A rapid and sensitive method to measure the enzymatic activity of ribosome-inactivating proteins

Maurizio Brigotti, Luigi Barbieri, Paola Valbonesi, Fiorenzo Stirpe, Lucio Montanaro and Simonetta Sperti*

Dipartimento di Patologia sperimentale dell’Università degli Studi di Bologna, Via San Giacomo 14, I-40126 Bologna, Italy

Received May 22, 1998; Revised June 30, 1998; Accepted July 22, 1998

ABSTRACT

A method is described in which the adenosine-N-glycosidase activity of ribosome-inactivating proteins (RIPs) is measured using as substrate a 2251 bp [3H]DNA obtained by PCR amplification of the 731–2981 region of the pBR322 plasmid. The DNA, labelled in the purine ring of adenine, proved a good substrate for all three RIPs tested (PAP-S, ricin and shiga-like toxin I). The method, which measures directly the [3H]adenine released, is highly specific, extremely rapid and quantitative in a wide range of RIP concentrations.

Ribosome-inactivating proteins (RIPs), widely distributed in the plant kingdom and produced also by some bacteria (shiga toxin and shiga-like toxins), are N-glycosidases which irreversibly inactivate ribosomes by removing a specific adenine from a highly conserved loop (GAGA loop) present in the large RNA of the large ribosomal subunit (1). Three methods are commonly used to assess the RNA-N-glycosidase activity of RIPs: (i) quantification of the inactivation of ribosomes in cell-free protein synthesising systems (2), (ii) isolation of rRNA and visualisation in gel electrophoresis of the fragment produced by aniline cleavage at the RNA-5′-glycosidase activity; (iii) direct measurement by HPLC of the fragment produced by aniline cleavage at the RNA-N-glycosidase activity (3). The three RIPs tested for RNA-5′-glycosidase activity were: PAP-S, from the seeds of Phytolacca americana, a member of the type-1 RIP (single-chain) class (1); ricin, the toxin from Ricinus communis, a member of the type-2 RIP (two-chain) class (1); and shiga-like toxin I (SLT-I), the first example of an RIP of bacterial origin shown to act on DNA (6). For the glycosidase

*To whom correspondence should be addressed. Tel: +39 051 354700; Fax: +39 051 354746; Email: gaggia@alma.unibo.it

A rapid and sensitive method to measure the enzymatic activity of ribosome-inactivating proteins

Maurizio Brigotti, Luigi Barbieri, Paola Valbonesi, Fiorenzo Stirpe, Lucio Montanaro and Simonetta Sperti*

Dipartimento di Patologia sperimentale dell’Università degli Studi di Bologna, Via San Giacomo 14, I-40126 Bologna, Italy

Received May 22, 1998; Revised June 30, 1998; Accepted July 22, 1998

ABSTRACT

A method is described in which the adenosine-N-glycosidase activity of ribosome-inactivating proteins (RIPs) is measured using as substrate a 2251 bp [3H]DNA obtained by PCR amplification of the 731–2981 region of the pBR322 plasmid. The DNA, labelled in the purine ring of adenine, proved a good substrate for all three RIPs tested (PAP-S, ricin and shiga-like toxin I). The method, which measures directly the [3H]adenine released, is highly specific, extremely rapid and quantitative in a wide range of RIP concentrations.

Ribosome-inactivating proteins (RIPs), widely distributed in the plant kingdom and produced also by some bacteria (shiga toxin and shiga-like toxins), are N-glycosidases which irreversibly inactivate ribosomes by removing a specific adenine from a highly conserved loop (GAGA loop) present in the large RNA of the large ribosomal subunit (1). Three methods are commonly used to assess the RNA-N-glycosidase activity of RIPs: (i) quantification of the inactivation of ribosomes in cell-free protein synthesising systems (2), (ii) isolation of rRNA and visualisation in gel electrophoresis of the fragment produced by aniline cleavage at the site of depurination (3) and (iii) direct measurement by HPLC of the adenine released after its conversion to the fluorescent derivative ethenoadenine (4). More recently, RIPs have also been shown to act on DNA releasing more than one adenine and the denomination of polynucleotide:adenosine glycosidases has been proposed (5). Method (iii) was used to assess such activity, but it is time consuming and labour intensive for routine work.

A rapid and sensitive method to determine the polynucleotide:adenosine glycosidase activity would be useful in the search and purification of new RIPs and in assessing the activity of RIPs after conjugation to antibodies or to other carriers specific for target cells. A simple method applied to the detection of shiga and shiga-like toxins in biological materials would also help in the diagnosis of enteric infections.

The method described here uses as substrate a [3H]DNA (2251 bp, Fig. 1) labelled in the purine ring of adenine obtained by PCR amplification in the presence of [8-3H]dATP of the region 731–2981 of the pBR322 plasmid (New England Biolabs). 5′-ATGACTGTCTTCCTTATCAT-3′ was used as forward primer and 5′-TACTGTCTTCATAGTGAGCC-3′ as reverse primer. PCR was in a 100 µl volume containing 1 fmol of the pBR322 plasmid linearized with BamHI, 1 µM of each primer (Promega), 200 µM each dATP, dGTP, dTTP and dCTP (Pharmacia), 30 µl [8-3H]dATP (19.7 Ci/mmol, 1 mCi/ml), 2 U Taq DNA polymerase (Promega) and buffer (10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100 and 2 mM MgCl2). The labelled dATP (DuPont NEN) was in 20 mM Tricine, pH 7.6. The use of commercial sources of labelled dATP containing ethanol was dropped since even traces of ethanol (1.25%) strongly interfere in PCR, and taking the product to dryness under nitrogen depressed the yield of the reaction. Conditions for amplification were 95°C for 5 min followed by 30 cycles of 95°C for 30 s (denaturation), 45°C for 30 s (annealing) and 72°C for 150 s (extension). Thirty more cycles were performed after a further addition of 2 U of Taq DNA polymerase. At the end of PCR, primers and labelled and unlabelled nucleotides were removed on pre-equilibrated MicroSpin Sephacryl-S300 HR columns (Pharmacia Biotech) according to the manufacturer’s instructions. The final yield of the 2251 bp substrate was ~9 µg with a specific radioactivity of 2 × 106 d.p.m./µg. This amount of [3H]DNA allows to perform 30 polynucleotide:adenosine glycosidase assays (see below). Rather than scaling up the volume of the PCR amplification reaction, a procedure that raises the time necessary to reach target temperatures thus increasing the overall cycling time, larger amounts of substrate were obtained by performing several simultaneous 100 µl reactions. Eight such reactions exhaust the [3H]dATP present in a 250 µCi commercial sample and produce an amount of labelled substrate sufficient to perform 240 polynucleotide:adenosine glycosidase assays. Storage of the [3H]DNA was at –20°C in small aliquots.

Preliminary experiments were also performed with other DNA substrates, differing in size (100–3500 bp) and adenosine content (21–32%), obtained by PCR amplification of various regions of the same plasmid. The 2251 bp DNA proved the best substrate for PCR yield and reproducibility of adenine release.

Incubation of trace amounts of the substrate (20 ng) in the conditions of the polynucleotide:adenosine glycosidase assay (pH 4.0, 40 min at 30°C, see below) showed that most of the radioactivity was lost upon centrifugation unless carrier salmon sperm DNA was added. The minimum quantity of carrier DNA required to avoid losses was 0.3 µg. In order to maximise the sensitivity of the method, we chose to avoid the addition of carrier and use in the standard assay 0.3 µg (0.215 pmol) of the labelled 2251 bp substrate. This amount contains 205.5 pmol of [3H]adenine with a specific radioactivity of 3066 d.p.m./pmol.

The three RIPs tested for polynucleotide:adenosine glycosidase activity were: PAP-S, from the seeds of Phytolacca americana, a member of the type-1 RIP (single-chain) class (1); ricin, the toxin from Ricinus communis, a member of the type-2 RIP (two-chain) class (1); and shiga-like toxin I (SLT-I), the first example of an RIP of bacterial origin shown to act on DNA (6). For the glycosidase
activity on DNA the multi-chain SLT-I required activation, performed as described by Brigotti et al. (7), behaving unlike the two-chain RIP ricin for which removal of the receptor-binding B-chain has no effect on the depurination rate of DNA (5). Enzymatic reactions were performed in Eppendorf tubes containing the RIP and 0.3 µg of the 2251 bp substrate in 50 µl of 50 mM sodium acetate buffer pH 4.0/100 mM KCl. The substrate was thawed just before use and added last to the reaction mixtures. At the end of incubation (40 min at 30°C) the samples were diluted 5-fold with the sodium acetate/KCl buffer and applied to Bond Elut® NH₂ columns (Varian, CA, USA) equilibrated with the same buffer. Free adenine was washed out of the columns by brief low-speed centrifugation at 4°C, the columns were washed with 150 µl of the above buffer and the radioactivity in the combined flow-through and column) are critical to ensure full retention of the negatively charged molecules and avoid unduly high blank values.

Figure 1 shows the [3H]adenine release as a function of the amount of PAP-S, ricin and SLT-I. The rank order of RIPs is as previously reported for their activity on herring sperm DNA. The adenine release is in fact much higher for PAP-S than for ricin (5) and the activity of SLT-I is amongst the highest reported for RIPs (5,6). Comparing the results obtained with SLT-I acting on different DNAs (6), the 2251 bp DNA appears a very good substrate approaching the depurination rate observed with herring sperm DNA.

Several factors contribute to the non-linearity of the plots in Figure 1A. The conditions described were chosen to achieve maximal sensitivity with minimum waste of substrate. The amount of adenine released in a fixed period of time (40 min), rather than initial velocities, was measured and at the higher concentrations of RIPs the enzyme was in excess over substrate (0.215 pmol). Moreover, depurination of the DNA substrate containing several adenines involves the simultaneous or consecutive splitting of many N-glycosidic bonds, resulting in an extremely complicated kinetics. The data do not fit a simple equation, and the amount of RIP must be read off empirically from a standard experimental kinetics. The data do not fit a simple equation, and the amount of RIP must be read off empirically from a standard experimental equation the amount of RIP can readily be calculated.

The present method, which takes advantage of the use of a good substrate and of the great sensitivity of radioactivity detection, measures RIPs in the same range (0.01–10 pmol) as the HPLC method (4) which owes its sensitivity to the conversion of adenine into the fluorescent derivative ethenoadenine (4). The present method avoids such conversion and opposes to multiple HPLC runs the simultaneous processing of many samples throughout the procedure, with a large gain in simplicity and rapidity. Both methods are highly specific since they measure directly the product of the adenosine-N-glycosidase reaction.

Of the two methods using ribosomes as substrate, quantitation of the inhibition of protein synthesis by RIPs is more sensitive than the above ones, with an ID₅₀ on the rabbit reticulocyte lysate of 0.0025 pmol for PAP-S (1) and of 0.006 pmol for both ricin A (1) and SLT-I (7). The method, however, is not specific for RIPs and cannot be used to detect polynucleotide:adenosine glycosidase activity. The method which isolates rRNA from treated ribosomes and visualises in gel electrophoresis the fragment produced by aniline cleavage (3) has been quantitated by using 32P-labelled ribosomes (8). The method is specific and highly sensitive, but besides sharing with the previous one the exclusive use of ribosomes as substrate, it is extremely laborious and time consuming.

The method described here is proposed because: (i) PCR affords a substrate rapid to prepare and highly reproducible as for yield and specific radioactivity, (ii) DNA is a substrate much easier to handle than ribosomes or naked RNA, (iii) the assay is quantitative in a wide range of RIP concentration and (iv) the procedure is extremely rapid (results are obtained in <2 h). The method has been successfully applied to measure PAP in crude extracts of Pamerica.