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Optimization study of the catalytic activity of DNAzymes based on telomeric G-quadruplexes

Research Article

Joanna Kosman, Bernard Juskowiak*

Faculty of Chemistry, Adam Mickiewicz University, 60-780 Poznan, Poland

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Abstract: Optimization studies of the procedure for peroxidase activity measurements with DNAzymes based on telomeric sequences and colorimetric indicator reactions are reported. Effect of metal cation, nature and concentration of surfactant, as well as thermal treatment of G-quadruplex sample are investigated. Nature of metal cation exhibited modest influence on the system performance. Great improvement of enzymatic activity of the telomeric quadruplexes in the presence of Brij 58 surfactant was observed. Further improvement of catalytic activity of the system based on human telomeric sequence was attained by applying a thermal treatment (heating/rapid cooling) procedure to prepare G-quadruplex/hemin complexes.

Keywords: *DNAzyme • G-quadruplex • Peroxidase activity • Brij58 • Telomerase assay*

1. Introduction

DNAzymes are deoxyribooligonucleotides that show enzymatic activity and are exploited in medicine, biology and material sciences. Advantages of DNAzymes over conventional protein enzymes include their thermal stability and simpler preparation [1].

DNAzymes with peroxidase-like activity have recently attracted great interest. To assure such catalytic activity, guanine-rich oligonucleotides have to adopt a G-quadruplex structure, which can bind the hemin molecule. This system facilitates a redox reaction between the target molecule (ABTS, luminol) and hydrogen peroxide, which results in the appearance of oxidized products [1-3]. DNAzymes with peroxidase-mimicking activity have great potential in bioassays as nucleic acid probes for the detection of specific DNA sequences with colorimetric or chemiluminescent approaches. Other applications include determination of metal cations such as Ag^{+}, K^{+}, Hg^{2+}, Pb^{2+} or Cu^{2+} and amplified detection of small molecules such as adenosine, cocaine or AMP or proteins such as lysozyme or thrombin [1].

Applications of DNAzymes with peroxidase activity also involve the determination of activity of some DNA enzymes, for example, telomerase. The single stranded 3'-end of telomeric DNA possesses many repeats of a TTAGG sequence that maintains the intact structure of chromosome ends. However, this protecting mechanism is not sufficient and the genetic information is lost after a certain number of cell divisions, which finally leads to the cell death. But there are two exceptions to this rule, stem cells and cancer cells, in which telomerase maintains the proper length of telomeres, causing immortality of cancer cells [4]. Hence, the detection of telomerase activity in cells can be regarded as a simple test in cancer diagnosis [2,3]. Two recently reported indirect telomerase assays exploited the DNAzyme as labels and used chemiluminescent and colorimetric detection approaches [2,3]. The colorimetric assay was based on the molecular beacon as a DNA sensing scaffold [2]. In the presence of telomerase and dNTPs (mixture of deoxynucleotide triphosphates), the nucleic acid strand was lengthened, which caused rearrangement of the elongated hairpin and releasing of segments capable of forming DNAzyme unit. Indicator reaction of ABTS and H_2O_2 was monitored spectrophotometrically. Second proposed telomerase assay was a heterogeneous approach and used chemiluminescence readout [3]. Primer for telomerase was attached to a gold surface and treated with telomerase in the presence of dNTPs, which led to elongation of primer strands. In the next step, hybridization was carried out with a complementary DNA probe that contained DNAzyme unit as a label. After washing out the unhybridized probe molecules from the Au surface, the chemiluminescence reaction with luminol was carried out.

* *E-mail: juskowia@amu.edu.pl*

It should be noted that most of the reported DNAzyme-based biosensor systems exploited a PS2.M sequence (5'-GTGGGTAGGGCGGGTTGG-3') that was selected in vitro because of its enhanced peroxidase activity. There are, however, examples of genomic guanine-rich sequences that can form G-quadruplex structures. Therefore, DNAzymes generated with G-quadruplexes from genomic sequences are expected to exhibit peroxidase-like activity. Such DNAzymes could be exploited for the direct detection of these genomic sequences. This approach has been recently reported by Willner *et al.* [5] for the determination of telomerase activity. They have exploited a weak catalytic activity of DNAzymes produced from the telomerase-elongated human telomeric sequences to oxidize colorimetric substrate (3,3',5,5'-tetramethylbenzidine). On the other hand, our preliminary optimization studies on the telomeric DNAzyme/ABTS system revealed that tuning of certain experimental conditions could enhance peroxidase activity of this DNAzyme.

This contribution reports on the results of the optimization study of the catalytic reaction between ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) and hydrogen peroxide, which lead to colored product in the presence of DNAzymes based on the human telomeric sequence. The schematic representation of the DNAzyme formation is shown in Fig. 1. Effect of DNA strand length was tested on the example of two oligonucleotides ($d(AG_3(TTAG_3)_3)$ and $d(AG_3(TTAG_3)_7)$). Efforts were made to enhance activity of telomeric DNA-based DNAzymes by changing reaction conditions: (i) nature of metal cation (K^+, Sr^{2+}, NH_4^+), (ii) presence of organic modifiers at three different concentrations (Tween 20, Brij 56, Brij 58, Triton X-100), and (iii) thermal treatment of the enzymatic system.

2. Experimental procedure

2.1. Materials

All oligonucleotides (PS2.M: 5'GTGGGTAGGGCG-GGTTGG3', Tel1: 5'AGGGTTAGGG TTAGGG-TTAGGG3', and Tel2: 5'AGGGTTAGGGTTAGG-GTTAGGGTTAGGG TTAGGGTTAGGGT-TAGGG3') were purchased from Genomed (Poland). Human telomeric sequences were chosen to possess one G-quadruplex structure (Tel1) and two G-quadruplex units (Tel2). The concentrations of oligonucleotides are reported in strand units. Oligonucleotides were dissolved in 10 mM Tris-HCl buffer of pH 8. Hemin, 2,2'-azinobis(3-ethylbenzothiozoline)-6-sulfonic acid (ABTS), Triton X-100, Brij 58, Brij 56, and Tween 20 were obtained from Sigma-Aldrich. All chemicals were of reagent grade purity and were used without further purification.

2.2. Standard procedure for the peroxidase activity measurement

Aqueous solution of oligonucleotide at 0.86 μM concentration in buffer (10 mM Tris-HCl, pH 8.0) containing 0.8 mM $MgCl_2$, surfactant at chosen concentration, and metal cation (16 mM) was heated at 95°C for 5 min and then slowly cooled to room temperature (sample was left in the heating block for 3 hours to reach the room temperature). After 30 min, hemin (1.72 μM) was added and solution was held at room temperature for another 60 min, then ABTS (1.72 mM) was added. Finally, the indicator reaction started after addition of H_2O_2 (1.72 mM) and absorbance was monitored at λ = 417 nm for 10 min using a Pharo 100 Spectrophotometer or Tecan M200 Infinite microplate reader. Absorption spectra were also recorded in the

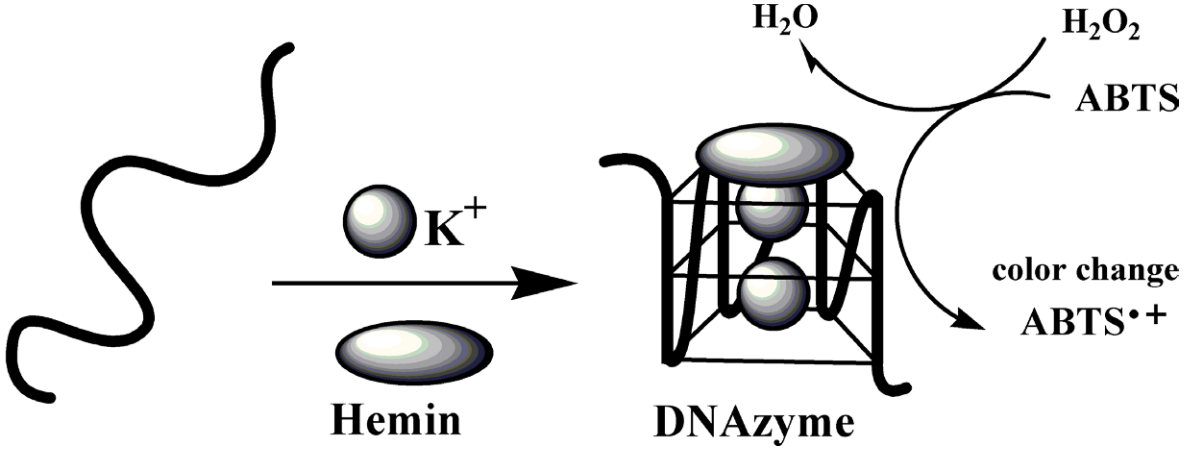

Figure 1. Schematic representation of the DNAzyme based on the G-quadruplex of telomeric repeat sequence and the indicator reaction with ABTS.

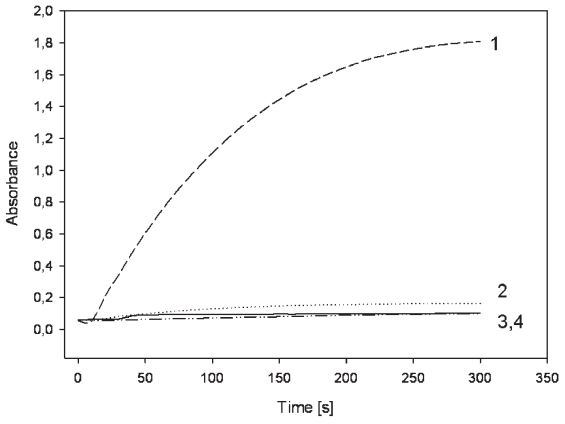

Figure 2. Absorbance changes at 417 nm, of the color product of ABTS oxidation catalyzed by DNAzyme based on: PS2.M (1), Tel2 (2), Tel1 (3), and hemin itself (4).

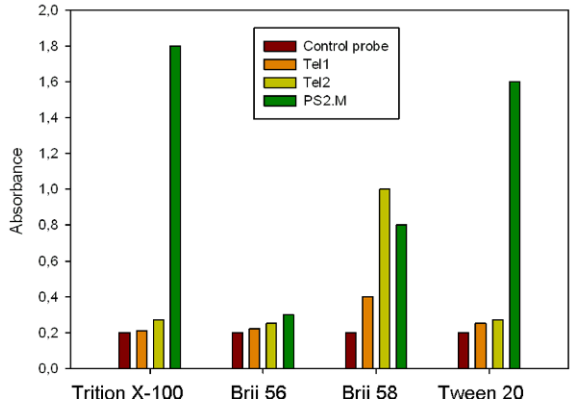

Figure 3. Comparison of absorbances at 417 nm obtained for indicator ABTS reaction catalyzed by DNAzymes in the presence of different surfactants.

300 – 500 nm spectral range before and after completion of the ABTS reaction. The cation radical of ABTS (the oxidation product) exhibits absorption maxima at wavelengths around 415 nm, 645 nm, 734 nm and 815 nm [6]. Because of the highest molar absorptivity, the selection of monitoring wavelength at the band of 415 nm is advantageous especially for colorless samples. The absorbance level attained by the system in the reaction time of 10 min was arbitrary chosen as a measure of the relative catalytic activity of DNAzyme. A control probe was prepared as above and contained all reagents (buffer, metal cation, surfactant and hemin) except for the G-quadruplex forming oligonucleotide.

2.3. Alternative procedure for the peroxidase activity measurement with rapid cooling step

Aqueous solution of oligonucleotide at 0.86 μM concentration in buffer (10 mM Tris-HCl, pH 8.0) containing 0.8 mM $MgCl_2$, 0.1 mM Brij 58, 16 mM KCl and 1.72 μM hemin was heated at 95°C for 5 min and then rapidly cooled to room temperature in a water/ice bath (approx. 15 min). ABTS (1.72 mM) was added to the sample solution and the indicator reaction started after addition of H_2O_2 (1.72 mM). Monitoring of the indicator reaction was carried out as described for the standard procedure.

3. Results and discussion

To exhibit peroxidase-like activity, the oligonucleotide has to adopt a G-quadruplex structure that is able to bind hemin molecule [1,2]. Such a tetraplex structure is crucial for the high activity of the system since the specifically bound hemin can form an activated intermediate with hydrogen peroxide. G-quadruplex structure is stabilized by hydrogen bonds between guanosine residues arranged in G-tetrads [7]. Different topologies of G-quadruplexes, classified as parallel or antiparallel, can be formed depending on the direction of DNA strands in quadruplex and the number of DNA molecules involved. Quadruplex topology is mainly determined by DNA sequence but environmental conditions may also play important roles [8,9]. All oligonucleotides investigated can form G-quadruplexes that should be able to bind hemin molecules.

Fig. 2 shows absorbance changes with time of indicator reaction carried out for DNAzyme systems based on different G-quadruplexes. The reference probe containing PS2.M oligonucleotide (plot 1) shows very high increase in absorbance that agrees with the reported high peroxidase activity of this system [7,10]. Activity of PS2.M oligonucleotide is about 10 times higher than that observed for Tel2 (plot 2) and telomeric sequences show only slightly higher activity then for the control system with hemin itself. Low peroxidase activity of quadruplexes based on telomeric sequences is consistent with literature reports [5] and may be regarded as a good starting point for the optimization study.

The presence of metal cations is crucial for quadruplex formation. Early studies implied that potassium ion is responsible for high catalytic activity of PS2.M sequence [11] but recent studies proved that other cations (Sr^{2+}, Na^+, NH_4^+) could also be used for this purpose [12]. Cheng and co-workers noticed the relationship between quadruplex topology and enzymatic activity of oligonucleotides [13]. This was related to different abilities of particular quadruplexes to bind hemin properly. Unimolecular parallel quadruplexes exhibit the highest peroxidase-like activity, which is

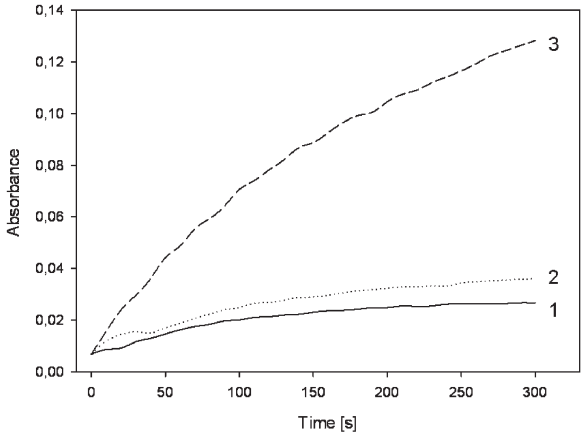

Figure 4. Effect of thermal treatment of G-quadruplex samples on the absorbance changes at 417 nm for ABTS reaction catalyzed by the Tel2/Brij 58 DNAzyme system: 1 – standard procedure with slow cooling, 2 – fast cooling before hemin addition, 3 – fast cooling after hemin addition.

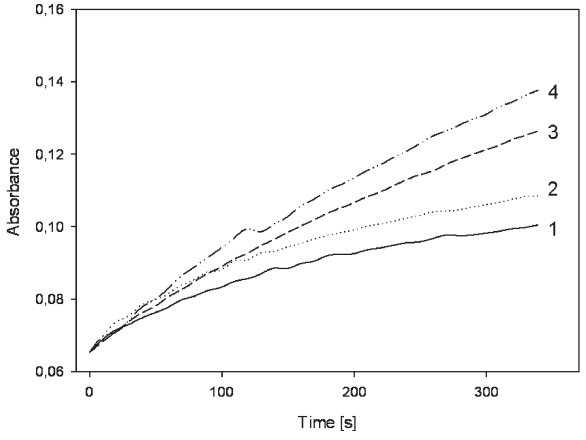

Figure 5. Temperature effect on the ABTS indicator reaction catalyzed by the Tel2/Brij 58 DNAzyme system, shown as absorbance changes at 417 nm: 1 – 25˚C, 2 – 30˚C, 3 – 35˚C, 4 - 40˚C.

probably connected with the availability of their exposed guanine tetrads for the hemin end-stacking binding mode [14]. The effect of different cations (K^+, NH_4^+, Sr^{2+}) on the enzymatic activity of particular DNAzyme systems was studied. Although literature reports suggested formation of parallel quadruplexes by telomeric oligonucleotides in the presence of NH_4^+ or Sr^{2+} cations [12], we have not observed any improvement effects upon replacing potassium with ammonium or strontium cations. Therefore, further studies were carried out in the presence of potassium cation.

On the other hand, the protocol for determination of peroxidase activity of DNAzyme requires addition of the surface-active agent, Triton X-100 [7]. Since the concentration of this nonionic surfactant used in the assay is above its CMC value (critical micelle concentration), one can assume an active role of micelles in quadruplex formation or quadruplex/hemin interaction mechanisms. Four nonionic surfactants were tested that differed in CMC values as shown in Table 1. Preliminary studies were carried out using three different surfactant concentrations: below CMC, at CMC, and at concentration above CMC. Best results were observed for concentrations of surfactants that were close to CMC values. Fig. 3 shows a bar plot that represents relative peroxidase activity of particular systems and are compared with the efficiency of the standard approach based on Triton X-100. The systems containing Brij 56 and Tween 20 have not exhibited significant changes in absorbance level for Tel1 and Tel2 systems, but in the case of reference sequence (PS2.M), the presence of Brij 56 resulted in marked decrease in peroxidase activity. Only the Brij 58-based systems showed noticeable effects with a significant improvement of Tel 2 activity, which exceeded even that for a PS2.M-based system. It should be noted however, that activity of the Tel2/Brij 58 DNAzyme was still lower than that for the PS2.M/Triton X-100 classic system.

Table 1. Surfactants used in the study and their critical micelle concentration (CMC) values.

Surfactant	Formula	CMC [mol dm^{-3}]
Triton X-100	$C_{14}H_{22}(C_2H_4O)_{10}$	0.2×10^{-3}
Tween 20	$C_{18}H_{34}O_6(C_2H_4O)_{20}$	8.0×10^{-5}
Brij 56	$C_{16}H_{33}(C_2H_4O)_{10}$	0.35×10^{-4}
Brij 58	$C_{16}H_{33}(C_2H_4O)_{20}$	0.77×10^{-4}

Thermal treatment of quadruplexes during sample preparation and temperature effect on the indicator ABTS reaction were also tested. A general tendency of guanine-rich oligonucleotides toward formation of multistranded assemblies at higher strand concentrations and in low temperature is well known [8,9]. To ensure that any existing structures formed during oligonucleotide synthesis, preservation and resuspension were dissociated, the standard sample preparation protocol involved heating of the DNA sample at 95˚C for 5 min followed by slow cooling. Assuming that oligonucleotides can form different quadruplex structures, the slow cooling allows thermodynamic equilibration between them. On the contrary, rapid cooling may result in the formation of a kinetically preferred topology that may exhibit different catalytic properties. We examined the effect of cooling rate on the peroxidase activity of the Tel2/Brij58 system and results are shown in Fig. 4. Plot 1 shows results obtained according to the standard procedure (slow cooling) and addition of hemin after the heating/cooling steps. Rapid cooling of the quadruplex solution enhanced activity of the system (plot 2), which suggested that the kinetically arrested quadruplex

structure is more active in enzymatic reactions. To clarify if a particular topology possesses this active structure, additional investigations are needed including CD spectroscopy. Also, we have checked whether the oligonucleotide must first adopt a quadruplex structure before hemin addition or the complex between them can be formed dynamically during quadruplex formation. Plot 3 in Fig. 4 shows that significant enhancement of DNAzyme activity is observed when hemin is added before the cooling step. Such a result may suggest that the binding of hemin with a Tel2 sequence mediates formation of a quadruplex structure, which possesses very high enzymatic activity. Interestingly, similar experiments with the PS2.M/Brij58 system did not revealed any specific effects of thermal treatment (fast against slow cooling) of the sample solution. Altering hemin addition sequence in fast cooling procedure gave similar results for the PS2.M system as with the standard protocol. Beside significant improvement of the assay sensitivity for telomere DNA, the developed procedure allows shortening of the assay duration from 5 hours to only 1 hour. The effect of temperature on the ABTS indicator reaction was also examined. Experiments were carried out according to the standard procedure, but the final ABTS indicator reaction was carried out at different temperatures. Reactions were performed in the thermostated accessory of spectrophotometer at four temperatures (25˚C, 30˚C, 35˚C, 40˚C) and results are shown in Fig. 5. The increase of peroxidase activity of Tel2 with temperature rising is evident and indicates that the complex between G-quadruplex and hemin does not undergo noticeable dissociation in the temperature range tested and the rate of the reaction increases with temperature as observed for most enzymatic reactions.

4. Conclusions

Effect of metal cation, nature and concentration of surfactant, as well as impact of thermal treatment of G-quadruplex sample on the peroxidase-mimicking activity of human telomere DNAzyme were investigated. Results confirmed the importance of environmental conditions on the activity of peroxidase-mimicking DNAzymes. Great improvement in enzymatic activity of the telomeric quadruplexes in the presence of Brij 58 surfactant was observed. Further improvement of catalytic activity of the system was attained by applying a thermal treatment (heating/rapid cooling) procedure to prepare the G-quadruplex/hemin complex. These optimization studies indicated that an efficient and direct assay for telomerase activity can be successfully developed. Further studies with cell extracts are in progress.

References

[1] I. Willner, B. Schlyahovsky, M. Zayats, B. Willner, Chem. Soc. Rev. 37, 1153 (2008)
[2] Y. Xiao, V. Pavlov, T. Niazov, A. Dishon, M. Kotler, I. Wilnner, J. Am. Chem. Soc. 126, 7430 (2004)
[3] V. Pavlov, Y. Xiao, R. Gill, A. Dishon, M. Kotler, I. Willner, Anal. Chem. 76, 2152 (2004)
[4] K. Dhaene, E.V. Marck, R. Parwaresch, Virchows Arch. 437, 1 (2000)
[5] R. Freeman, E. Sharon, C. Teller, A. Henning, Y. Tzfati, I. Willner, ChemBioChem 11, 2362 (2010)
[6] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Free Radic. Biol. Med. 26, 1231 (1999)
[7] P. Travascio, Y. Liu, D. Sen, Chem. Biol. 5, 505 (1998)
[8] A. Włodarczyk, P. Grzybowski, A. Patkowski, A. Dobek, J. Phys. Chem. B 109, 3594 (2005)
[9] S. Nagatoishi, Y. Tanaka, K. Tsumoto, Biochem. Biophys. Res. Commun. 352, 812 (2007)
[10] A.T. Phan, FEBS J. 277, 1107 (2010)
[11] Y. Li, D. Sen, Biochem. 36, 5589 (2001)
[12] S. Nakayama, H.O. Sintim, J. Am. Chem Soc. 131, 10320 (2009)
[13] X. Cheng, X. Liu, T. Bing, Z. Cao, D. Shangguan, Biochem. 48, 7817 (2009)
[14] D. Kong, W. Yang, J. Wu, C. Li, H. Shen, Analyst 135, 321 (2010)