. The high sensitivity of firefly luciferase to ATP, near-unity quantum yield of the reaction, and simple detection of the enzymatic activity made it possible to develop an efficient and fast method for determination of ultrasmall amounts of ATP. The ATP bioluminescence method is increasingly widely used in biochemistry, medicine, biotechnology, and environmental monitoring [2].

So-called ATP reagents—lyophilized multicomponent systems containing soluble recombinant firefly luciferase, luciferin, components of a buffer solution necessary for proceeding of the bioluminescent reaction, and stabilizing additives—are used for bioanalytical purposes [3]. The source of ATP for this system is a tested sample. A current tendency in biotechnology is to use reagents in solution. This not only simplifies the analysis procedure but also excludes the expensive and energy- and time-consuming stage of lyophilization from the synthesis of the ATP reagent. A rather high content of hydrophobic amino acid residues and a predominance of β layers in the secondary structure of luciferase determine its low stability in aqueous solutions, especially at elevated temperatures [4]. Luciferase is inactivated mainly due to aggregation of enzyme molecules [5] and their adsorption on the surface [6, 7]. Oligosaccharides [8], albumin [7], nonionic detergents, and polyols [9] are most frequently used as enzyme stabilizers.

This paper deals with (i) study of the possibility of using different polyols to stabilize ATP reagents in solution with retention of the enzymatic activity of luciferase and (ii) optimization of the composition of a water-soluble stabilizing composition containing polyols.

MATERIALS AND METHODS

Reagents and solutions. The following reagents were used: D-luciferin (Lyumtek, Russia); adenosine 5'-triphosphate (ATP), dithiothreitol (DTT), tris(hydroxymethyl)aminomethane, and magnesium sulfate (MP Biomedicals, Unitied States); disodium ethylenediaminetetraacetate (EDTA), bovine serum albumin (BSA), and polyethylene glycol (PEG-8000) (Sigma, United States); Triton X-100 (Merck, Germany); freshly distilled ethyl alcohol, ethylene glycol, and glycerol (Khimmed, Russia); deionized water obtained on a Milli Q water system (Millipore, France).

Recombinant *Luciola mingrelica* luciferase was isolated from *E. coli* cells (strain LE392) carrying the pLR plasmid was purified as described in [10] and stored at –70°C.

Preparation of ATP reagents. The initial concentrated solution of luciferase was diluted to an enzyme concentration of 0.1 mg/mL with a 0.1 M TRIS–acetate buffer solution (pH 7.8) containing 20 mM $MgSO_4$, 2 mM EDTA, 1 mM LH_2, 5 mM DTT, 180 mg/mL sucrose (control ATP reagent), and different polyols, namely, ethylene glycol, PEG-8000, or glycerol (2–20 vol %). The mixture was slowly stirred taking care to avoid foaming and then filtered through a sterile filter with a pore size of 0.22 μm. The resulting ATP reagents were kept at room temperature for 1 h until the background signal decreased to 5–10 arb. units. In each

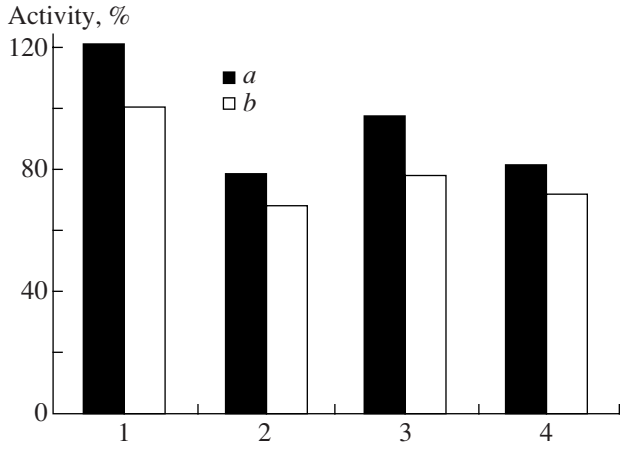

Fig. 1. Specific activity of ATP reagents at 20°C in the (*a*) absence and (*b*) presence of Triton X-100: (1) control and (2–4) ATP reagents containing (2) ethylene glycol, (3) glycerol, and (4) PEG-8000.

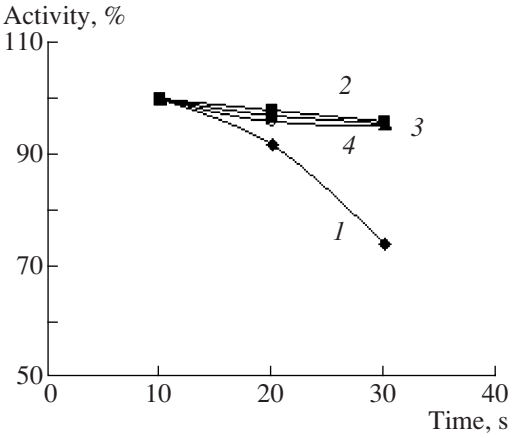

Fig. 2. Stability of the bioluminescence signal in the course of measurement for ATP reagents of different composition: (*1*) control ATP reagent, and (*2–4*) ATP reagents containing (*2*) ethylene glycol, (*3*) glycerol, and (*4*) PEG-8000.

series of experiments, freshly prepared ATP reagents were used.

Measurement of the luciferase activity in solution. A 0.4-mL portion of 0.05 M TRIS–acetate buffer (pH 7.8) containing 2 mM EDTA, 10 mM $MgSO_4$, 0.3 mL of 4 mM ATP solution in the same buffer, and 0.1 mL of a luciferase solution were placed in a cell. Background luminescence was measured, and then 0.3 mL of a 1 mM D-luciferin solution in the same buffer was rapidly introduced by means of an injector, and a maximal bioluminescence intensity proportional to the enzymatic reaction rate was recorded on a Femtomaster FB 12 luminometer (Zylux Corp., Germany). The luciferase activity was expressed in arb. units (1 arb. unit/mL = 1 mV = 10^9 quanta/s).

Measurements of the bioluminescence signal for ATP reagents. A luminometric microcell 0.3 mL in volume was placed with the help of pincers into the cell compartment of a 3560 microluminometer (New Horizons Diagnostic Corp., United States), and 50 μL of an ATP reagent solution and 50 μL of a 10^{-8} M ATP solution were introduced into the cell. The solution in the cell was rapidly and thoroughly stirred, and a biochemical signal was measured. Measurements were repeated no less than twice.

Thermoinactivation of ATP reagents. Solutions of ATP reagents were incubated at 37, 20, and 4°C on a Gnom thermostat (DNK-Tekhnologiya, Russia). Aliquots (100 μL) of an ATP reagent solution were taken at certain intervals and a bioluminescence signal was measured as described above. For ATP reagents inactivated at 37°C, bioluminescence signals were measured after additional incubation of the sampled aliquots on ice for 10 min.

RESULTS AND DISCUSSION

Influence of polyols on the activity of ATP reagents. We obtained samples of ATP reagents containing, in addition to standard components of a buffer system (luciferase, D-luciferin, magnesium sulfate, and sucrose), polyols, such as ethylene glycol, glycerol, and PEG-8000. The concentration of active luciferase in the ATP reagents studied was 0.1 mg/mL. The concentration of polyols was varied in the range 2–20%. The bioluminescent reaction was initiated by adding a 10^{-8} M ATP solution in water to an ATP reagent solution. Polyols inhibited the activity of ATP reagents by 20–40% as compared with the control (Fig. 1). As is known [12], the luciferase activity increases in the presence of nonionic micelle-forming surfactants: luciferase and its substrate, luciferin, turn out to be enclosed in micelles, which increases the efficiency of their interaction. Indeed, the specific activity of ATP reagents containing 0.75 mM Triton X-100 as an extra component increased by 15–20% whatever the type of polyol used (Fig. 1). In additon, polyols led to a ~20% increase in the stability of the bioluminescence signal (Fig. 2), which improves the accuracy of analysis.

After 70 min of incubation at 37°C, the highest residual activity (more than 70%) was observed for ATP reagents containing 10% of polyols (table), whereas the activity of the control ATP reagent was only 13%. An increase in the ethylene glycol or glycerol concentration to 20% led to a twofold decrease in the activity of ATP reagents, and 20% PEG-8000 almost completely inactivated the ATP reagent. It is likely that high concentrations of polyols increase the viscosity of the solution, which reduces the accessibility of the active site of luciferase for both substrates. The observed decrease in the enzymatic reaction rate can be due to both hindered diffusion and deceleration of conformational transitions of the enzyme, which are

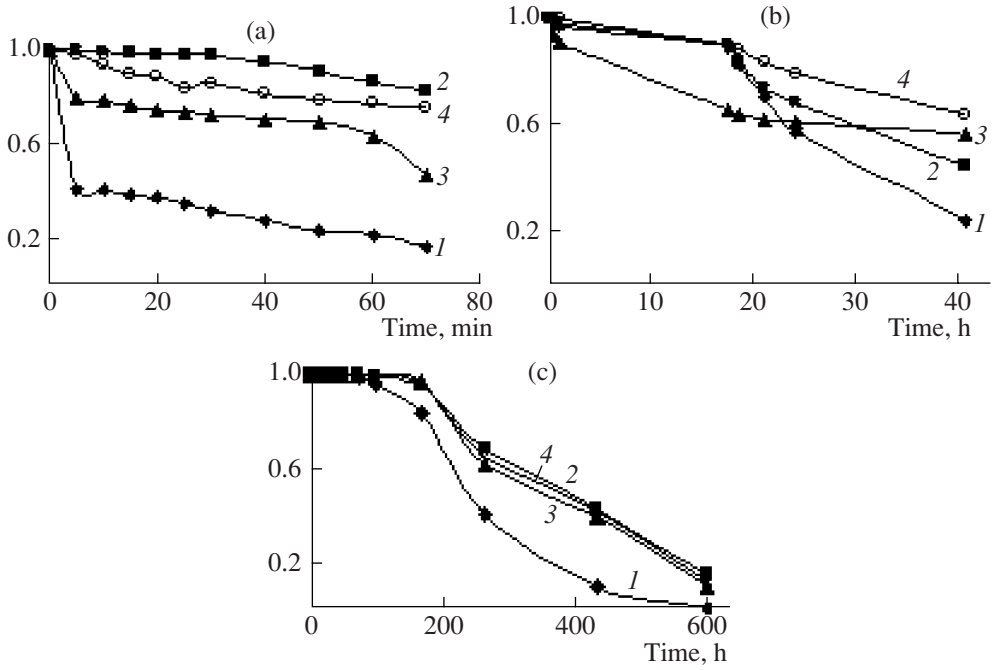

Fig. 3. Thermoinactivation of ATP reagents containing 10% of polyols at (a) 37, (b) 20, and (c) 4°C: (*1*) control ATP reagent, and (*2–4*) ATP reagents containing (*2*) ethylene glycol, (*3*) glycerol, and (*4*) PEG-8000.

necessary for the formation of the enzyme–substrate complex [13]. Thus, for studying the thermoinactivation, we used the ATP reagents containing 10% of ethylene glycol, glycerol, or PEG-8000.

Kinetics of thermoinactivation of ATP reagents at different temperatures. The kinetic curves of thermoinactivation of ATP reagents at 37, 20, and 4°C are shown in Fig. 3. The curve shape does not obey the first order and changes as a function of incubation temperature. At 37°C, all kinetic curves show two stages of inactivation, fast and slow (Fig. 3a). In the presence of polyols, the inactivation rate at the fast stage decreased, whereas the inactivation rate at the slow stage remained virtually unaltered. The greatest stabilizing effect was observed in the presence of ethylene glycol. After 1 h, 80% of the original activity of the ATP reagent was retained; for the control ATP reagent, only 20% of activity was retained. After 18 h at 20°C in the presence of PEG or ethylene glycol, 90% of the original activity was retained as compared with 60% for the control ATP reagent (Fig. 3b). After the induction period, inactivation was accelerated; nevertheless, after 40 h, the ATP reagents containing polyols retained 40–70% of their activity, whereas the control ATP reagent retained only 25% of its original activity. At 4°C, the induction period with complete retention of the activity of ATP reagents in the presence of polyols increased to 200 h, whereas for the control ATP reagent, this period was two times shorter (Fig. 3c). After one week of storage, the ATP reagents under consideration retained 40% of their original activity, which is five times higher than the residual activity of the control ATP reagent under analogous conditions.

Thus, our findings show that polyols (ethylene glycol, glycerol, and PEG-8000) are good candidates for stabilization of recombinant firefly luciferase and make it possible to enhance the efficiency of the ATP bioluminescence method. From the economic viewpoint, the use of ethylene glycol is most efficient.

Residual activity of ATP reagents after incubation at 37°C for 70 min

Polyol concentration, %	Percentage of original activity			
	control	ethylene glycol	glycerol	PEG-8000
0	13	–	–	–
2	–	66	54	53
5	–	74	61	67
10	–	76	71	75
20	–	47	46	3

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