Analytical Biochemistry xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

A simple assay for the ribonuclease activity of ribonucleases in the presence of ethidium bromide

Debi Ranjan Tripathy, Amit Kumar Dinda, Swagata Dasgupta *

Department of Chemistry, Indian Institute of Technology, Kharagpur, Kharagpur 721302, India

ARTICLE INFO

Article history:
Received 9 January 2013
Received in revised form 28 February 2013
Accepted 1 March 2013
Available online xxxx

Keywords:
Ribonucleolytic assay
Ribonucleic acid
Ethidium bromide
Ribonuclease A
Angiogenin

ABSTRACT

The ribonuclease (RNase) activity of ribonucleases has been assayed by observing the change in fluorescence intensity of ethidium bromide on binding with yeast RNA. The binding of EtBr with RNA was monitored via UV–vis and fluorimetric methods. The degradation of RNA by RNase A was monitored by the change in fluorescence emission intensity of ethidium bromide at 600 nm on excitation at 510 nm. The ribonucleolytic activity of RNase A and angiogenin at various pH values was determined by this method. From this technique we have also determined the macroscopic pK_a values of active site residues of these enzymes. This assay permits the evaluation of the catalytic efficiency of enzymatic proteins ranging from high ribonucleolytic activity to low ribonucleolytic activity toward the natural substrate RNA.

The ribonucleolytic activity of certain ribonuclease family members is correlated with their biological properties. For example, the biological activity of angiogenin is dependent on its ribonucleolytic activity [1]. Eosinophil-derived neurotoxin (EDN) has neurotoxic and antiviral activities that are dependent on its ribonucleolytic activity [2,3]. The ribonuclease activity is also required for the antiviral and neurotoxic activity of eosinophil cationic protein (ECP) [4]. Since most of these proteins show an elevated level under disease conditions [5], a rapid assay to monitor increased levels would be of immense use.

Ribonucleases catalyze the degradation of RNA to smaller fragments, namely the nucleotides. In this cleavage process two His residues present at the active site of ribonucleases behave as proton donors and acceptors. His residues in the active site are well conserved for ribonucleolytic proteins [6]. The proton at the 2′-OH of the sugar ring is taken up by His that acts as a base, while the other His acts as an acid to protonate the oxide of the phosphate backbone. The intermediate thus formed (the cyclic phosphodiester) is hydrolyzed in the presence of one molecule of water in which the roles of the His residues are reversed [6]. This cleavage process plays a vital role in cellular pathways. Since almost all proteins are synthesized from DNA via RNA, the relative activity of these ribonucleolytic enzymes has a profound effect on the cellular system. This degradation process is also crucial for various biological processes. Thus knowledge of certain parameters of this class of enzymes related to the active site residues, such as the ionization constant, is of interest for the effect they have on the catalytic mechanism of the enzymes [7].

Ethidium bromide is known to bind with DNA and RNA. Many fluorescent dyes are available to determine the concentration of nucleic acids such as SYBR Green II [8] and ethidium bromide that can be used to quantify the concentration of RNA [9]. Richard et al. have reported a qualitative fluorometric method for the assay of RNase activity [10]. In this present study, the ribonucleolytic assay of RNase A and angiogenin has been optimized via a fluorescence assay. Initially, the change of absorbance spectra of EtBr in the presence of RNA was monitored via UV–vis spectroscopy. The degradation rate of RNA in the presence of EtBr was studied fluorimetrically for RNase A and angiogenin. The fluorescence enhancement of this dye in the presence of RNA facilitated the fluorescence kinetic study. Finally, the ribonucleolytic activity of RNase A and angiogenin has been investigated at various pH values. From the pH vs log (k_{cat}/K_m) plots, the macroscopic pK_a value of active site His of both ribonucleases was determined.

It has been observed that ribonuclease activity increases in cancer cells attaining a level much higher than the control both in humans and in animals [11]. The serum and urine of the affected individuals have elevated levels of certain ribonucleolytic proteins as well due to pathological disorders. Interestingly, the activity of the ribonuclease inhibitors was found to decrease in cancer tissues [12]. It appears therefore that a rapid assay that is also sensitive for the ribonuclease activity is essential. The method proposed here is a simple yet effective method that is also inexpensive.

* Corresponding author. Fax: +91 3222 282252.
E-mail address: swagata@chem.iitkgp.ernet.in (S. Dasgupta).

0003-2697/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2013.03.005

Please cite this article in press as: D.R. Tripathy et al., A simple assay for the ribonuclease activity of ribonucleases in the presence of ethidium bromide, Anal. Biochem. (2013), http://dx.doi.org/10.1016/j.ab.2013.03.005

Materials and methods

Materials

Bovine pancreatic RNase A and yeast RNA MW 5000–8000 (Sigma–Aldrich, Catalog No. 83850), were purchased from Sigma–Aldrich (St. Louis, MO, USA). Angiogenin was isolated from buffalo milk according to Bond et al. [13]. EtBr and all other reagents were from SRL India. Buffers were prepared using diethyl pyrocarbonate (DEPC)-treated autoclaved water. A Shimadzu UV–vis spectrophotometer (UV-2450) was used for spectrophotometric determination. All fluorimetric measurements were performed on a Horiba Jobin Yvon Fluoromax-4 spectrofluorimeter. Concentrations of species were determined using the following data: RNase A $\varepsilon_{278.5}$ = 9800 M^{-1} cm^{-1}; EtBr ε_{480} = 5450 M^{-1} cm^{-1} in water [14,15]. The concentration of angiogenin was measured by ε_{280} = 9200 M^{-1} cm^{-1}. All experiments were performed at least three times.

UV–vis spectroscopic study of EtBr with RNA

To determine the binding efficiency of EtBr with RNA, UV–vis spectra of EtBr in the presence and absence of RNA were monitored. The stock concentration of EtBr was measured in water. A 63 μM EtBr was taken in 0.1 Mes-NaOH buffer containing 0.1 M NaCl. The UV–vis spectra of 63 μM EtBr was monitored on addition of varying concentrations of RNA (from 0 to 0.33 mg/ml).

Fluorescence study of EtBr in the presence of RNA

Fluorimetric titrations were performed in a 3 ml quartz cuvette. A 3 ml aliquot of 1 μM EtBr solution was excited at 510 nm and emission monitored from 520 to 700 nm using a 2/5 nm slit width in 0.1 M sodium phosphate pH 6.5 buffer containing 0.1 M NaCl. This EtBr solution was titrated by successive addition of 10 μl of 1 mg/ml RNA each time.

Degradation of RNA with enhancement of RNase A concentration

The degradation of RNA with respect to change in the concentration of RNase A was monitored at pH 7.5 (0.05 M Tris, acetic acid and Mes-NaOH buffer) containing 0.1 M NaCl. The RNA and EtBr solutions were mixed and incubated for 30 min. This mixture was used for the kinetic studies. In the cuvette 2.7 ml buffer with 0.2 ml (1 mg/ml RNA with 0.077 mM EtBr) was mixed and incubated for 100 s. To this solution 20 to 200 μl of 2 μM RNase A was added and the change in fluorescence intensity monitored for 300 s using an excitation and emission wavelength of 510 and 600 nm and a slit width of 5/5 nm.

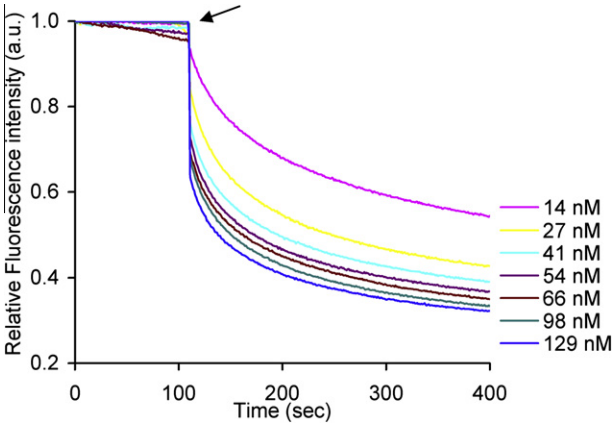

Fig.2. Relationship between the changes in fluorescence intensity of the EtBr–RNA system with increasing concentrations of RNase A (14 to 129 nM). The arrow indicates the addition of RNase A to the solution. EtBr = 5.12 μM; RNA = 66.67 μg/ml. λ_{ex} = 510 nm.

pH metric assay for RNA cleavage

Before the kinetic experiment RNA was mixed with EtBr for 30 min. A 100 μl 2 μM RNase A aliquot was added after 100 s in the 66.67 μg/ml RNA and 5.12 μM EtBr (final concentration in 3.0 mL) for the kinetic measurements. Degradation of RNA was monitored via a change in fluorescence intensity of EtBr. The excitation and emission wavelengths were 510 and 600 nm, respectively. Integration time and slit width were 0.1 s and 5/5 nm, respectively. The various pH values from 4 to 9 were adjusted by addition of 1 M HCl or 1 M NaOH in 0.05 M Tris, acetic acid, and Mes buffer solution containing 0.1 M NaCl. Only EtBr fluorescence intensity was measured at each pH under the same experimental conditions. We performed the enzymatic assay for RNase A and angiogenin at different pH values. The effective concentrations of RNase A and angiogenin are 67 and 400 nM, respectively, in the respective reaction mixtures.

Results and discussion

Ribonuclease like proteins comprises a large superfamily of proteins, many of which also have special biological properties such as angiogenin. There are many enzymes within the ribonuclease family. Members such as onconase [16,17], bovine seminal RNase [18], eosinophilic cationic protein [19], and angiogenin are known for their biological activities that range from cytotoxic and antitumor effects as well as antiviral, antimicrobial, and neovascularization.

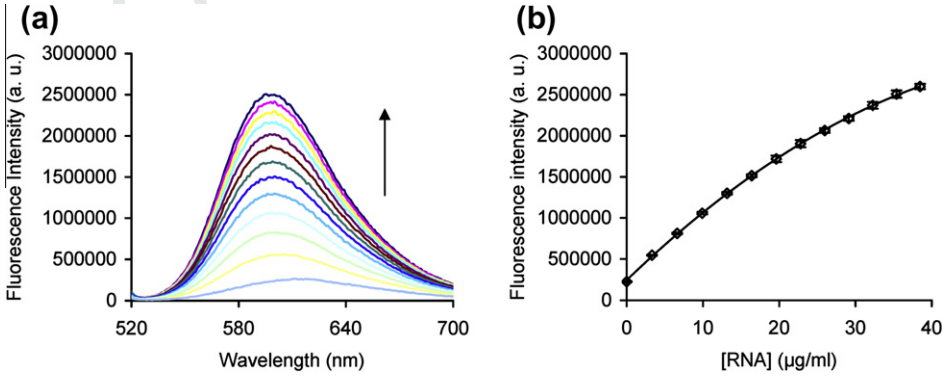

Fig.1. (a) Fluorescence spectra of EtBr (1 μM) in the presence of RNA (0 to 38 μg/ml); the arrow indicates increasing concentrations of RNA. (b) Fluorescence intensity of EtBr (1 μM) in the presence of varying concentrations of RNA on excitation at 510 nm.

Q1 Please cite this article in press as: D.R. Tripathy et al., A simple assay for the ribonuclease activity of ribonucleases in the presence of ethidium bromide, Anal. Biochem. (2013), http://dx.doi.org/10.1016/j.ab.2013.03.005