Luminescence 2003; 18: 224–228
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bio.731

ORIGINAL RESEARCH

Effect of quinones and phenols on the triple-enzyme bioluminescent system with protease

N. S. Kudryasheva,[1] E. N. Esimbekova,* N. N. Remmel,[1] V. A. Kratasyuk,[2] A. J. W. G. Visser[3] and A. van Hoek[3]

[1]Institute of Biophysics, Akademgorodok, 660036 Krasnoyarsk, Russia
[2]Krasnoyarsk State University, 79 Svobodny Av., 660041 Krasnoyarsk, Russia
[3]Wageningen Agricultural University, Laboratory of Biochemistry, PO Box 8128, 6700 ET Wageningen, The Netherlands

Received 27 March 2002; revised 8 April 2003; accepted 15 April 2003

ABSTRACT: The study addressed the effects of redox-active compounds on trypsin activity. Series of organic oxidizers (quinones) and reducers (phenols) were chosen as model redox-active compounds. Trypsin activity was quantified by bioluminescent technique. Interactions of these compounds with trypsin were studied by fluorescent and light absorption methods. Luminescence intensity decay constants in the reduced nicotinamidadeninedinucleotide (NADH): flavinmononucleotide (FMN)-oxidoreductase (R)–luciferase (L)–trypsin (T) (R + L + T) triple-enzyme system were calculated and compared in the presence of different concentrations of quinones and phenols. The triple-enzyme system was shown to be sensitive to quinones and not sensitive to phenols. It has been found that the effects produced by quinones on the coupled enzyme system (R + L) and on the trypsin molecule (T) are not related. The conclusions were extrapolated to the properties of other proteases and antiproteases. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: bioluminescence; man-made pollutants; trypsin activity

INTRODUCTION

It is well known that humans and animals are exposed to the simultaneous action of numerous chemical substances, and their combined damaging effect is not equal to the sum of their separate impacts. Poisoning by chemical reagents leads to changes in biochemical processes, triggering the chain reaction that often ends in pathology. Organisms of humans and animals respond to the action of any damaging factor by compensatory reactions aimed at maintaining their homeostasis. The damaging effect of man-made pollutants on living organisms can be determined by bioassay methods. They are based on the influence of man-made pollutants on the functions of living organisms (paramecia, luminous bacteria, algae, fish, lichens, etc.). It is assumed that the results of bioassay testing can be directly extended to humans—hygeinic norms of environmental pollution (maximum allowable concentration, maximum allowable discharge, etc.). The advantages of bioassays (rapidity, integrated response to many pollutants, high sensitivity, low cost, etc.) have made them very popular among researchers. As a consequence, hundreds of bioassays have been devised. To estimate the pollution of a medium, authors of bioassays usually use one method and one organism only. However, it is not possible to use only the bioassay for adequate estimation of environmental media quality.

We previously proposed a concept of creating a universal battery of bioassays for ecological monitoring (1, 2). Polyenzymic assays were constructed, employing different types of interactions with the bacterial luciferase-catalysed reaction: elongation of enzyme coupling chain, competitive relations and proteolytic interactions. Currently the battery of enzyme assays includes the coupled enzyme system, R + L, and the triple enzyme reactions R + L + alcohol dehydrogenase (ADH) and R + L + T.

To estimate the sensitivity of the bioassays and to define the spheres of their application, we use an original approach: we monitor enzyme activity in the presence of a series of model homologous compounds with various physicochemical characteristics. In our earlier work we

*Correspondence to: E. N. Esimbekova, Institute of Biophysics, Akademgorodok, 660036 Krasnoyarsk, Russia.
Email: lucifer@ibp.ru

Contract/grant sponsor: Ministry of Education, Russian Federation, and American Foundation of Civil Research; Contract/grant number: CRDF REC 002.
Contract/grant sponsor: Krasnoyarsk Regional Scientific Foundation; Contract/grant number: 10F084N.
Contract/grant sponsor: NWO–Russia; Contract/grant number: 047-007-005.
Contract/grant sponsor: NATO Collaborate Linkage; Contract/grant number: CLG-974984.
Contract/grant sponsor: Russian Foundation of Basic Research; Contract/grant number: N01-03-32843.
Contract/grant sponsor: INTAS; Contract/grant number: N2001-562.
Contract/grant sponsor: Russian Academy of Science; Contract/grant number: N223.

thoroughly investigated the inhibition of bioluminescence in the coupled enzyme reaction R + L and in the triple enzyme reaction R + L + ADH by redox-active manmade pollutants (3–5). This paper continues the publication series; here we address the effects of various substances with different redox characteristics on trypsin activity in the triple enzyme reaction R + L + T.

Trypsin activity often has to be determined in media with different redox characteristics, e.g. in clinical investigations the content of ions with variable valence in blood and disordered urine sediment can affect the course of reaction and considerably reduce the accuracy of analysis (6). In ecological investigations trypsin activity is also measured in the presence of redox-active pollutants. It is therefore essential to know how redox-active compounds influence the bioluminescent triple-enzyme system R + L + T, which is used for monitoring trypsin activity in different media. This is even more important because redox-active compounds are also frequently occurring man-made pollutants.

Series of phenols and quinones were chosen as model redox compounds. Phenolic compounds are ubiquitous organic pollutants. Quinones—products of phenol oxidation—are usually more toxic than corresponding polyphenols (7).

Variations in trypsin activity were measured as variations in the bioluminescence intensity decay constant in the triple-enzyme system R + L + T (8). The bioluminescent method of measuring trypsin activity, as well as the activity of other proteases and antiproteases, is based on the ability of these enzymes to hydrolyse peptide bonds of L and R. Additionally, to determine whether phenols are bound to trypsin, we measured the fluorescence anisotropy decay of 1,4-naphthalendiol in the presence of trypsin. Since quinones are not fluorescent, we could not use fluorescence techniques to investigate these compounds, we used spectrophotometric methods to study the interactions of quinones with trypsin molecules.

MATERIALS AND METHODS

Trypsin activity measurement by bioluminescent technique

Lyophilized reagents of L and R from *Photobacterium leiognathi* were produced by the Biotechnology Sector of the Institute of Biophysics (Russian Academy of Sciences, Krasnoyarsk). One bottle of enzymes contained 0.15 mg/mL of L and 0.069 units of activity/mL of R. Trypsin from swine pancreas was produced by Sigma (USA), NADH, FMN by Serva (Germany), and tetradecanal and acetaldehyde by Merck (Germany). Quinone and phenol samples were presented by the Institute of Biology of Irkutsk State University. Solutions were made in 0.1 mol/L potassium phosphate buffer (pH 6.8).

The reaction mixture for measuring trypsin activity comprised 10 μL R + L enzyme solution, 50 μL 0.002% solution of myristic aldehyde, 10 μL 0.5 mmol/L FMN solution, 400 μL 0.1 mol/L phosphate buffer (pH 6.8) and 50 μL 0.4 mmol/L NADH solution. This reaction mixture was used to calculate the luminescence intensity (I), induction period (P), time of passage to bioluminescent maximum (t_{max}), and decay constant ($k_{d\ backgr}$) in the coupled enzyme system R + L. Then, 5 μL 0.01% trypsin solution and 20 μL solution under study were added to the above solution. Under these conditions, the values I, P, t_{max} and $k_{d\ exp}$ were determined. The real decay constant (k_d) of the triple-enzyme reaction was calculated as follows: $k_d = k_{d\ exp} - k_{d\ backgr}$ (8).

Concentrations of water or water–ethanol solutions of quinones and phenols were varied ($10^{-7} - 10^{-3}$ mol/L).

The work was carried out with a BLM8801 bioluminometer (Nauka Design Bureau, Institute of Biophysics, Krasnoyarsk). The experiment error was less than 10%.

Measurements of anisotropy decay

Time-resolved fluorescence measurements were carried out using mode-locked continuous wave lasers for excitation and time-correlated photon counting as the detection technique (9). The pump laser was a mode-locked CW yttrium lithium fluoride (YLF) laser Coherent, Model Antares 76-YLF (10). The repetition rate of excitation pulses was 951.2 kHz, the duration about 4 ps full-width at half-maximum (FWHM), and the pulse energy in the tens of pJ range. The samples were in 1 mm and 10 mm light path fused silica cuvettes (Hellma, Model 114F-QS). The fluorescence was collected at an angle of 90° with respect to the direction of the exciting light beam. A monochromator was used for wavelength selection of emission. Detection electronics were standard time-correlated single-photon counting modules containing some additional improvements (11).

Measurements were carried out in water–ethanol (20:1) solution with 1,4-naphthalindiol ($C = 2 \cdot 10^{-6}$ mol/L) under the excitation wavelength 343 nm (absorption maximum of 1,4-naphthalendiol is about 345 nm). Light filter KV389 + Schott 441.7 nm was used for detection. Measurements were done in the presence and absence of trypsin $C = 10^{-5}$ mol/L. For obtaining a dynamic instrumental response of the experimental set-up, the single exponential fluorescence decay of 1,4-bis(5-phenyloxasol-2-il)benzene (POPOP) in ethanol was measured.

Anisotropy decay of aqueous solution of trypsin $C = 10^{-5}$ mol/L was studied at the excitation wavelength of 300 nm and detection wavelength 348.8 nm. The single exponential fluorescence decay of *p*-terphenyl

in a mixture of cyclohexane and CCl_4 (50/50% volume ratio) was measured and used as a reference.

Data analysis was performed using a home-built computer program (12, 13). Rotational correlation time, φ, and its amplitude, β, were calculated and compared for: (a) the water–ethanol solution of 1,4-naphthalendiol; (b) the water–ethanol solution of 1,4-naphthalendiol in the presence of trypsin; (c) solution of trypsin. The average, maximal and minimal values of φ were calculated.

Light absorption measurements

Changes in the spectral characteristics of trypsin solution in the presence of quinones were estimated by measuring absorbance (A) of the enzyme solution, using a UVIKON-943 spectrophotometer (Kontron Instruments, Italy). A-values were recorded at $\lambda = 278$ nm, corresponding to absorption of tryptophan residues. Absorbance of 0.01 mmol/L solutions of trypsin and trypsin with 1,4-benzoquinone (1 and 0.1 mmol/L) was measured and compared. The differential spectra were derived by deduction of A-values of quinone solutions from analogous values for solutions of the quinone with trypsin.

RESULTS AND DISCUSSION

Variations in kinetic bioluminescence parameters of the triple-enzyme system R + L + T (induction period P and the time of passage to the bioluminescent maximum t_{max}) were studied in the presence of a series of quinones. P and t_{max} values increased with quinone concentration (Figs 1, 2) in the coupled enzyme system R + L and the triple-enzyme system R + L + T. Figs 1 and 2 show the influence of toluquinone on the coupled and triple-enzyme systems. The presence of other quinones (1,4-benzoquinone, 1,4-naphthoquinone, thymoquinone)

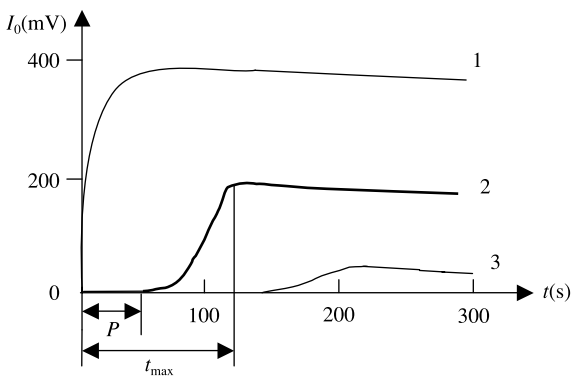

Figure 1. Bioluminescence intensity (I_0) of the coupled enzyme system R + L vs. time under different toluquinone concentrations: 1, 0; 2, 1.7 μmol/L; 3, 64 μmol/L. P, induction period; t_{max}, time of passage to the bioluminescence maximum.

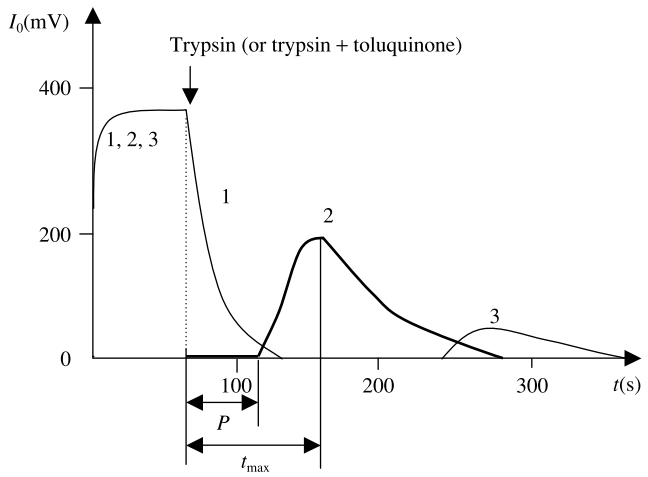

Figure 2. Bioluminescence intensity (I_0) of the triple enzyme system R + L + T vs. time under different toluquinone concentrations: 1, 0; 2, 1.7 μmol/L; 3, 64 μmol/L. P, induction period; t_{max}, time of passage to the bioluminescence maximum

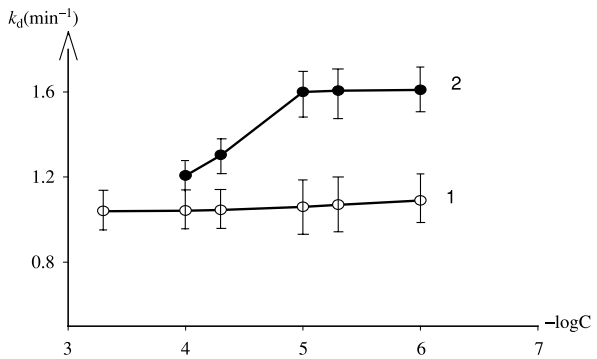

Figure 3. Bioluminescence decay constants k_d vs. quinone concentration: 1, 1,4-benzoquinone in the R + L coupled enzyme system; 2, 1,4-benzoquinone in the triple enzyme system R + L + T

yielded similar dependences. Analogous dependences were earlier found for the coupled system R + L in the presence of the series of quinones (3). The variation in these parameters can be accounted for by the influence of the quinones on the coupled enzyme reaction R + L, i.e. by competition between the quinones and FMN for NADH (4).

The bioluminescence intensity of the triple-enzyme system R + L + T in the presence of quinones was lower than that of the coupled enzyme system R + L.

Constant k_d describes differences between the effects of quinones on the coupled and triple-enzyme systems. Fig. 3 shows that k_d values slightly increase with the quinones' concentration in the coupled system R + L, while in the triple-enzyme system R + L + T, k_d-values decrease with increasing quinone concentration. Similar dependences were found in the presence of

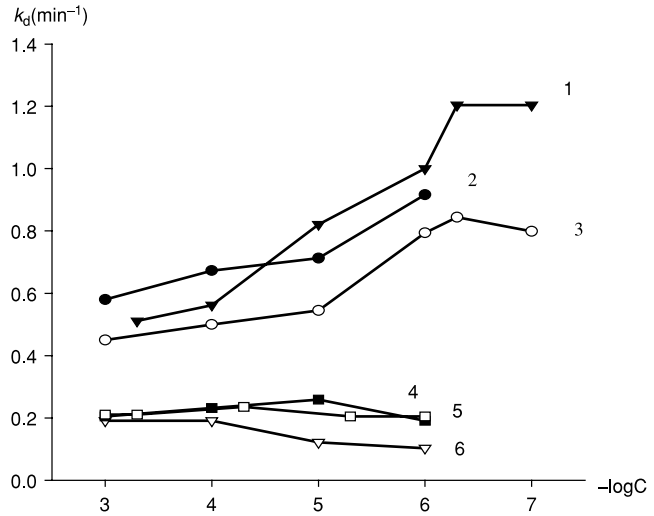

Figure 4. The R + L + T triple enzyme system bioluminescence decay constant k_d vs. concentration (–log C) of some quinones (1–3) and phenols (4–6): 1, toluquinone; 2, 1,4-naphthoquinone; 3, thymoquinone; 4, resorcine; 5, tetrachloropyrocatechol; 6, 1,4-hydroquinone

the other quinones (toluquinone, 1,4-naphthoquinone, thymoquinone). Variations in the k_d of the triple-enzyme system caused by quinones may be accounted for by deactivation of the trypsin molecule. SH-groups and reactive amino acids in the active centre of the enzyme might be modified by oxidizers (14). A proof of this assumption was changes of the absorbance values (A) of trypsin solution measured spectrophotometrically in the presence or absence of a quinone. The differential spectra recorded in the presence of 1,4-benzoquinone were compared to the spectrum of trypsin solution without the quinone. It was found that A-values of trypsin solution decreased as the quinone concentration increased. This is a sign of changes in the state of the trypsin molecule interacting with 1,4-benzoquinone.

Unlike quinones, phenols are weak reducers. They produced an insignificant effect on the bioluminescence kinetics of the triple-enzyme system R + L + T. Fig. 4 demonstrates that k_d of the triple-enzyme system did not vary when concentrations of some phenols increased.

Phenols are fluorescent compounds. To illustrate possible interactions between the trypsin molecule and phenols, the fluorescent characteristics of 1,4-naphthalendiol were studied in the presence and absence of trypsin using a fluorescence anisotropy decay technique. Table 1 shows the rotational correlation time, φ, and its amplitude, β, calculated and compared for: (a) water–ethanol solution of 1,4-naphthalendiol; (b) water–ethanol solution of 1,4-naphthalendiol in the presence of trypsin; and (c) trypsin solution. From the residual plot, which is randomly around zero, and the autocorrelation plots, which show no oscillation, as well as from the near-unity value of χ^2, it can be assumed that the theoretical fits are excellent. The fluorescence anisotropy decay is a double exponential function for all solutions under study:

$$r(t) = \beta_1 \exp(-t/\varphi_1) + \beta_2 \exp(-t/\varphi_2)$$

Table 1 shows that the rotational correlation time φ of the 1,4-naphthalendiol molecules in the water–ethanol solution and that of the 1,4-naphthalendiol molecules in trypsin solution are comparable and much smaller than φ of the trypsin solution in the absence of 1,4-naphthalendiol. So, the interaction of the phenol with the trypsin molecule is negligible.

Thus, the kinetics of the triple-enzyme bioluminescent system R + L + T was shown to vary in the presence of redox-active compounds. It was demonstrated that the effect of phenols on the bioluminescence parameters of the triple system R + L + T was negligible. This means that phenols do not influence trypsin activity. Variation of bioluminescence decay constant in the presence of quinones (organic oxidizers) is associated with their effect on the trypsin molecule. Quinones independently affect the coupled enzyme system (R + L) and the trypsin molecule (T). These conclusions might be extrapolated on the behavioural properties of other proteases and antiproteases if these were used instead of trypsin in the triple-enzyme bioluminescence system.

It is reasonable to compare the triple-enzyme system R + L + T with another triple-enzyme system, R + L + ADH, which includes an ADH-catalysing redox

Table 1. Parameters describing anisotropy decay of 1,4-naphthalendiol, trypsin and 1,4-naphthalendiol with trypsin solutions

β_1	φ_{1min}(ns)	φ_1(ns)	φ_{1max}(ns)	β_2	φ_{2min}(ns)	φ_2(ns)	φ_{2max}(ns)
(1) Water–ethanol solution of 1,4-naphthalendiol							
0.097	0.044	0.062	0.069	0.030	1.02	1.24	1.37
(2) 1,4-Naphthalendiol solution in the presence of trypsin							
0.168		0.076		0.025	1.51	1.75	1.99
(3) Trypsin solution							
0.12	1.02	1.55	2.23	0.14	9.68	13.85	26.12

$\varphi_{min, max}$ are the lower and upper variations of parameter φ.

NADH-dependent reaction. These two triple-enzyme systems are both suggested for use in the universal battery of bioassays (1, 2). In previous work (5) we investigated the effects of quinones and phenols on the triple-enzyme system R + L + ADH. Changes of the kinetics bioluminescent parameters (P and t_{max}) in the presence of quinones in this triple-enzyme system, R + L + ADH, were explained by shift of the NAD^+/NADH balance in the chain of the redox enzymatic reactions. It was found that modifications of enzymatic groups of ADH by quinones were not responsible for the changes of the bioluminescence kinetics parameters. Thus, the mechanisms of influence of the quinones on the triple-enzyme systems R + L + T and R + L + ADH are different. The triple-enzyme systems R + L + T and R + L + ADH both appeared not to be sensitive to the phenols. The general conclusion can be made that the method of coupling enzymic reactions in a bioluminescence system (i.e. elongated enzyme coupling chain, competitive relations or proteolytic interactions) governs the mechanisms of the pollutants' influence on these bioassay systems.

So, the protease activity in the solutions is governed by the redox characteristics of the media. The reported data could be useful for the development of new bioassays based on bioluminescent enzyme systems and for correct evaluation of the results of these assays.

Acknowledgments

This work was supported by Grant CRDF REC 002 (program 'Basic Research and High Education' of the Ministry of Education of the Russian Federation and the American Foundation of Civil Research); Krasnoyarsk Regional Scientific Foundation, Grant 10F084N; a Grant providing scientific cooperation between The Netherlands and the Russian Federation (NWO–Russia), No. 047-007-005; a NATO Collaborate Linkage Grant, No. CLG-974984; a Grant from Russian Foundation of Basic Research, No. 01-03-32843; a Grant from INTAS, No. N2001-562; and a Grant from the Russian Academy of Science for young scientists, No. N223.

REFERENCES

1. Kratasyuk VA. Bioassays for monitoring of aquatic ecosystems. Materials from International Conference, 'Biodiversity and Dynamics of Ecosystems in North Eurasia', Novosibirsk, Russia, 2000; **5**(1): 13–15.
2. Kratasyuk VA, Esimbekova EN, Gladyshev MI *et al*. The use of bioluminescent biotests for study of natural and laboratory aquatic ecosystems. *Chemosphere* 2001; **42**(8): 907–915.
3. Kudryasheva NS, Kratasyuk VA, Beloborov PI. Bioluminescent analysis. The action of toxicants: physical–chemical regularities of the toxicants' effects. *J. Anal. Lett.* 1994; **27**: 2931–2948.
4. Kudryasheva NS, Shalaeva EV, Zadorozhnaya EN *et al*. Patterns of inhibition of bacterial bioluminescence *in vitro* by quinones and phenols—components of sewage. *J. Biofizika* 1994; **39**: 455–464.
5. Kudryasheva NS, Kudinova IY, Esimbekova EN, Kratasyuk VA, Stom DI. Effects of quinones and phenols on the NAD (H)-dependent triple enzyme systems. *Chemosphere* 1998; **28**: 751–758.
6. Kozinets GI. *Interpretation of Analyses of Blood and Urine and their Clinical Meaning*. Triada-X: Moscow, 1998.
7. Stom DI. Influence of polyphenols and quinones on aquatic plants and their blocking of SH-groups. *J. Acta Hydrochim. Hydrobiol*. 1977; **5**: 291–298.
8. Petushkov VN, Kratasyuk VA, Fish AM, Gitelson JI. A method of evaluating protease activity. Author's certificate N1027615, published 07.07.83. Bulletin N25:159.
9. Hoek A, Visser AJWG. Artifact and distortion sources in time correlated single photon counting. *Analyt. Instrument*. 1985; **14**: 359–378.
10. Reed E, Frangineas G. Design and performance of a high-power mode-locked Nd:YLF laser. In *Solid State Lasers. SPIE Proceedings* 1990: 1223.
11. Vos K, van Hoek A, Visser AJWG. Application of a reference deconvolution method to tryptophan fluorescence in proteins. A refined description of rotational dynamics. *Eur. J. Biochem*. 1987; **165**: 55–63.
12. Novikov EG, van Hoek A, Visser AJWG, Hofstraat JW. Linear algorithms for stretched exponential decay analysis. *Opt. Commun*. 1999; **166**: 189–198.
13. Digris AV, Skakun VV, Novikov EG, van Hoek A, Claiborne A, Visser AJWG. Thermal stability of a flavoprotein assessed from associative analysis of polarized time-resolved fluorescence spectroscopy. *Eur. Biophys*. 1999; **28**: 526–531.
14. Dodson G, Wodawer A. Catalytic triads and their relatives. *TIBS* 1998; **3**: 347–352.

