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Relief of Amplification Inhibition in PCR with Bovine Serum Albumin or T4 Gene 32 Protein

CAROL A. KREADER*

Microbiology Research Division, Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268

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The benefits of adding bovine serum albumin (BSA) or T4 gene 32 protein (gp32) to PCR were evaluated with reaction mixtures containing substances that inhibit amplification. Whereas 10- to 1,000-fold more $FeCl_3$, hemin, fulvic acids, humic acids, tannic acids, or extracts from feces, freshwater, or marine water were accommodated in PCR when either 400 ng of BSA per μl or 150 ng of gp32 per μl was included in the reactions, neither BSA nor gp32 relieved interference significantly when minimum inhibitory levels of bile salts, bilirubin, EDTA, NaCl, sodium dodecyl sulfate, or Triton X-100 were present. Use of BSA and gp32 together offered no more relief of inhibition than either alone at its optimal level, and neither protein had any noticeable effect on amplification in the absence of inhibitors.

Substances that inhibit enzyme activity are present in many materials of interest and can limit the use of PCR. The identities of several inhibitors are known. Heme, the oxygen carrier in blood, as well as its derivatives, can inhibit PCR amplification of target DNA in samples containing blood (5). The breakdown products of heme, such as bilirubin, as well as bile salts can inhibit PCR in samples containing feces (20). In addition, many of the reagents used to cultivate microorganisms or to prepare samples for PCR can inhibit PCR when present at contaminating levels (15, 19). Other sources of inhibition are chemically ill defined. Humic substances, which can also inhibit PCR (18), are a mixture of complex polyphenolics produced during the decomposition of organic matter. As such, humic substances are ubiquitous in soil and water and can contaminate any material exposed to the environment. In many cases, however, the source (or sources) of inhibition is unknown.

As a consequence of inhibition, extensive purification is often required to generate PCR-compatible material. However, because purification adds to the time and expense of sample preparation, as well as to the loss of target nucleic acids, a more satisfying approach to the problem of PCR inhibition would be to relieve interference rather than attempt to remove all of the offending substances. To this end, various additives have been included in PCR to relieve inhibition (4, 13, 14). Among these, bovine serum albumin (BSA) has had widespread use for relieving interference in PCR (1, 6, 7, 13), as well as in a variety of other enzymatic reactions (9). In addition, T4 gene 32 protein (gp32), a single-stranded DNA-binding protein, has shown promise in facilitating PCR (11, 20).

Known inhibitors. To identify inhibitory substances affected and to determine the extent of relief provided by BSA and gp32, several known or suspected inhibitors were tested in PCR assays with and without BSA (Serological Research Institute, Richmond, Calif.) or gp32 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). EDTA (molecular biology grade), sodium dodecyl sulfate (SDS), Triton X-100, bile salts (sodium cholate plus deoxycholate), and hemin (equine) were from Sigma (St. Louis, Mo.); bilirubin, the bile salts sodium glycocholate and sodium taurocholate, tannic acids, and humic acids were from Fluka (Ronkonkoma, N.Y.); $FeCl_3 \cdot 6H_2O$ (American Chemical Society reagent grade) was from Aldrich (Milwaukee, Wis.); and NaCl (molecular biology grade) was from Fisher (Itasca, Ill.). In addition, reference samples of humic and fulvic acids from both peat and the Suwannee River were obtained from the International Humic Substance Society (Patrick MacCarthy, Department of Chemistry and Geochemistry, Colorado School of Mines, Golden) and were tested in PCR. Stock solutions of hemin, bilirubin, fulvic acids, and humic acids were prepared at 1 mg/ml in 10 mM NaOH or KOH. Others were prepared in water. Serial 10-fold dilutions were prepared in water for all, and each dilution series was tested by PCR. Inhibitors were mixed with template DNA from 10× solutions, and the inhibitor-DNA mixtures were added last to the reactions. PCR assays were run with primers and template for *Bacteroides distasonis*, as described previously (8), and all substances whose inhibition was relieved by BSA or gp32 were retested at least once with the same primer-template combination. All assays were repeated with freshly prepared solutions of inhibitor with primers and template for *Bacteroides vulgatus* (8).

The optimum concentration of BSA for relief of inhibition from humic acids was 200 to 400 ng/μl in PCR, and that of gp32 was 100 to 150 ng/μl. Higher levels, up to 3 μg of BSA per μl or 250 ng of gp32 per μl, or the addition of both BSA and gp32 to the same reaction provided no more relief of inhibition than either alone at its optimum concentration (data not shown). Therefore, each known inhibitor was tested, as illustrated in Fig. 1, in reactions lacking BSA or gp32 (panel A) and in reactions containing 400 ng of BSA per μl (panel B) or 150 ng of gp32 per μl (panel C). An inhibitory level was defined as the lowest concentration of the inhibitor that reproducibly reduced the yield of PCR product, as observed on agarose gels. EDTA inhibited PCR at 1 mM but not at 0.1 mM, regardless of whether BSA or gp32 was included in the reaction. On the other hand, $FeCl_3$ was 100-fold less inhibitory in reactions containing either BSA or gp32 than in their absence (1 mM versus 10 μM). Above 1 mM, the pH of the PCR mixture dropped rapidly with increasing $FeCl_3$ concentrations because of the formation of insoluble hydroxides and release of H^+. None of the other inhibitors affected the pH of the PCR

* Present address: Johnson & Johnson Clinical Diagnostics, Nucleic Acid Diagnostics, Building 82, 4th Floor, RL, Rochester, NY 14650-2117. Phone: (716) 453-5780. Fax: (716) 453-5689.

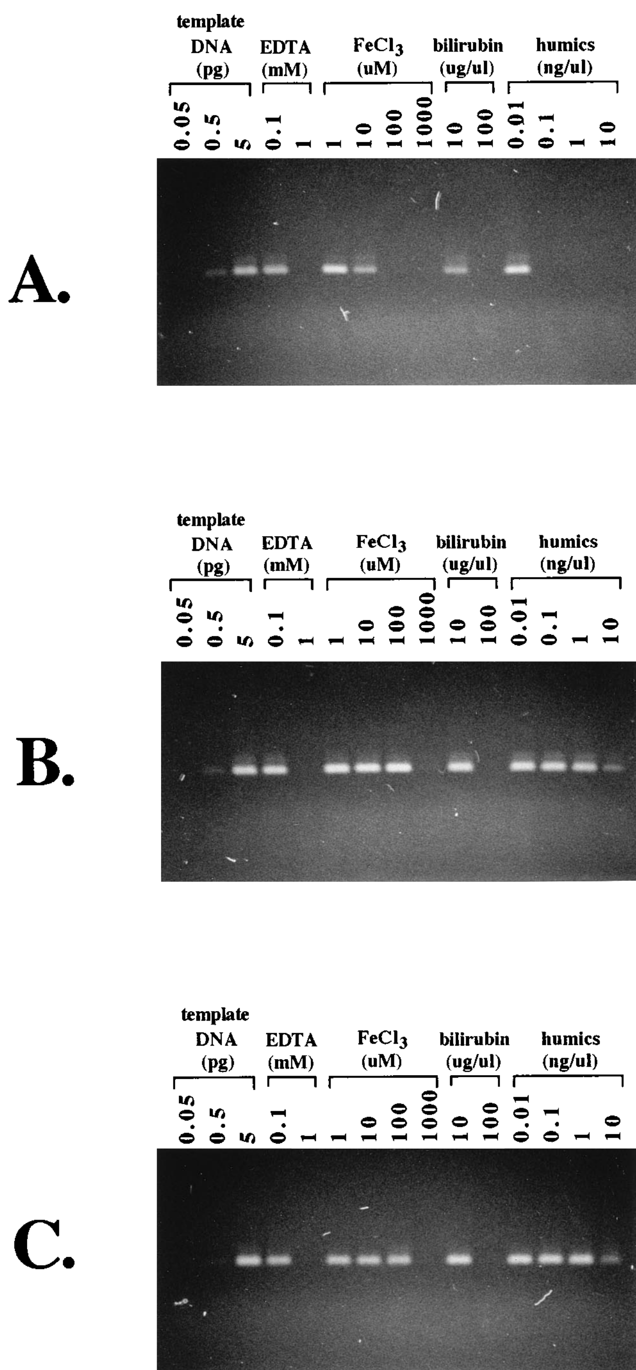

FIG. 1. Relief of interference from known inhibitors in PCR with BSA or gp32. The indicated concentrations of inhibitors were included in reaction mixtures containing 5 pg of template DNA and PCR primers for *B. distasonis*. In addition, 0.05, 0.5, and 5 pg of *B. distasonis* DNA were assayed without added inhibitor. Five microliters of product from each 25 μl of PCR mixture was fractionated on a 1.5% agarose gel and visualized by staining with ethidium bromide. (A) Standard PCR conditions without BSA or gp32; (B) PCR with 400 ng of BSA per μl; (C) PCR with 150 ng of gp32 per μl.

mixture, even at the highest concentrations tested. Bilirubin was only slightly less inhibitory when BSA or gp32 was included in the PCR. This slight relief of inhibition from bilirubin was reproducible, but it was less than 10-fold and therefore not considered to be significant. Humic acids inhibited PCR at 0.1 ng/μl. However, at least 100-fold more humic acid was accommodated when either BSA or gp32 was included in the reaction. Similar results were obtained with humic acids from both commercial and noncommercial sources, prepared from either soil or water. The yields of PCR product in the absence of added inhibitor were similar with and without BSA or gp32 (Fig. 1, first three lanes in each panel, and data not shown), indicating that neither acts by enhancing amplification in general.

Several other known or suspected inhibitors of PCR were tested, as illustrated in Fig. 1. Hemin and tannic acids inhibited PCR at 0.1 ng/μl, while fulvic acids were not inhibitory until 1 ng/μl. As for humic acids, at least 100-fold more hemin, tannic acids, or fulvic acids were accommodated in PCR with BSA or gp32 than without. On the other hand, neither BSA nor gp32 relieved the interference from minimum inhibitory levels of NaCl (0.1 M), SDS (0.1 mM), Triton X-100 (10%), or any of the bile salts (1 to 10 μg/μl), as shown in Fig. 1 for EDTA. In most cases the inhibitory levels determined in the present study are similar to published values, where available (15, 18–20).

It is noteworthy that the inhibition from hemin is relieved by both BSA and gp32, whereas that of its degradation product, bilirubin, is not. The minimum inhibitory concentration (MIC) of hemin was 0.1 ng/μl (0.15 μM), whereas that of bilirubin was 100 ng/μl (170 μM). Therefore, hemin is over 1,000-fold more potent than bilirubin. However, the MICs were the same for both hemin and bilirubin in the presence of BSA or gp32 (100 ng/μl). These results suggest that hemin inhibits PCR by more than one mechanism—one that is relieved by BSA and gp32 and a second, in common with bilirubin, that is not affected by BSA or gp32. Hemin and bilirubin differ in that the closed-ring structure of hemin coordinates with iron, whereas the open structure of bilirubin does not. Therefore, adding hemin contributes iron to the reaction, and as shown in Fig. 1, iron inhibition would be relieved by BSA or gp32. However, at its minimum inhibitory level, 0.15 μM, hemin would not supply enough iron to inhibit PCR since $FeCl_3$ did not inhibit PCR until 10 μM.

BSA obtained from several different sources provided similar relief of inhibition from humic acids (data not shown). The ultrapure and molecular biology grade preparations from Boehringer Mannheim Biochemicals; the molecular biology grade (B2518), ethanol-precipitated (A2153), fatty acid-free (A6003), and heat shock-treated (A7906) preparations from Sigma; and the ultrapure-grade preparation from Ambion (Austin, Tex.) were tested. Of these, the only preparation that did not relieve inhibition, the molecular biology grade BSA from Sigma, had been treated with acetic anhydride to acetylate and thereby inactivate nucleases. This preparation inhibited PCR itself unless it was diluted 1,000-fold (0.4 ng/μl in the PCR). Furthermore, the inhibition from acetylated BSA was relieved by including nonacetylated BSA in the reaction (data not shown). Similarly, McKeown found that an acetylated BSA from Gibco BRL inhibited amplification in PCR assays (10); however, the source of inhibition has not been identified.

Unknown inhibitors. The effects of BSA and gp32 on interference from unknown inhibitors was tested with extracts from feces and from natural water samples. Extracts of bacterial fractions from chicken, cow, hog, horse, and sheep manure, as well as extracts of filter retentates collected from the Ohio River, the Atlantic Ocean, Delaware Bay, a marsh, a freshwater creek, and a canal, all inhibited PCR. However, 10- to 1,000-fold more of these extracts was accommodated when either BSA or gp32 was included in the PCR.

Examples of results with unknown inhibitors in PCR are shown in Fig. 2 for a manure sample, a marine water sample,

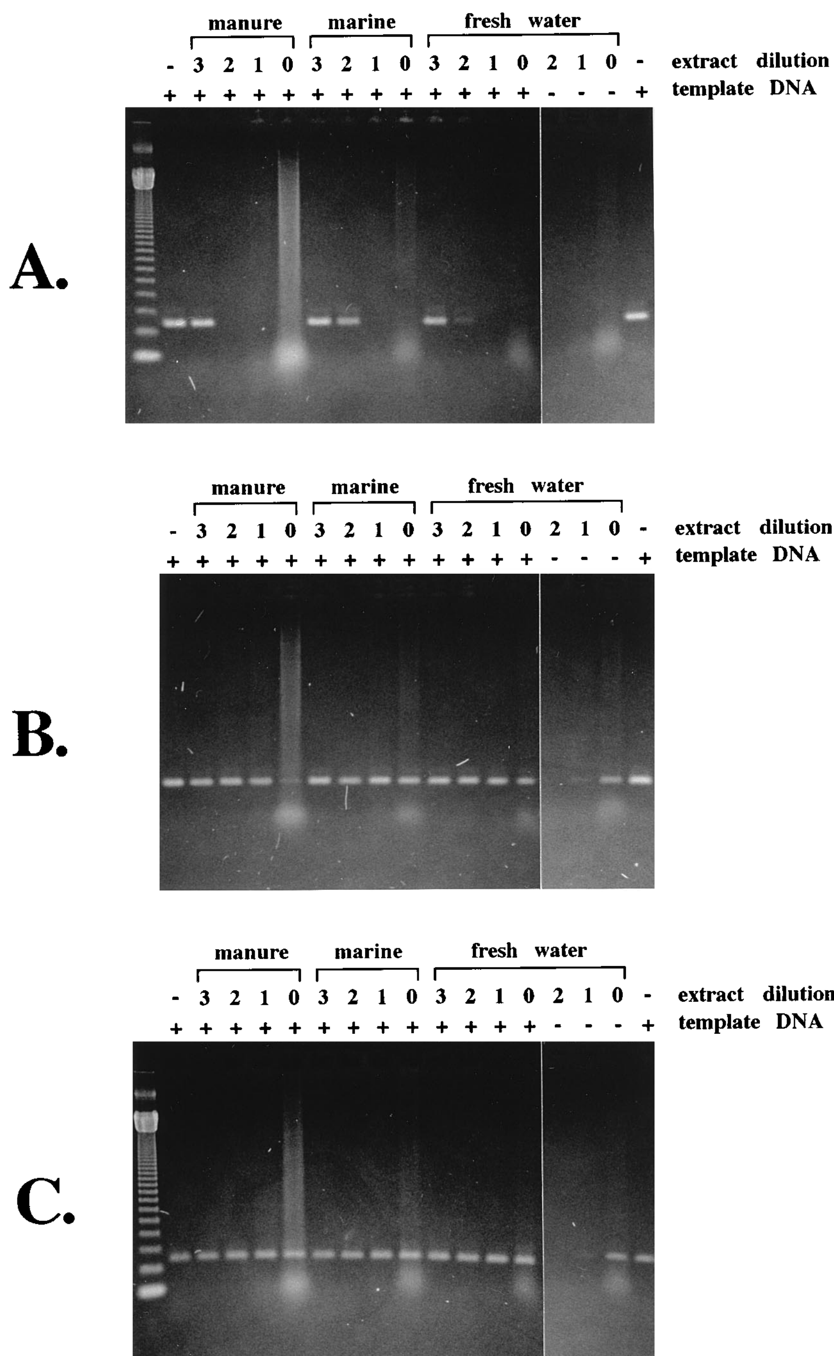

FIG. 2. Relief of interference from unknown inhibitors in PCR with BSA or gp32. A bacterial fraction was prepared from cow manure by differential centrifugation, and DNA was extracted as described previously (8). Water samples from the Delaware Bay (marine) or the Ohio River (fresh water) were filtered through Sterivex-GS units (Millipore Corp., Bedford, Mass.), and the material collected was lysed directly in the filter housing essentially as described by Sommerville et al. (17), except that extraction conditions were as for fecal bacteria. DNA concentrations were determined with a fluorescent dye-binding DNA assay kit as specified by the manufacturer (Pierce, Rockford, Ill.) and verified by fractionation on 1% agarose gels. The undiluted extracts contained 1.1, 0.12, or 0.14 μg of total DNA, respectively, in the 2.5 μl added per 25 μl of reaction mixture. Serial 10-fold dilutions of each extract were tested for inhibition in PCR. Dilutions: lanes 3, 10^{-3}; lanes 2, 10^{-2}; lanes 1, 10^{-1}; lanes 0, 10^{0} (undiluted) (lanes −, no extract was added). Either 5 pg of *B. distasonis* DNA (+) or no DNA (−) was added for the template, and 5 μl of each PCR product is shown on a 1.5% agarose gel. A 123-bp ladder (GibcoBRL Life Technologies, Gaithersburg, Md.) was used for molecular size standards. The PCR product from *B. distasonis* is 301 bp. (A) Standard PCR conditions without BSA or gp32; (B) PCR with 400 ng of BSA per μl; (C) PCR with 150 ng of gp32 per μl.

and a freshwater sample. All three extracts shown were prepared by SDS and proteinase K lysis followed by hexadecyltrimethyl ammonium bromide (CTAB) and phenol-chloroform-isoamyl alcohol (PCI) extractions as described by Wilson (21). Serial dilutions of each extract were added along with target DNA to standard PCR mixtures (panel A), to mixtures containing 400 ng of BSA per μl (panel B), or to mixtures containing 150 ng of gp32 per μl (panel C). At least 100-fold more of these three extracts could be analyzed without inhibition when reaction mixtures contained BSA or gp32. Extracts

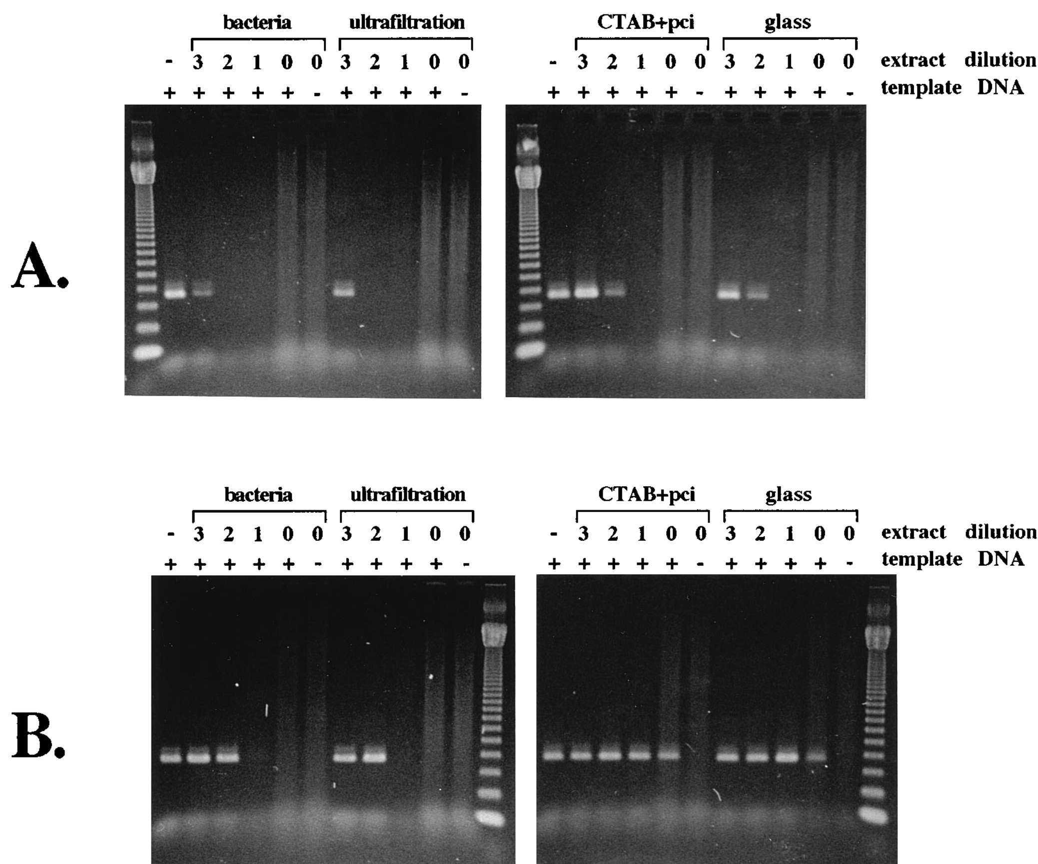

FIG. 3. Relief of interference from residual inhibitors after use of alternate purification methods. One-fourth of a bacterial fraction from sheep manure was reserved and tested for inhibition of PCR after no further treatment (bacteria). The remaining bacteria were lysed, split into three equal portions, and tested after ultrafiltration, CTAB plus PCI extraction, or glass purification. For ultrafiltration, the lysate was heated at 80°C for 15 min and chilled on ice for 1 min to inactivate proteinase K, and DNA was concentrated and washed twice with 10 mM Tris-HCl–0.1 mM EDTA, pH 8.0, in Centricon 100 units as specified by the manufacturer (Amicon, Beverly, Mass.). For glass purification, DNA was concentrated from the lysate by alcohol precipitation and resuspended in 100 μl of 50 mM Tris-HCl–50 mM EDTA, pH 8.0. Two hundred microliters of GuSCN lysis buffer (5 M guanidinium thiocyanate, 50 mM Tris-HCl [pH 6.4], 20 mM EDTA, 1% Triton X-100) (2) was added, and the mixture was loaded into a SpinBind cartridge (FMC BioProducts, Rockland, Maine). After 1 min, the lysate was spun through the cartridge in a microcentrifuge. Flowthrough material was reloaded twice to recover any residual DNA. The cartridge was washed twice with 5 M guanidinium thiocyanate and twice with ice-cold 70% ethanol. DNA was eluted by adding 50 μl of 10 mM Tris-HCl–0.1 mM EDTA, pH 8.0, at 56°C and incubating the mixture at 56°C for 10 min. Eluate was collected by centrifugation into a fresh tube, and the elution was repeated. Equal proportions of each preparation were assayed. DNA concentrations were determined as for Fig. 2. The undiluted fractions contained 540, 400, 380, or 290 μg of total DNA, respectively, in the 2.5 or 5.0 μl added per 25 μl of PCR assay mixture. Extract dilutions: lanes 3, 10^{-3}; lanes 2, 10^{-2}; lanes 1, 10^{-1}; lanes 0, 10^{0} (undiluted) (lanes −, no extract was added). Either 5 pg of *B. vulgatus* DNA (+) or no DNA (−) was added for the template, and 5 μl of each PCR product is shown on a 1.5% agarose gel. A 123-bp ladder (GibcoBRL Life Technologies) was used for molecular size standards. The PCR product from *B. vulgatus* is 445 bp. (A) Standard PCR conditions without BSA; (B) PCR with 400 ng of BSA per μl.

from the other water samples and most of the manure samples tested gave results similar to those shown in Fig. 2. However, some manure extracts inhibited the standard PCR at 10-fold-higher or 10-fold-lower concentrations than did the extract from cow manure shown in Fig. 2. It should be noted that the extract from freshwater shown in Fig. 2 contained endogenous target DNA (template DNA lanes −). The level of target DNA was too low to detect after diluting to reduce inhibition; however, it was readily detected by PCR with BSA or gp32. No endogenous target DNA was detected in either the manure or the marine water sample (data not shown).

As shown in Fig. 3, residual inhibitory substances whose interference is relieved by BSA were present in samples prepared by several different methods. The prelysis bacterial fractions from feces, as well as lysates purified by ultrafiltration, CTAB plus PCI extraction, or glass adsorption, all inhibited PCR (panel A), and this inhibition was relieved to some extent by including BSA in the reactions (panel B). Extracts from other feces and water samples tested gave results similar to those shown in Fig. 3, except that most were 10-fold, and many fecal extracts were 100-fold, less inhibitory in the standard PCR than that from the sheep shown in Fig. 3. It should also be noted that extracts from separate aliquots of these manure samples, purified by CTAB plus PCI extraction and glass adsorption, consecutively, did not inhibit amplification of target DNA in previous studies (8).

Several of the substances whose inhibition is relieved by including BSA or gp32 in the PCR contain phenolic groups, which suggests a mode of action. Phenols are known to bind to proteins by forming hydrogen bonds with peptide bond oxygens, and BSA has been widely used during isolation of organelles and enzymes from plants to scavenge endogenous phenolic compounds that can bind to and inactivate proteins of interest (9). Tannic acids are one type of plant phenolic compound, whereas humic and fulvic acids are mixtures of polyphenolic substances produced during the degradation of plant material. All three can be abundant in natural waters. In addition, feces, especially from herbivores, contain copious quan-

tities of degraded plant material as well. BSA is also known to bind lipids via hydrophobic forces and anions by virtue of its high lysine content (9). Therefore, BSA may be able to scavenge a variety of substances and thereby prevent their binding and inactivation of *Taq* DNA polymerase.

BSA has also been added to PCR to relieve inhibition from samples containing endogenous protease activity (12). Since all manure and water samples used in the present study were lysed with proteinase K, both endogenous and added protease are possible sources of inhibition. BSA or gp32 may provide an alternate substrate and thereby protect the *Taq* DNA polymerase.

gp32, a single-stranded DNA-binding protein, may facilitate PCR by binding with denatured strands of DNA to retard reannealing and perhaps by stimulating the DNA polymerase (3). gp32 has been reported to enhance PCR product yield, especially for long amplicons (16), as well as to relieve inhibition (11, 20). In the present study, gp32 was more effective than BSA at low levels, since some relief of inhibition from humic acids was observed with 20 to 50 ng of gp32 per μl but not with this same amount of BSA (data not shown). However, with optimum levels, the pattern of relief from inhibition was the same for both proteins. Therefore, gp32 may act as a scavenger as well. Since the use of gp32 adds $2 to $3 per reaction whereas BSA can be added for less than a penny per reaction, BSA is by far the more cost-effective additive to use.

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