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DETECTION OF TOTAL DNA WITH SINGLE-STRANDED DNA BINDING PROTEIN CONJUGATES

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SUMMARY. We have developed a rapid and sensitive method for total DNA measurement using single-stranded DNA binding protein from E. coli conjugated with horseradish peroxidase or urease. To detect DNA, the sample is heated or alkali treated to denature the DNA and then filtered through nylon or nitrocellulose membranes. After the single-stranded DNA is bound to the membrane, single-stranded DNA binding protein enzyme-conjugate is incubated with the membrane. Next, the unbound conjugate is washed off the membrane and the bound conjugate detected colorimetrically. The assay can detect 10 pg of DNA in <3 hr. This method can be applied to the detection of DNA contamination in therapeutic proteins produced by recombinant DNA or hybridoma techniques.

The amount of DNA in a sample has usually been determined by spectrophotometric measurement of absorbance at 260 nm (1) or fluorometrically with the use of fluorochromes, such as ethidium bromide (1) or Hoechst 33258 (2,3). The UV absorbance method can not detect DNA concentrations less than 0.5 μg/ml. The Hoechst dye and ethidium bromide techniques, respectively, have sensitivity limits of 10 ng/ml and 1-5 ng.

In the past ten years methods have been introduced for the mass production of a broad spectrum of therapeutic proteins by recombinant DNA methods in microorganisms and mammalian cell lines (4). In addition, therapeutic monoclonal antibodies have been produced using murine hybridomas (5). The advent of these methods has produced concern about DNA contamination, especially DNA of oncogenic or retroviral origin (6). Detection methods using hybridization techniques (7,8) are capable of detecting small amounts of DNA but are limited to finding only specific nucleic acid sequences. In addition, hybridization is time-consuming and

ABBREVIATIONS

SSB, single-stranded DNA binding protein; GMCSF, granulocyte macrophage colony stimulating factor; MBS, N-maleimidobenzoic acid N-hydroxysuccinimide; HRP, horseradish peroxidase; SPDP, N-succinimidyl 3-(2-pyridyldithio) propionate; BSA, bovine serum albumin; PBSE (50 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1 mM EDTA).

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typically involves the use of radioisotopes. The need has become apparent for a general method that can detect DNA in picogram amounts with broad sequence specificity and is easy to perform.

To measure small amounts of DNA in a few hours, we have developed a novel method that uses a DNA binding protein covalently coupled to a signal generating enzyme. The protein that binds the DNA is SSB from Escherichia coli. SSB is composed of four monomers of 18,840 daltons each and binds to single-stranded DNA with high affinity ($>10^8$ M^{-1}) (9). The tetramer binds 33 ± 3 nucleotides to 65 ± 5 nucleotides depending on the salt concentration (10,11). To measure DNA in samples, the DNA is first denatured by heating. The samples are then filtered through a nitrocellulose or a nylon membrane to immobilize the DNA. Subsequently, the membrane-bound DNA is incubated with an SSB-enzyme conjugate. Specifically bound conjugates are detected by observing the colored products of the enzyme catalyzed reactions (12,13,14).

MATERIALS AND METHODS

Materials: Proteinase K, calf thymus DNA, bovine serum albumin (RIA grade), E. coli rRNA, baker's yeast RNA, urease (type VII), Triton X-100, Tween-20, Ficoll (type 400), polyvinylpyrolidone (PVP-40), N,N-dimethylformamide, Sephadex G-25, MBS ester and SPDP were from Sigma Chemical Company (St. Louis, MO). Dithiothreitol and horseradish peroxidase were from Boehringer-Mannheim (Indianapolis, IN). SSB from E. coli was obtained from United States Biochemical Corporation (Cleveland, OH). Porcine insulin was purchased from Calbiochem (La Jolla, CA). Sephacryl S-400 HR was from Pharmacia (Piscataway, NJ). GMCSF was obtained from Biogen Research Corporation (Cambridge, MA). Nitrocellulose membrane (0.45 μ, BA 85) and the minifold(TM) were purchased from Schleicher and Schuell (Keene, NH). Nylon membrane (Genatran 45™) was from Plasco, (Woburn, MA). HPLC-grade water was obtained from Fisher Scientific (Springfield, NJ). E. coli: strain 25922 was from American Type Culture Collection (Rockville, MD).

SSB-Urease Preparation: SSB from E. coli was coupled to urease with the hetero-bifunctional reagent MBS. One hundred μl of 0.25% (w/v) MBS in dimethylformamide was added to 2 mg of SSB in 2 ml of 0.1 M sodium phosphate buffer, pH 6.8, and stirred for 30 min. at room temperature. Maleimido-SSB was separated from unreacted MBS by Sephadex G-25 chromatography, then combined with 20 mg urease. After 20 min, the reaction was stopped with the addition of 2-mercaptoethanol to a final concentration of 2mM. Urease-conjugated SSB was purified by elution from Sephacryl S-400 in 50 mM Na_2SO_3, 20 mM NaH_2PO_4, 2 mM dithiothreitol, 1 mM EDTA, 0.05% (v/v) Tween 20, pH 7.3 and stored at 4°C until use.

SSB-HRP Preparation: HRP was thiolated with SPDP for coupling to SSB. 7.5 mg SPDP in 1 ml dimethylformamide was added to 50 mg HRP in 5 ml of 0.1 M sodium phosphate buffer pH 7.5 and 0.2 M NaCl, and stirred for 30 min at ambient temperature. The mixture was chromatographed on Sephadex G-25 in 0.1 M sodium phosphate buffer

pH 7.0 and 0.2 M NaCl. Thiols on HRP were unblocked by adding dithiothreitol to 50 mM for 30 min. Excess dithiothreitol was then separated from the enzyme on Sephadex G-25 in the same buffer. The reduced HRP was added to 2 mg SSB that had been reacted with MBS as described above. After 20 min the reaction was stopped as previously described. SSB-HRP was stored at 4°C.

Detection of Pure DNA with SSB-Urease: Samples containing 0-100 pg calf thymus DNA in PBSE were denatured to single-stranded DNA by heating at 100°C for 10 min followed by chilling on ice. The denatured samples were filtered through 0.45 μ nitrocellulose membrane using a minifold™. The membranes were then baked at 80°C for 1 hour to fix the DNA. The membranes were dipped into a 0.2 ml/cm^2 of 0.3 μg/ml SSB-urease in 2% (w/v) BSA, 2% (w/v) Ficoll, 2% (w/v) polyvinylpyrolidone, 10 mM sodium phosphate, 40 mM sodium chloride, 2 mM EDTA, pH 7.5 and allowed to incubate for 1 hour. Unbound conjugate was removed by dipping the membranes three times for 3 min each in 0.5 ml/cm^2 of 0.15 M sodium chloride, 1 mM EDTA pH 6. The urease substrate, 100 mM urea, 0.15 M sodium chloride, 1 mM sodium phosphate, pH 6, 0.5 mM cresol red, was then added to the membrane. The change in pH due to the reaction catalyzed by urease (12) resulted in a color change of the cresol red from orange to purple-red (13).

Detection of DNA in protein samples: Porcine insulin (100 μl, 10 mg/ml) in 10 mM Tris-HCl, 1 mM EDTA spiked with 0, 10, 50, 100 or 200 pg of double-stranded calf thymus DNA was digested with proteinase K (5 μl, 2 mg/ml) for 16 hr at 55°C and then heated to 100°C for 5 min. GMCSF (300 μl, 1 mg/ml) spiked with 0 to 200 pg of DNA was heated to 100°C for 5 min and allowed to cool to room temperature. DNA standards were prepared by diluting calf thymus DNA in PBSE and heating to 100°C for 5 min. All samples were filtered through nylon at a rate of about 100 μl/min using the minifold. In a modification of a previously described method (15), DNA was detected by incubating the membrane with 0.2-0.5 ml/cm^2 SSB-HRP conjugate (150 μg/ml in PBSE, 0.1 mg/ml BSA, 5% (w/v) Triton X-100™) for 40 min at room temperature. Unbound conjugate was then removed by washing the membrane three times in 0.5 ml/cm^2 PBSE containing 1M urea, 1% (w/v) dextran sulfate for 3 min each wash. The membrane was then briefly rinsed with distilled water and incubated for 10 min in 0.2 ml/cm^2 of 10 mM sodium citrate 10 mM EDTA, 0.1 mg/ml tetramethyl benzidine, 5% (v/v) ethanol, 0.001% (v/v) hydrogen peroxide, pH 5.

Detection of Bacteria in a Water Sample: Cultures of *E. coli* strain 25922 were diluted in HPLC-grade water to 400, 2,000, 10,000 bacteria/100 μl. For one set of samples, the bacteria were lysed and denatured by treatment with 10 μl of 3 M NaOH, then neutralized with 50 μl of 1 M Tris-HCl pH 7.3. A second identical set of *E. coli* dilutions was not base treated to serve as a control for nonspecific conjugate binding. Both sets of samples and heat denatured pure calf thymus DNA standards were loaded onto a nylon membrane. Each well of the minifold was loaded with 160 μl of sample. The DNA was detected with SSB-HRP as described above.

Scoring of Colored Spot Intensities in Detection: We visually observed the colored spots and assigned values to the intensities according to the amounts of DNA added to the standards. For example, we scored the 0 pg standard as "-", the 50 pg standard "1+", and the 100 pg standard as "2+". Experimental samples were scored by comparison with the standards.

RESULTS

Detection of DNA in buffer: To establish the ability of SSB-enzyme conjugate to detect DNA, different amounts of denatured calf thymus DNA were diluted into buffer, filtered through nitrocellulose, and fixed to the membrane. The membrane was incubated with SSB-urease and then washed to remove unbound conjugate. Conjugate bound to DNA was detected by the addition of urease substrate. The reaction catalysed by urease resulted in a pH shift that caused a color change. Thus, the presence of DNA is seen as a purple-red spot. As shown in Table 1, the visually determined spot intensity was correlated with the amount of DNA present. To test the cross-reactivity of RNA in the assay, 3000 pg of *E. coli* rRNA and baker's yeast RNA were tested in the same experiment and yielded no signal above the negative control, confirming that SSB binds RNA with much less affinity than DNA (10).

Detection of DNA in proteins: DNA was diluted into two protein samples and into buffer. The insulin sample was digested with proteinase K overnight and heated to 100°C for 5 min to denature the DNA. GMCSF was heated to 100°C for 5 min and allowed to cool to room temperature. DNA in buffer was heated to 100°C for 5 min. All of the samples were filtered through a nylon membrane and incubated with SSB-HRP. After unbound enzyme conjugate was washed away, bound conjugate was detected by addition of HRP substrate solution. The HRP-catalysed oxidation resulted in a color change. Thus, the presence of DNA was indicated by a blue spot. The intensities of the spots in the protein samples were the same as in the corresponding buffer samples. As shown in Table 2, 10 pg of DNA can be detected in these protein-containing samples.

Detection of DNA in bacteria: *E. coli* was diluted into buffer, lysed with base, neutralized, and filtered through nylon. As a control for nonspecific binding of conjugate, unlysed *E. coli* was

TABLE 1

Detection of DNA in Buffer with SSB-urease

DNA (pg/sample)	Color Intensity
0	-
50	1+
100	2+

TABLE 2

Detection of DNA in Protein Samples with SSB-HRP

DNA (pg/sample)	Color Intensity		
	Insulin	GMCSF	Buffer
0	-	-	-
10	+/-	+/-	+/-
50	1+	1+	1+
100	2+	2+	2+
200	4+	4+	4+

also diluted into buffer and filtered through nylon. DNA standards were prepared by diluting heat-denatured calf thymus DNA in buffer and filtering these samples through nylon. The DNA on the membrane was detected as described above. The results suggest that the DNA from 2 x 10^3 bacteria can be detected. Since the amount of DNA per E. coli has been estimated to be $\geq$4 fg (1), this agrees with the 10 pg limit of sensitivity seen with the standards, as shown in Table 3. DNA was not detected from unlysed E. coli.

DISCUSSION

We have demonstrated a novel assay concept: a high affinity DNA binding protein, such as SSB, can be conjugated to an enzyme and used to detect DNA. Other DNA binding proteins that interact strongly with DNA, such as anti-DNA antibodies (15), topoisomerase I (16), or T4 gene 32 protein (11) have the potential of being used in a similar manner. This assay concept may be used in screening for general DNA contamination of therapeutic proteins produced by recombinant DNA on hybridoma technology. Compared to hybridization, the total DNA assay concept has two key advantages: lack of DNA sequence specificity (17,18) and rapidity (less than

TABLE 3

Detection of DNA from E. coli with SSB-HRP

Number of E.coli/sample	Color Intensity		Standard DNA (pg/sample)	Color Intensity
	lysed	unlysed		
4 x 10^2	-	-	0	-
2 x 10^3	+/-	-	10	+/-
10^4	1+	-	50	1+

three hours). DNA can be measured in the presence of large amounts of at least two therapeutic proteins with minimal pretreatment. In addition, the total DNA assay can detect DNA from lysed bacteria. Also, we have used SSB as a DNA binding protein in a similar assay to detect mammalian DNA in chromatin without measurable interference (19).

This assay may have other uses such as detecting microbial contamination of process water, buffers, reagents, and chromatographic resins. For the purpose of qualifying process material, the DNA assay is more rapid than microbial culture methods and may detect the DNA of microorganisms which are dead or hard to culture. There are also possible applications in molecular genetics research. For example, materials used in molecular cloning could be screened for DNA contamination. In addition, the assay may have application in measuring the amount of cDNA produced from an mRNA template by reverse transcriptase.

Although the present assay can detect DNA, improvements can be envisioned that can provide more sensitivity and better quantitation. A light-addressable potentiometric sensor that measures pH changes in small volumes rapidly with high sensitivity (20) is currently being coupled to this assay to achieve higher sensitivity and better precision (19).

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