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Improved Fluorometric Assay Method for Ribonuclease Activity

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Abstract : A simple quantitative assay method for ribonuclease activity has been developed. This method is based on the decrease of fluorescence intensity emitted by the ethidium bromide bound to RNA due to the degradation of RNA by ribonuclease. The substrate RNA was reacted with ribonuclease A, and the fluorescence intensity was measured after the addition of ethidium bromide. The intensity difference was calculated using a blank reaction mixture containing no RNase. Whole cellular RNA substrate produced a significant error and was not suitable for this assay method possibly because of local microheterogeniety caused by high molecular weight rRNA, but satisfying results were obtained with tRNA substrate. The intensity difference increased linearly by raising enzyme concentration up to 2×10^{-4} Kunitz Units of ribonuclease A. More refined and reliable results were obtained by use of initial reaction velocities which were calculated from the plots of intensity difference vs. time. A linear relationship between initial velocities and enzyme concentrations was observed up to 0.01 Kunitz Units of enzyme.
Key words : ethidium bromide, fluorometric assay, ribonuclease

Ribonucleases (RNases) catalyze the hydrolysis of RNA molecules, and are involved in various biological functions including angiogenic, antitumor, or antibiotic activities (D'Alessio, 1993). Ribonucleolytic activity is the essential part of these functions. Typically, ribonucleolytic activity can be determined by release of perchloric acid-soluble nucleotides (Blank and Dekker, 1981). In this assay method, RNA is reacted with RNase, and the undegraded RNA is removed by centrifugation after precipitation with acid. This precipitation step is susceptible to the concentration of acid, temperature, and centrifugal force, affecting sensitivity of this assay method. Moreover, it makes this assay cumbersom and limits the number of samples to be handled. More sensitive quantitave assay methods were also reported. Lee *et al.* (1983) utilized the degradation of f[^{3}H]Met-tRNA and the extraction of radioactive amino acid with an organic solvent. In another method, the ring opening of cytidine 2',3'-phosphate to cytidine 3'-phosphate by RNase was measured spectrophotometrically (Crook *et al.*, 1960). Both of these methods require specific substrates and are not used widely.

The present work is based on the fact that the fluorescence intensity of ethidium bromide is greatly enhanced by binding to nucleic acids. Ethidium bromide is a fluorescent dye which absorbs ultraviolet light at 300 nm and emits light at 590 nm. It intercalates between the stacked bases of nucleic acids and yields a much higher intensity of fluorescence (Le Pecq and Paoletti, 1967). Nonintercalative binding to tRNA was also reported (Liebman *et al.*, 1977). Therefore, a very low concentraton of nucleic acids can be detected by measuring fluorescence intensity of bound ethidium bromide (Le Pecq and Paoletti, 1966). The dye has a higher affinity for double-stranded nucleic acids than single-stranded ones. When degraded by nucleases, DNA or RNA molecules are converted to nucleotides or shorter fragments with a lower affinity for ethidium bromide.

This observation led to the development of a quantitative assay method for RNase activity (Kamm *et al.*, 1970). A reaction mixture containing RNase, RNA, and ethidium bromide was placed in a cuvette and the fluorescence was recorded over time. Although this method is much simpler than the acid-precipitation method, there are several drawbacks such as insensitivity, possible interference of ethidium bromide with the reaction, and difficulty in the assay of multiple samples. Other fluorometric asay methods were also developed for the quantitative determination of endonucleases (Marusyk *et al.*, 1975) or mitochondrial nuclease (Dake *et al.*, 1988), or for the qualitative detection of nucleases (Burke and Slinker, 1980).

This report describes a more sensitive and reliable flu-

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orometric assay method for ribonucleolytic activity. The reaction mixtures containing RNase A and tRNA were mixed with ethidium bromide after incubation for a fixed time, and were directly subjected to fluorometric measurements. The notable improvements in this procedure are: 1) Simultaneous assay of multiple samples was made easier by using the photometric mode instead of the time-scanning mode. 2) The possible interference of ethidium bromide with RNase activity was excluded by adding ethidium bromide after the reaction. 3) The sensitivity was greatly enhanced, thus enabling a precise estimation of nanograms of RNase A.

Materials and Methods

Materials

Ethidium bromide was purchased from Aldrich (Milwaukee, USA). Calf liver RNA (type IV), yeast tRNA (Type X), bovine pancreatic RNase A (type II-A), and other chemicals for buffers were obtained from Sigma (St. Louis, USA).

Standard assay procedure

Reaction mixtures containing 200 μl of 3X reaction buffer (0.1 M Tris·Cl, pH 8.5, 150 mM sucrose, 450 mM NaCl, autoclaved), 200 μl of 0.1 mg/ml tRNA, and 200 μl of RNase A were prepared. They were incubated at 37°C with shaking for 1 h, and were transferred to an ice bath. An equal volume (600 μl) of 2 μg/ml ethidium bromide solution in TE buffer (10 mM Tris · Cl, pH 7.4, 1 mM EDTA, pH 8.0) was added to each reaction mixture. The whole mixture was transferred to a cuvette and the fluorescence intensity was measured with a fluorescence spectrophotometer, Hitach F-2000, under the following conditions: excitation wavelength, 300 nm; emission wavelength, 590 nm; integration time, 2 sec; response time, 0.1 sec; excitation bandpass, 20 nm; emission bandpass, 20 nm; PM voltage, 700 V. The intensity difference was defined as a difference between the intensity of a given reaction mixture and that of a blank reaction mixture containing no RNase.

Results and Discussion

Determination of a maximum substrate concentration

Two sets of reaction mixtures containing various amounts of yeast tRNA were prepared, one set of which was fully hydrolyzed by incubating with 8.2 Kunitz Units of RNase A at 37°C for 4 h. Ethidium bromide solution was added and the fluorescence intensities were measured. The intensity of the intact sample reached a maximum (3240 units) at 50 μg/ml of tRNA (Fig. 1). Similar results were obtained with whole RNA from calf liver (data not shown). On the other hand, the hydrolyzed tRNA gave approximately the same intensity of 690 units for all the concentrations of tRNA tested, suggesting that the completely hydrolyzed tRNA does not enhance the fluorescence intensity of ethidium bromide. There was a linear relationship between the intensity and the concentration of tRNA in the range of 0~40 μg/ml (inset of Fig. 1). Therefore, the upper substrate concentration defined by the instrument used for the analysis was determined to be about 40 μg/ml.

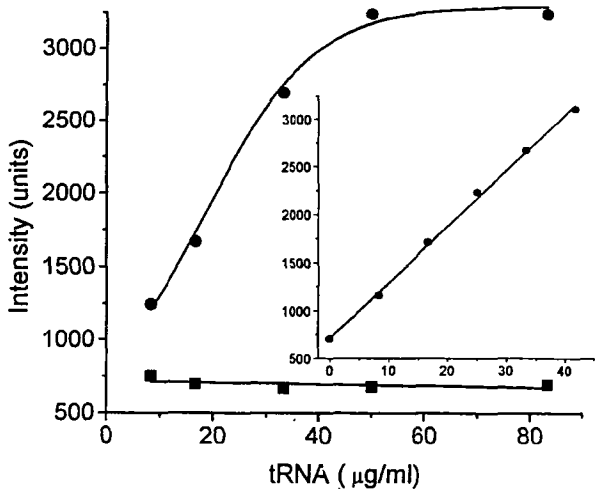

Fig. 1. The plot of fluorescence intensity vs. concentration of tRNA. Fluorescence intensities were measured from two sets of reaction mixtures containing various amounts of tRNA as described in Materials and Methods; one set was fully hydrolyzed with RNase A (■), and the other was not (●). Fluorescence intensities were again measured with 0~40 μg/ml of tRNA (inset; data fit by linear regression analysis).

Determination of a suitable substrate

When the calf liver whole RNA was used as a substrate, the intensity difference increased upon raising either enzyme concentration or incubation time, but with significant deviations (data not shown). It is possible that the isolated high molecular weight rRNA molecules might assume different structures, thus causing fluctuations in the fluorescence measurements with ethidium bromide. In this sense, tRNA could be a better substrate. To examine this further, the fluorescence intensity of whole RNA or tRNA reacted with RNase was scanned over a period of time. While a steady and slow decrease of intensity was observed with tRNA, the whole RNA produced intensity fluctuations over time (Fig. 2). The fluctuation patterns varied greatly in different assays (data not shown). The result clearly indicates that tRNA is a better subtrate for this assay

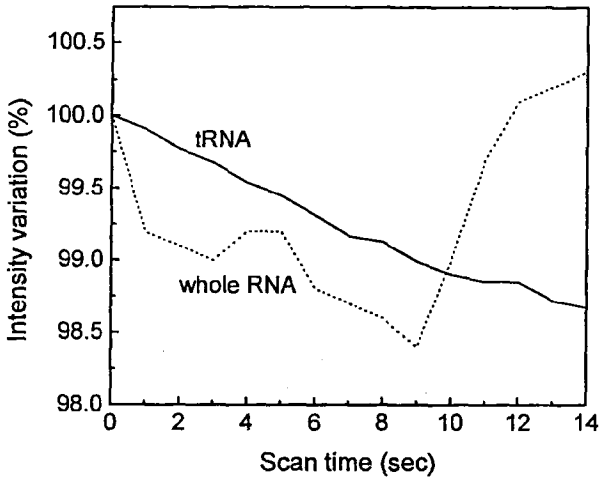

Fig. 2. Fluorescence intensity variation over time. Whole RNA or tRNA was reacted with RNase A under the standard assay protocol except that the incubation time was 30 min, and the fluorescence intensity of each reaction mixture was scanned for a period of time. The intensities at zero time were considered as 100% to calculate intensity variation. The solid line represents the intensity scanning of tRNA, and the dotted line does for whole RNA.

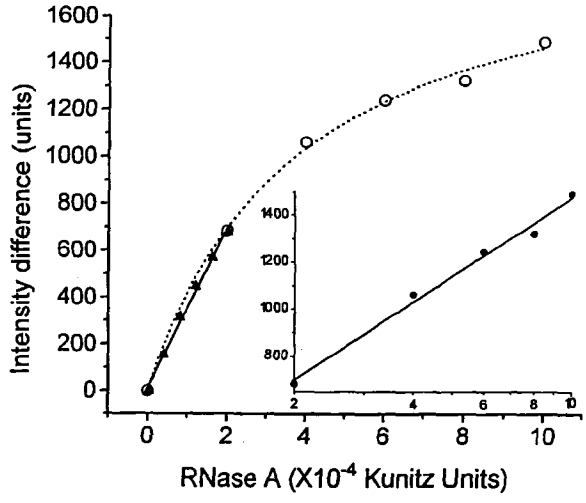

Fig. 3. The plot of intensity difference against enzyme units. The assay was performed under the standard assay condition with $0\sim10^{-3}$ Kunitz units of RNase A (○). It was reproduced in the narrow range of enzyme concentration (▲). The incubation time was 60 min in both cases. The hyperbolic curve was converted to a linear one by using a semi-log plot (inset).

method.

Standard assay with tRNA

The intensity difference was proportional to the enzyme concentration with tRNA substrate and produced a hyperbolic curve (Fig. 3). It was possible to convert it to a linear plot by taking logarithms of the enzyme amounts (inset of Fig. 3). Since the discrepancy from linearity is possibly due to substrate limitation or product inhibition, a linear relationship might be obtained either by using a lower enzyme concentration or by reducing the incubation time. A good straight line was obtained in the range of $0\sim2\times10^{-4}$ Kunitz Units of RNase A (Fig. 3). By the procedure of Kamm *et al.* (1970), a linear relationship was observed in the range of 64~256 Kunitz Units (1.7~6.7 mg) of RNase A. For the decrease of fluorescence intensity was recorded over a period of time with this method, the reaction time should be kept to a minimum for the rapid assay of many samples, and a large amount of RNase is required. By contrast, a precise estimation of nanograms of RNase A has been made possible by employing a longer incubation time in our procedure. The improvement in sensitivity is partly due to the change of the excitation wavelength from 546 nm to 300 nm. The fluorescence enhancement of ethidium bromide on binding to RNA was reported to be greater in 300 nm excitation rather than in 546 nm (Le Pecq and Paoletti, 1967; Burns, 1971). Another potential advantage of our method is that the ethidium bromide was added after the end of a reaction. Holbrook *et al.* (1975) pointed out problems in using nucleic acid-ethidium bromide complexes as substrates since the dye was shown to interfere with the activity of RNases.

Initial reaction velocity approach

Theoretically, the initial reaction velocities are expected to give better results than the measurements of the product accumulation for a fixed reaction time. This is especially true when problems arise from substrate limitation or product inhibition, because the con-

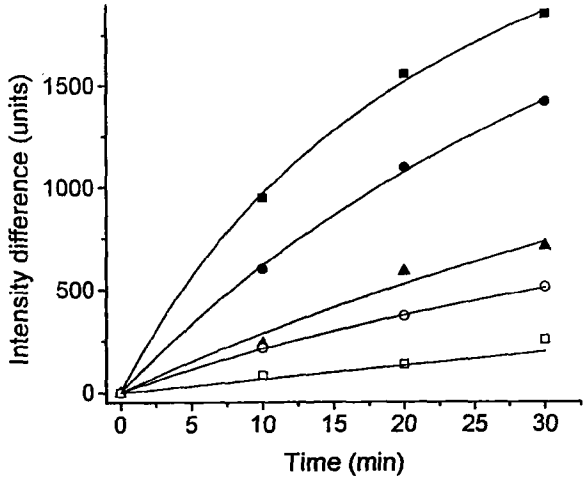

Fig. 4. Reaction progress over time with varying amounts of enzyme. Aliquots were taken from each reaction mixtures at 10 min interval and analyzed as described in the text. Each reaction mixture contained the following amounts of RNase A: □, 0.5; ○, 1; ▲, 2; ●, 5; ■, 10 ($\times10^{-3}$ Kunitz Units).

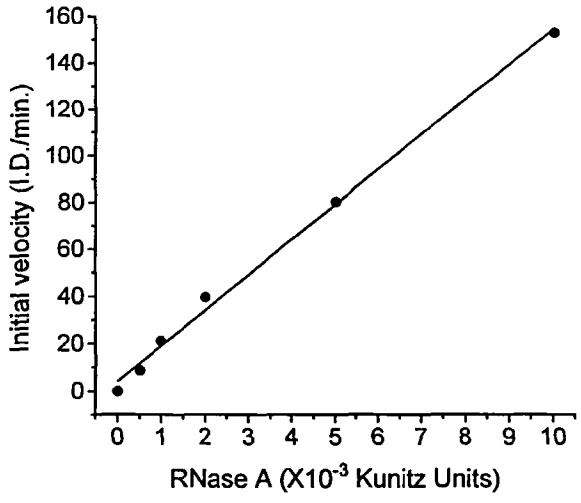

Fig. 5. The plot of initial velocity against the amount of enzyme. The initial velocity was calculated as described in the text. The data were fitted by the linear regression analysis. The initial velocity was defined as intensity difference (I.D.) per min.

centration of a substrate is highest and that of a product is lowest at the start of a reaction. Initial reaction velocities can be determined by drawing tangent lines at the origin in the graphs which represent the progress of reaction over time. In order to determine initial velocities, aliquotes were taken at 10 min intervals from each reaction mixture containing varying amounts of RNase A and subjected to the standard assay method. The intensity difference did not increase linearly over time, especially at high enzyme concentrations as expected (Fig. 4). Instead, they were shown to fit into the following hyperbolic equation by using a fitting function of a computer program;

$(x+a)(y-b)=-ab$, where x was time and y was intensity difference.

The point $(-a, b)$ represents the origin of the graph. The values of a and b for each graph were determined by the same computer program. Rearranging the above equation gave

$$y = \frac{bx}{a+x}.$$

The derivative of this equation produced a reaction velocity, v;

$$v=\frac{dy}{dx} = \frac{ab}{(a+x)^2}.$$

Because the initial velocity, v_i is a velocity at $x=0$,

$$v_i = \frac{b}{a}.$$

Using the values of a and b obtained from each graph, the initial velocity was calculated. The plot of initial velocity against enzyme amount was linear in the range of 0~0.01 Kunitz Units of RNase A (Fig. 5).

Thus a sensitive and simple assay method for the RNase A activity is established. This method may be applied to the assay of other RNases acting on tRNA as well. For more precise estimation of activity, the initial reaction velocity approach would be a method of choice. It should also be noted that this fluorometric assay method may be modified to use a fluorescence microplate reader, where a lot of samples could be analyzed simultaneously with ease.

References

Blank, A., and Dekker, C. A. (1981) *Biochemistry.* **20**, 2261.

Burke, W. F. Jr. and Slinker, B. S. (1980) *Anal. Biochem.* **108**, 320.

Burns, V. W. F. (1971) *Arch. Biochem. Biophys.* **145**, 248.

Crook, E. M., Mathias, A. P. and Rabin, B. R. (1960) *Biochem. J.* **74**, 234.

Dake, E., Hofmann, T. J., McIntire, S., Hudson, A. and Zassenhaus, H. P. (1988) *J. Biol. Chem.* **263**, 7691.

D'Alessio, G. (1993) *Trends Cell Biol.* **3**, 106.

Holbrook, D. J., Wichard, L. P. and Washington, M. E. (1975) *Eur. J. Biochem.* **60**, 317.

Kamm, R. C., Smith, A. G. and Lyons, H. (1970) *Anal. Biochem.* **37**, 333.

Lee, C. C., Trotman, C. N. A. and Tate, W. P. (1983) *Anal. Biochem.* **135**, 64.

Le Pecq, J. B. and Paoletti, C. (1966) *Anal. Biochem.* **17**, 100.

Le Pecq, J. B. and Paoletti, C. (1967) *J. Mol. Biol.* **27**, 87.

Liebman, M., Rubin, J. and Sundaralingam, M. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4821.

Marusyk, R. G., Morgan, A. R. and Wadell, G. (1975) *J. Virol.* **16**, 456