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Enzyme-Mediated Individual Nanoparticle Release Assay

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Abstract

Numerous methods have been developed to measure the presence of macromolecular species in a sample, however methods that detect functional activity, or modulators of that activity are more limited. To address this limitation, an approach was developed that utilizes the optical detection of nanoparticles as a measure of enzyme activity. Nanoparticles are increasingly being used as biological labels in static binding assays; here we describe their use in a release assay format where the enzyme-mediated liberation of individual nanoparticles from a surface is measured. A double stranded fragment of DNA is used as the initial tether to bind the nanoparticles to a solid surface. The nanoparticle spatial distribution and number are determined using dark-field optical microscopy and digital image capture. Site specific cleavage of the DNA tether results in nanoparticle release. The methodology and validation of this approach for measuring enzyme-mediated, individual DNA cleavage events, rapidly, with high specificity, and in real-time is described. This approach was used to detect and discriminate between non-methylated and methylated DNA, and demonstrates a novel platform for high-throughput screening of modulators of enzyme activity.

Keywords

solid phase enzyme assay; plasmon resonant particle; nanoparticle; nuclease; cleavage; susceptible linker; inhibitor

Introduction

Assays that measure the functional activity of minute quantities of biomolecules, rather than their physical presence, are of increasing interest. In part, this interest has already driven the shift from genomics, and measurement of RNA expression levels, to proteomics where protein amounts are directly determined. A next step in systems biology is to develop assays that can be used to measure the functional activity and specificity of differentially expressed proteins, and regulators of their activity. The specific action of proteases and nucleases, for example, is important for regulating many crucial cellular events such as DNA replication, cell cycle progression, differentiation, immunity, and apoptosis (1-6). Aberrant regulation of key activities is involved in the development of many pathological conditions including neurodegeneration, cardiovascular disease, arthritis, cancer metastasis, and infectious diseases, thus they are attractive therapeutic targets, and monitoring their activity can be diagnostic and prognostic of disease states.

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Historically, the detection of cleavage enzymes like nucleases or proteases required time intensive and laborious separation methods to monitor alterations in the physical structure of a substrate, or the use of radio-labeled substrates. Methods have been refined and several new techniques have been employed to measure cleavage enzyme activities that are based primarily on fluorescent detection technology. These techniques include measurement of a spectroscopic change (7,8), or the use of paired dye molecules that exhibit distance-dependent fluorescence resonance energy transfer (FRET) as substrates (9,10). While useful, FRET type assays, which are applicable to both *in-vitro* and *in-vivo* cell based assays, require extensive substrate optimization and are subject to the general limitations of fluorescent labels: photobleaching, autofluorescence from some biological samples, and the intrinsic signal-to-noise for detection of single fluorophores. In these traditional population-based methods, the measured signal is typically the accumulated sum of all reporter labels present in the sample volume, and are still limited by the sensitivity of the underlying label. Here, we describe a general method, based on monitoring individual nanoparticle release events, for measuring the functional activity of cleavage enzymes and modulators or regulators of their activity.

Materials and Methods

DNA Linker Preparation

The fragments used for restriction enzyme cleavage were prepared by polymerase chain reaction synthesis from the pBluescript II KS plasmid (Stratagene, La Jolla, CA) using an aminated M13 sequence reverse primer (Midland Certified Reagent Company, Texas) and biotin containing M13 sequence forward primer. The resultant fragments were purified using a Qiaquick column (Qiagen, CA) and size was determined following separation by electrophoresis in a 1% agarose gel. A doubled stranded 20 base pair oligonucleotide (Midland Chemical Company, Midland, TX) was ligated into the BamHI and EcoRI cloning sites of the pBluescript II plasmid, prior to PCR amplification, thus the final fragment size used in this study was 232 base pairs. DNA sequence analysis was used to confirm the accuracy of the PCR amplification.

Covalent Immobilization of DNA Fragments to a Solid Substrate

DNA containing arrays with 190 μm diameter spots were prepared using an automated microarrayer (Cartesian Instruments, Irvine, CA) functioning in a 60% humidity chamber (11). DNA fragments prepared by PCR amplification were suspended at specified concentrations in 1X print buffer (NoAb Diagnostics, Mississauga, ON). Several different positive and negative control materials were deposited onto pre-cleaned NHS-ester activated slides (TeleChem, Sunnyvale, CA), and include, non-biotinylated oligonucleotides (Midland Chemical Company, Midland, TX), biotinylated BSA (Pierce Chemicals, Rockford, IL), BSA (Boehringer Mannheim, Indianapolis, IN), and salmon sperm DNA (Ambion, Austin, TX). Phosphate buffered saline (PBS), and nuclease free water was purchased from Ambion. Spotted microarrays were left overnight at room temperature prior to use.

Preparation of PRP Protein Conjugates

Gold plasmon resonant particles (PRPTM) approximately 75 nm in diameter were fabricated using standard colloidal chemistry techniques (12). The PRPs were concentrated and purified by centrifugation for 15 minutes at $3000 \times g$. The goat anti-biotin antibody (Pierce Chemicals, Rockford, IL) conjugated PRPs were prepared by passive adsorption, following methods similar to those commonly used to prepare protein conjugated colloidal gold for electron microscopy observation (12). The PRPs are resuspended to a chosen concentration by adjusting the optical density at 430 nm to correspond to a calibrated value of nucleation centers per optical density.

Enzymatic Reactions and Detection

NHS-ester activated microarray slides were printed using the method described and a series of six array spots were demarcated with a silicone gasket. The silicone creates a barrier between different groups of spots on the slide allowing for analysis of multiple reaction conditions. Non-reacted NHS-ester was blocked by the addition of 1X NoAb (Mississauga, ON) blocking solution followed by an overnight incubation with 9X casein (Vector Laboratories, Burlingame, CA) in TNM buffer (50 mM Tris pH 7.4, 100 mM NaCl, 5 mM MgCl₂). The slides were washed in TNM buffer containing 9X casein, and goat anti-biotin PRPs were bound to the immobilized DNA fragments in 9X casein TNM buffer for 30 minutes at room temperature. Non-bound PRPs were removed by washing with restriction enzyme buffer. The PRPs were then viewed using dark field microscopy and time zero images were captured using a Spot2E color CCD. Restriction enzymes, in restriction enzyme buffer, were then added to the appropriate well and the samples were incubated for specified amounts of time at room temperature. During the time course of restriction enzyme mediated PRP release, slides were viewed and images from the array spots were captured using the CCD.

Solid Phase Methylase Assay

The methylase modification of the immobilized fragment occurred prior to the addition of the PRPs. Following the overnight incubation in 9X casein blocking buffer the wells were washed three times with EcoR1 methyltransferase buffer (50 mM Tris pH 8, 50 mM NaCl, 10 mM EDTA, 80 μ M S-adenosylmethionine) and reacted with specified concentrations of EcoR1 methyltransferase at 37° C for 1 hour. For the methyltransferase competition experiments, sinefungin (5'-deoxy-5'-(1,4-diamino-4-carboxybutyl) adenosine (Sigma-Aldrich, Rockford, IL) was included during each methyltransferase reaction. Each of the wells was then washed with EcoR1 restriction enzyme buffer (New England Biolabs, Ipswich, MA), and PRP release and detection performed as described.

Image Acquisition

The PRPs were detected by dark field illumination using an Olympus BX-40 equipped with 10 $\times$, 50 $\times$, and 100 $\times$ dark field/bright field objective lenses, and an Olympus TH3 100 Watt halogen light source. A three axis motorized stage (Prior Instrument) and Spot 2E color CCD (Diagnostic Instruments, Sterling Heights, MI) are controlled by custom Image-Pro macro routines (Media Cybernetics, Silver Spring, MD) that autofocus and image the particles within the microarray spots.

Results

Single Nanoparticle Release Assay

We have previously described the use of plasmon resonant nanoparticles (PRPs) as extremely sensitive labels for the detection of single nucleotide polymorphisms (SNPs) using a solid phase microarray based format. Individual anti-biotin PRPs, bound to biotinylated oligonucleotide and immobilized via a SNP dependent, sequence specific, hybridization reaction to an immobilized capture oligonucleotide, are counted following optical imaging of the microarray spot (13). Herein, we report an alternative and complementary approach that utilizes the enzyme mediated release of individual PRPs from a solid microarray surface. As shown in the schematic depicted in Figure 1B, PRPs, bound to the solid substrate via a linker molecule that contains enzyme specific cleavage sites, are released from the surface by the activity of a cleaving reagent. PRPs exhibit several unique properties that make them particularly suited for this application. Each individual plasmon resonant nanoparticle (PRP) is ultrabright (12), they are readily imaged, and each individual nanoparticle can be rapidly

counted using a common optical microscope configured for dark-field illumination (14). In addition, the PRPs do not photobleach allowing real-time data collection without loss of signal.

Enzymatic cleavage activity is measured by incubating the substrate, PRPs bound to the microarray surface via a double stranded DNA fragment, with the sample of interest. To demonstrate the feasibility of using this type of assay to measure specific endonuclease activity we have designed linker DNA molecules that incorporate single restriction sites. A DNA fragment is prepared (Figure 1A) that has a number of features which include; (1) a primary amine for covalent immobilization to a reactive glass slide, (2) a biotinylated-termini that functions as a ligand for immobilization of the anti-biotin PRPs, and (3) a single EcoRI or XbaI restriction enzyme recognition site. The release of bound PRPs after cleavage of the linker fragment with a specific restriction enzyme is illustrated in Figure 1B. Modification of the restriction site by a sequence specific DNA methylase blocks the restriction enzyme mediated cleavage and release of the PRP.

Preparation of Enzyme Cleavable Susceptible Substrates

Cleavable DNA Linker Fragments—A series of DNA fragments of different size were prepared in order to assess whether linker length affects the efficacy of restriction enzyme mediated release of PRPs from a solid substrate. A set of plasmids, each containing a cDNA fragment of defined length (I.M.A.G.E. Consortium) inserted into the polylinker site, were purchased from Invitrogen (Carlsbad, CA). Alternatively, the pBluescript KS II plasmid (Stratagene, San Diego, CA) containing a multiple cloning site was used. Sufficient quantity of insert was produced from each plasmid by PCR amplification using a pair of primers (Midland Chemical Company, Texas) complementary to sequences flanking the insert. One primer corresponding to the M13 reverse sequence has a 5'-biotin, and the other primer, corresponding to the M13 forward sequence, has a 5'-amino termini. Internal to each of the PCR products are single recognition sites for a number of restriction enzymes including EcoRI and XbaI. The integrity of the restriction enzyme cleavage sites, following PCR amplification and purification, was confirmed by agarose gel electrophoresis analysis of the fragments produced following a 1 hour digestion at 37 °C. In the PRP release assay, there was no significant difference in the efficacy of the assay when fragments that ranged in size from 200-1250 base pairs were used, thus, a 232 base pair PCR fragment was used for the experiments reported.

Microarray Surfaces—Each of the different length DNA fragments was attached to the surface of a microarray slide, incubated with anti-biotin PRPs, and washed. The binding of anti-biotin PRPs to the microarray surface was confirmed using dark field microscopy (Figure 2A) (13). A variety of capture surfaces were evaluated to determine whether they satisfied several important PRP release assay criteria including: (1) efficient and stable immobilization of the 5' amino terminated DNA substrate, (2) robust and reproducible antibody coated PRP binding to the biotinylated DNA, and minimal non-specific binding of PRPs to non-biotinylated control DNA, (3) stability of the interaction between the nucleic acid linker and the solid substrate, with no detectable release of PRPs in the absence of DNA cleavage reagents, and (4) reliable release of captured PRPs from the surface after nucleic acid linker cleavage. After testing primary amine reactive SMA-25 super aldehyde (Telechem International, Sunnyvale, CA), aldehyde, epoxide, aldehyde-hydrogel, epoxide-hydrogel, NHS-ester hydrogel slides (NoAb BioDiscoveries, Canada) and NHS activated agarose beads (Affi-Gel 15; Bio-Rad, Hercules, CA) with several different binding, washing and release conditions, we chose NHS ester activated glass slides (NoAb BioDiscoveries, Canada). Typically, the DNA substrate concentration was titrated in each array of spots to be tested, and positive (biotinylated BSA) and negative (non-modified DNA linkers) controls were included in each reaction slide. Up to 24 capture spots per reaction condition were analyzed by sequestering

each group of spots using silicone gaskets. Routinely, eight different reaction conditions are tested for each microarray slide.

Binding Stability of Anti-biotin Conjugated PRPs—An important criterion for determining the specificity of PRP release is the maintenance of immobilized PRPs in the absence of a cleaving reagent. The stability of the PRP-anti-biotin antibody interaction was determined by measuring anti-biotin antibody conjugated PRP binding to biotinylated bovine serum albumin (b-BSA) microarray spots. The stability of the antibody-PRP and antibody-biotin binding interactions were determined by measuring the release of PRPs from the b-BSA spots as a function of time. The antibody-PRP linkage is highly stable and no change in the surface density of PRPs after incubation for 24 hours under standard buffer conditions was observed (data not shown).

The specificity and stability of the bond made between antibody conjugated PRPs and the biotinylated DNA linker fragment was also measured. The specificity of PRP binding to the DNA linker fragment is demonstrated by the release of microarray surface bound PRPs following the addition of DNase I to a final concentration of 0.1 U/ μ l in each reaction. After 2 minute incubation with DNase I at room temperature, PRPs are observed to stream away from the surface. After 4 minutes, a majority of PRPs have been released from each microarray spot, and within 10 minutes less than 5 percent of the original particles remain bound. To assess the stability of DNA fragment mediated PRP binding, microarray substrates were incubated with antibody conjugated PRPs, rinsed to wash away unbound PRPs, incubated in buffer for periods up to 24 hours and imaged. No significant change in the number of PRPs bound to the surface was observed (data not shown).

Restriction Enzyme Mediated PRP Release

Specific restriction enzyme mediated release of PRPs from the microarray surface is shown in Figure 2. In Figure 2A (elapsed time = 0 minutes; T₀) dark field images of PRPs bound to the biotinylated DNA fragment attached to the microarray surface are shown. The PRPs are imaged with a 50X dark field objective and images are captured using a Spot 2E digital camera. In order to highlight individual PRP locations, the samples shown in Figure 2A are magnified views of regions taken from individual microarray spots (the microarray spot diameter is 120 micron). Addition of EcoR1 (120 U per 100 μ l reaction volume) and incubation at room temperature results in rapid release of PRPs from the surface (elapsed time = 20 minutes; T₂₀). Released particles float away from the capture spot and are out of the image focal plane. Since the susceptible linkers are efficiently cleaved and the PRPs presumably have a large number of their antibody binding sites occupied with the cleaved linker molecule there is no detectable rebinding of the liberated PRPs to the capture spots. In the absence of EcoR1, cleavage of the linker DNA does not occur, and PRPs are not released from the substrate surface. By identifying and counting the individual PRPs, present as distinct bright point sources in each image, it is possible to quantitatively monitor the time course of the cleavage reaction. In Figure 2B the number of PRPs released from a defined optical imaging area as a function of incubation time with EcoR1 is shown. These data show that release is very rapid, with the majority of particles liberated within the first two minutes of incubation with the enzyme. The number of PRPs released is determined by counting the number of particles in a field of view immediately prior to enzyme addition and subtracting the number of particles remaining at specified times after addition of the enzyme.

Detection of DNA Modifying Enzyme Activity

Nanoparticle release assays can also be used to measure the presence of molecules, or the activity of enzymes, that modify the linker substrate in a manner that results in a change, either inhibition or enhancement, in the rate of the cleavage reaction. We have used our nanoparticle

release assay to demonstrate DNA modification and DNA methyltransferase activity detection. In prokaryotes, DNA methylases have been identified as elements of the restriction enzyme system and function to protect the microbial genome from cleavage by the corresponding restriction endonuclease (15), whereas, methylation of DNA in eukaryotes is thought to play a role in differentiation and gene expression (16,17). Recent results have implicated aberrant methylation of tumor suppressor genes in a number of different types of cancers (18,19). Standard DNA methyltransferase activity assays either require the use of radioactive incorporation of methyl groups into the substrate, or measure protection from restriction enzyme cleavage using gel electrophoresis analysis (20-22).

Linker DNA substrates were incubated for 60 minutes with several different concentrations of EcoR1 methyltransferase, washed with restriction enzyme buffer, PRPs were added and allowed to bind to the tether for 30 minutes. After washing to remove non-bound PRPs, either 120 U of EcoR1, or 360 U of Xba 1 was added to the 100 μ l reaction volumes. After 30 minutes, in the absence of EcoR1 methyltransferase, the linker fragment is cleaved, resulting in release of PRPs from the microarray surface (Figure 3). In contrast, if EcoR1 methyltransferase is used to modify the linker substrate, prior to the addition of EcoR1, the number of PRPs released is significantly reduced. The concentration dependent modification of the linker substrate is shown in Figure 3 (shaded boxes). Maximal inhibition of PRP release is seen using >0.6 U of EcoR1 methyltransferase per reaction. This inhibition of EcoR1 mediated cleavage events is due to specific modification of the EcoR1 cleavage site since pre-treatment of the DNA substrates with EcoR1 methyltransferase has a minor effect on Xba1 mediated cleavage events (Figure 3, open bars). The small number of PRPs released even in the presence of a high concentration of EcoR1 methyltransferase may be due to incomplete washing and removal of non-specifically bound PRPs.

Detection of Inhibitors of Enzyme Activity

Specific inhibitors or activators of enzyme activity can also be detected using the nanoparticle release assay. The generality of this approach is demonstrated by measuring the concentration dependent inhibition of the EcoR1 methyltransferase reaction using sinefungin, a nonreactive analogue of the essential cofactor S-adenosyl-L-methionine (AdoMet). The presence of sinefungin (100 μ M) during the EcoR1 methyltransferase linker modification reaction (1U methylase) partially blocks (~66% inhibition) methylation, and results in DNA fragments that are cleaved by EcoR1 (Figure 4). The concentration dependent inhibition of methyltransferase by sinefungin observed is consistent with that measured when using a standard solution phase based assay and gel electrophoresis analysis (Figure 4; inset).

Discussion

The development of ultrasensitive assays based on release of molecules from solid substrates that are suited for use with a microarray platform is of current interest, especially for high-throughput screening (HTS) applications. Typically, in such assays the only signal measured is the amount of released radioactive or fluorescently labeled material (23,24), since it is difficult to quantitatively measure the remaining material bound to the surface. This is primarily due to contributions of the measured signal from released labels present in the assay solution. Although fluorescence assays based on illumination by total internal reflection (TIR), where only surface bound fluorophores are excited, have shown promise, extensive and time-consuming wash steps are normally required, and measurements in real-time are limited. In contrast, when optically detectable nanoparticles such as fluorospheres, quantum beads, or plasmon resonant particles (PRPs) are used as labels for use in assays based on cleavage and release, the surface bound particles can be readily imaged using a standard microscope. The nanoparticles can be individually identified, spatially mapped, distinguished from released

particles, and automated algorithms can provide quantitative real-time particle counts. The assay we have described demonstrates the feasibility of this approach.

In the nanoparticle release assay the activity of a restriction enzyme endonuclease was determined by the cleavage of a nucleic acid based susceptible linker and release of a nanoparticle label. In contrast, a standard method to measure endonuclease activity relies on nucleic acid digestion, followed by agarose gel electrophoretic analysis of the generated DNA fragments. Another versatile technology that is used to monitor hydrolytic enzyme reactions makes use of fluorogenic (organic fluorophores or green fluorescent protein (GFP)) susceptible substrates that contain an internally quenched fluorophore that produces fluorescent emission upon cleavage based on a distance-dependent fluorescence resonance energy transfer (FRET) mechanism (9,25-27). These type of fluorescent substrates have been used in both *in vitro* and cell based assays (10,28). In a related approach to that which we describe, a fluorescent or phage associated substrate is linked to an immobile support matrix, such as a bead, and enzyme mediated cleavage of a linking substrate molecule results in a change in fluorescence emission properties, or release of bound fluorophores or phages (24,29-31). Although fluorescent quenched substrate libraries are a very powerful tool for investigation of enzymatic activity and specificity, potential disadvantages of using fluorophore based assays are the tendency of chromophores to interact with either the active site of the enzyme or the substrate (32), the limitations to short intramolecular distances due to the efficiency of the fluorescence resonance energy transfer (FRET) as a function of the Forster distance (9), and the necessity for many cleavage reactions to occur to get a reasonable signal (33). In contrast to fluorophore based assay, the described nanoparticle release assay has several potential advantages that include the ability to accommodate long substrates (ie. susceptible linkers) which is important for determining the extended substrate specificities of enzymes, or for assaying structure-specific proteases. The optically detectable nanoparticle labels are non-bleaching, ultrabright – therefore, easy to visualize, and improved optical microscope based imaging allows for the design and implementation of single nanoparticle counting based assays. For our system, the theoretical limit on detection sensitivity is the action of an enzyme on a single susceptible linker, since the cleavage and release of an individual Plasmon Resonant Particle (PRP) reporter is detected. Additionally, the action of enzymes on hundreds or even thousands of individual linker bound PRPs can be observed in parallel and in real time, permitting statistical validation of the kinetic process. In addition, the availability of nanoparticle labels of different colors (12,34) enables the simultaneous monitoring of multiple different cleavage assays, and the incorporation of negative and positive internal controls, in a single reaction image area. Measurement of the ratio of different colored nanoparticles bound by unique susceptible linkers has the potential to provide improvements to assay reliability and specificity.

High sensitivity of detection is particularly important when sample quantities are limited, such as isolates from small numbers of cells, scarce patient samples, or small-scale preparations of individual molecules in chemical libraries. Therefore, increased sensitivity—the ability to reliably detect ever smaller absolute amounts of the entity of interest, coupled with increased speed, and ease of use—are key assay development goals. The nanoparticle based release assay is rapid, requires minimal substrate material, and small quantities of enzyme activity can be detected.

An assay appropriate for high throughput screening applications should be suited for miniaturization and have a sensitivity of detection such that the small quantities of drugs present in combinatorial chemical libraries are in sufficient quantity to modulate the activity of their intended target (35). In addition, recent advances in lithography and microfluidics have created the need for assay methods compatible with miniaturized and/or automated systems, such as “laboratory on a chip” designs. An assay system which offers improvements in these areas, and that can be produced at a competitive cost will gain wide scale use. The enzyme-mediated

individual nanoparticle release assay described meets these criteria and thereby has potential broader applicability as a drug discovery platform for high throughput screening (HTS) applications.

We believe the PRP-based assay system described here presents significant advantages in terms of speed, sensitivity, and ease of use, as compared with currently available technology for ultrasensitive detection of biologically active molecules. As an example of the application of the assay format for drug screening applications we have demonstrated that the assay can be used to detect DNA methyltransferase activity, and measure the activity of drugs that inhibit DNA methyltransferases. The identification of new drugs that modulate the activity of DNA methyltransferases is important as they are novel targets for development of both anti-microbial and anti-cancer therapeutics (15,17).

The described solid-phase nanoparticle release assay could be used to test the activity of restriction enzymes, and has potential broad applicability as a diagnostic tool (36), for detection of biowarfare agents (26), in addition to the generation of new therapeutic inhibitors to control nuclease or protease activity (37,38). Finally, the described endonuclease activity monitoring approach could be extended to other hydrolytic enzymes, such as proteases, polysaccharidases, lipases, and the molecules that influence these activities on a designated target.

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Figure 1.

Schematic diagram of the solid-phase nanoparticle release assay. (A) A 232 base pair DNA fragment containing single restriction enzyme sites is generated by PCR from a plasmid using primers that are modified with either a 5' primary amine or biotin. The EcoR1 and Xba1 sites are 114 and 149 base pairs, respectively, from the 5' amine substrate immobilization site. (B) PRPs are used to label covalently immobilized DNA fragments and enzymatic activity is detected by measuring the release of the tethered PRP during the reaction. Site specific methyltransferase activity modifies the substrate resulting in a non-cleavable substrate, inhibiting enzyme digestion and limiting PRP release.

Figure 2.

Restriction enzyme mediated release of PRPs. Panel A shows dark field images of PRPs bound to biotinylated DNA fragment attached to a solid surface. Addition of EcoR1 results in the release of PRPs from the surface (T20 = 20 minutes, +EcoR1). In the absence of specific enzyme cleavage of the linker DNA, PRPs are retained on the surface (T20, -EcoR1). The time course of PRP release following addition of restriction enzyme is shown in Panel B. The error bars calculated for each time point are based on the detection and counting of the number of PRPs released from three separate designated imaging areas.

Figure 3.

Detection of EcoR1 methyltransferase activity using PRP release assay. Immobilized linker DNA is incubated with varying concentrations of EcoR1 methyltransferase, followed by PRP binding, and then digested with EcoR1 (solid bars) or Xba1 (open bars). Increasing concentrations of EcoR1 methyltransferase inhibit enzyme-mediated PRP release. Agarose gel electrophoresis shows that in a solution phase assay EcoR1 cleaves the DNA fragment in the absence of methyltransferase, however, the methyltransferase inhibits EcoR1 digestion of the fragment (EcoR1+) and has no effect on Xba1 cleavage (Xba1+) (inset).

Figure 4.

Detection of an enzyme inhibitor using PRP release assay. The immobilized linker DNA is incubated with 1 U of EcoR1 methyltransferase per reaction in the presence of increasing concentrations of sinefungin, a specific methyltransferase inhibitor. The inhibition of methyltransferase is detected by the larger number of PRPs that are released from samples incubated with higher concentrations of the inhibitor. The inset shows an ethidium bromide stained gel of fragments generated after solution phase incubation with 1 U EcoR1 methyltransferase, in the presence or absence of 100 μM sinefungin, followed by EcoR1 digestion.