A Sensitive, High-Volume, Colorimetric Assay for Protein Phosphatases

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Protein phosphatases are intimately involved in a variety of cellular processes, many of which are of interest to the pharmaceutical industry. Phosphatase assays generally employ radioisotopes, making them tedious to perform, costly, and hazardous, while other procedures require antibodies and/or are unsuitable for mass screening efforts. To facilitate screening for inhibitors of the CD45 protein tyrosine phosphatase (PTPase), we have developed a sensitive colorimetric assay, using small volumes in 96-well microtiter plates and read on a standard ELISA plate reader. The assay was sensitive down to 0.5 nmol of released phosphate and can be easily run by robotics to assay thousands of compounds in a day. The assay is sparing of reagents and has been successfully used with all classes of phosphatases. The reagents are nonradioactive, readily obtainable, and minimal in cost. This assay should facilitate the search for specific inhibitors of phosphatases.

KEY WORDS: sensitive; high volume; colorimetric phosphatase assay.

INTRODUCTION

Protein phosphatases fall into two general categories: those with specificity for phosphoserine or phosphothreonine residues and those with specificity for phosphotyrosine residues. Because of their involvement in many cellular processes, both classes of enzymes have received considerable interest by the academic and pharmaceutical research communities. Protein tyrosine phosphatases (PTPases) are known to play a critical role in lymphocyte signaling, cell cycling, bacterial virulence, and, possibly tumorigenesis (1–3). The serine/threonine class of protein phosphatases is also involved in many aspects of cellular regulation including immunosuppression, shellfish poisoning, and control of the cell cycle (4,5). Since many of the cellular functions involving phosphatases are germane to inflammation, transplant rejection, and oncology, phosphatases have become attractive targets for drug intervention.

Phosphatases are often assayed with the nonspecific substrate, *p*-nitrophenylphosphate (pNpp), however, pNpp is not applicable to all phosphate releasing enzymes. Nor can pNpp be employed when one wishes to explore the phosphoprotein substrate selectivity of a phosphatase. The latter experiments rely on costly and hazardous radiolabeled substrates, which often must be generated by the researcher, generally with low yields. Furthermore, the increasingly stringent controls on the use and disposal of radioisotopes contribute to the unattractiveness of radioactive assays for high-volume screening. Other phosphatase assays require HPLC (6), antiphosphotyrosine antibodies (6,7), or specialized equipment (7) or follow changes in the fluorescence or absorbance of tyrosine or tyrosine-containing substrates (8,9). None of these assays except for particle concentration fluorescence immunoassay (PCFIA) (9) are suitable for mass screening efforts. However, the PCFIA procedure requires a substrate coupling step and antiphosphotyrosine antibodies and relies on instrumentation not readily available to most researchers.

To facilitate high-volume screening for inhibitors of the CD45 PTPase (10), we have developed a colorimetric assay using Malachite Green (11). This assay offers excellent sensitivity without the hazards and disposal liabilities of the radioactive assays. The Malachite Green assay was adapted to small volumes in a 96-well microtiter plate format and can be run efficiently either by hand or by robotics. The assay accommodates a variety of substrates including phosphoserine or phosphotyrosine peptides, phospho-amino acids or pNpp, and all classes of phosphatases. The Malachite Green assay can also be used for kinetic experiments to help characterize phosphatases and determine the class and K_i of inhibitors. Since the Malachite Green assay uses a standard enzyme-linked immunosorbent (ELISA) reader, this assay should be readily accessible to all laboratories.

MATERIALS AND METHODS

Materials

Ammonium sulfate, Malachite Green oxalate, and DL-phosphotyrosine were purchased from Aldrich and chromatography supplies were from Pharmacia. Dithiothreitol (DTT; ultrapure) was from Boehinger Mannheim and Brij 35 was purchased from Pierce. Sulfuric acid was from Baker and DL-phosphoserine was purchased from Fluka. Easy-wash microtiter plates (Corning No. 25882-96) were purchased from Corning and protein phosphatase type 1 was from Upstate Biotechnology Inc. (UBI). Phosphoserine and phosphotyrosine peptides were synthesized as described in Ref. 9 and obtained from Dr. R. Roeske. The phosphotyrosine peptide substrate [FTATEPQ(PO_4)YQPGENL] represents the regulatory region of the *fyn* kinase (12,13) while the phosphoserine peptide substrate [GRFDRRV(PO_4)SVAAE] is the cAMP-dependent kinase sequence preferred by calcineurin (14). All other reagents, including alkaline phosphatase (Catalog N. P-3895) were from Sigma.

Methods

Purification of CD45 Phosphatase

The recombinant CD45 phosphatase used for these studies was expressed and purified as described by Pacitti *et al* (15). Briefly, the cytoplasmic region of human CD45 (cCD45) containing the enzymatically active portion of the

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molecule was expressed in yeast. Yeast were lysed in a French press, cellular debris was removed by centrifugation, and cCD45 was precipitated with 70% ammonium sulfate. Purified cCD45 was obtained following phenyl Sepharose, Superdex 200, Mono Q, and Superose 12 chromatography. The enzyme was stored at −70°C in buffer containing 50 m*M* HEPES, 150 m*M* NaCl, 4 m*M* DTT, 0.00375% Brij 35, pH 7.0, and protease inhibitors.

Malachite Green Reagent

The stock reagent consists of 130 m*M* Malachite Green in 3.6 *M* sulfuric acid. Stock reagent can be stored in the dark for up to a year. The working reagent was made by combining the Malachite Green stock solution with 7.5% ammonium molybdate (w/v, in water) and 11% Tween 20 (v/v, in water) at a ratio of 1/0.25/0.02 (v/v/v). Ammonium molybdate must be added to the Malachite Green stock solution while vortexing to avoid precipitate formation. The working reagent is stable for at least 24 hr. The ammonium molybdate stock must be made fresh whenever a precipate forms.

Phosphatase Assays

Human placenta alkaline phosphatase was assayed in a buffer containing 100 m*M* glycine, 1 m*M* $ZnCl_2$, and 1 m*M* $MgCl_2$, pH 10.4. cCD45 was assayed in a buffer containing 100 m*M* imidazole, 5 m*M* DTT, 1 m*M* EDTA, and 0.05% Tween 20, pH 6.8, and PP-1 was assayed in 20 m*M* MOPS, 0.1 *M* NaCl, and 10 m*M* DTT, pH 7.5. One unit of activity is defined as the release of 1 μmol of phosphate/min from substrate. Standard curves were generated using potassium phosphate pH 6.8. Data from kinetic experiments were analyzed using a nonlinear curve-fitting computer program based in RS/1.

Natural Product Extracts

Microbial extracts were made from fungi and *Actinomyces* by extracting the entire culture with an equal volume of ethyl acetate. The extract was dried under reduced pressure and resuspended in dimethyl sulfoxide (DMSO) for assay. Plant homogenates were either steeped in cold water, then passed through a 5-kDa filter, or defatted with hexane, then extracted with methylene chloride. Extracts were dried under reduced pressure and resuspended in DMSO for assay.

RESULTS

Malachite Green Phosphate Assay

The Malachite Green assay described by Baykov *et al.* (11) was adapted to small volumes in microtiter plates to facilitate mass screening efforts. Initial experiments determined the most suitable microtiter plate for the assay and the optimal volumes for high-throughput screening. The Corning Easy-Wash plate was found to provide the lowest background reading at $A_{620\ nm}$, and the sloping walls at the bottom of the well readily accommodated small volumes. The Corning Easy-Wash microtiter plate was used throughout these studies.

The optimal reaction volume for assay was determined to be 40 μl, which allowed for the substrate, enzyme, and inhibitor to be added in volumes with reliable accuracy. We also observed that the total volume in the wells at the time of reading was critical for obtaining reliable values on the microtiter plate reader. For the Easy-Wash plate and our plate reader, optimal readings were obtained with a final volume of >100 μL, which was accomplished by the addition of distilled, deionized water. This step can be performed in either of two ways. At the end of reaction, 80 μL of water can first be added to each well, followed by 20 μL of Malachite Green reagent, or a mixture of Malachite Green reagent/water at a ratio of 1/4 (v/v) can be premixed, and 100 μL added per well to terminate the reaction. Identical results are obtained with either method, but the single addition was more suitable for terminating time-sensitive kinetic experiments. The premixed reagent should be made approximately 30 min prior to use to obtain the lowest background values. With either method, the plate was allowed to sit at room temperature for 25 min to allow color formation to stabilize before reading on a UV_{max} microtiter plate reader (Menlo Park, CA) at 620 nm.

As expected, color formation was dependent on enzyme concentration, substrate concentration, time, and temperature. The linear range of the assay was also dependent on the concentration of color reagent as shown in Fig. 1. Color formation was linear between 0.5 and 7 nmol phosphate, when 20 μL of Malachite Green reagent was added per well. Linearity can be extended to higher concentrations of phosphate with the use of more color reagent, but there is a concommitant loss of linearity at the low end of the concentration range. We used 20 μL/well of the Malachite Green reagent in our experiments, with standard curves ranging from 0.5 to 5 nmol phosphate/well.

Phosphatase Inhibitor Screening

The goal of this study was to produce a simple assay

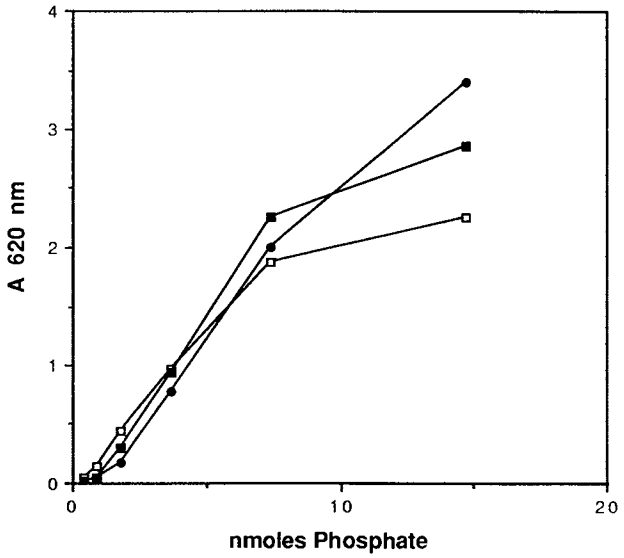

Fig. 1. Optimal Malachite Green reagent concentration. Potassium phosphate was serially diluted in an Easy-Wash microtiter plate, followed by 80 μL of water and various amounts of the Malachite Green reagent. Color formation was read 25 min later at $A_{620\ nm}$. Malachite Green reagent: (□) 20 μL; (■) 30 μL; (●) 40 μL.

suitable for the mass screening of chemical files and natural products for inhibitors of the CD45 phosphatase. For the high-volume screening assay, 20 μL of inhibitor and 10 μL of a suitable dilution of enzyme were added to the plate, the reaction was initiated by the addition of 10 μL of substrate, and the mixture was incubated for 1 hr at 37°C. CD45 is a tyrosine phosphatase, a class of phosphatases known to be inhibited by micromolar concentrations of vanadate, molybdate, and tungstate. Figure 2 shows a typical inhibition curve for cCD45 and ammonium molybdate. cCD45 shows a dose–response inhibition by molybdate, with an IC_{50} of 1 μ*M*. Similar inhibition was observed with sodium orthovanadate and sodium tungstate. The values obtained with the Malachite Green assay for inhibition of CD45 by these ions was consistent with the values reported by others using radioactive assays (16). The sensitivity and reproducibility of the Malachite Green assay with known phosphatase inhibitors has been excellent.

Applicability to Natural Products Screening

To be successful as a high-volume screening tool, the Malachite Green assay must withstand the solvents used to solubilize natural product extracts and be free of nonspecific interferences from plant and microbial extracts and/or culture media. Table I shows the tolerance of the Malachite Green assay for the solvents commonly used for solubilization of plant and microbial extracts and the lack of general interference by natural product extracts. The assay can tolerate concentrations of at least 5% DMSO, ethanol, or a 50:50 mix of methanol and water. At concentrations greater than 5% DMSO or ethanol, cCD45 showed a marked enhancement of enzyme activity but could still be inhibited by vanadate or molybdate. Enhancement of CD45 activity by

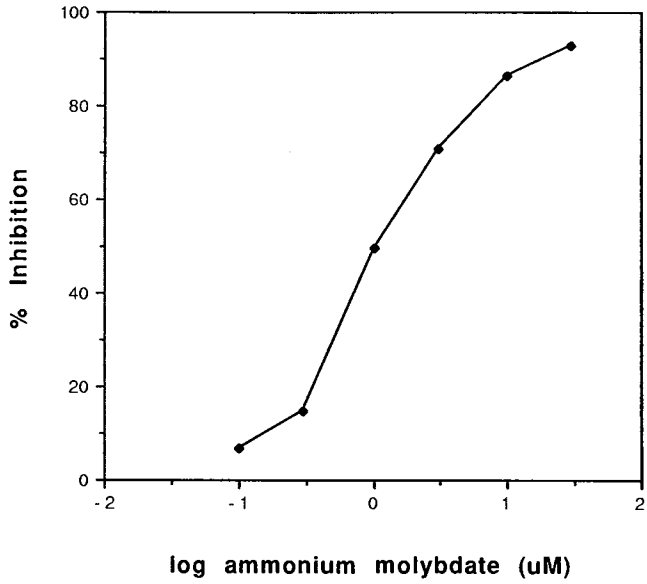

Fig. 2. Inhibition of cCD45 by ammonium molybdate. Ammonium molybdate was diluted in the microtiter plate in CD45 assay buffer, followed by the *fyn* phosphopeptide substrate, to a final concentration of 200 μ*M*. The reaction was initiated by cCD45 phosphatase and allowed to proceed at 37°C for 1 hr before termination with the Malachite Green reagent.

Table I. Natural Products Screening[a]

Sample	Concentration	% inhibition − Mo	% inhibition + Mo
DMSO	5%	−8	78
Ethanol	5%	−5	75
Methanol/H_2O (1/1)	5%	−3	72
Mo alone	10 μ*M*	—	73
Plant extracts			
1	75 μg/ml	−4	74
2	75 μg/ml	−14	78
Mo alone	10 μ*M*	—	78
Microbial extracts			
1	75 μg/mL	11	89
2	75 μg/mL	−11	79
Mo alone	10 μ*M*	—	89

[a] Reactions were run at 37°C/1 hr using 200 μ*M* (final concentration) of the *fyn* phosphotyrosine peptide. Mo, ammonium molybdate.

DMSO and ethanol appears to be more a characteristic of the CD45 enzyme than of the assay per se. Table I also shows two plant extracts and two microbial extracts with and without spiking of the inhibitor, ammonium molybdate. These results show that the Malachite Green assay was not subject to general interference by plant or microbial extracts and that the presence of an inhibitor could be readily detected in either type of extract. Extracts of four common microbial growth media did not contain detectable levels of phosphate or other components which interfere with the assay. Using the Malachite Green screening assay, we have detected inhibitors of CD45 phosphatase in both microbial and plant extracts with a "hit" rate similar to other enzyme screens. The Malachite Green assay also offers an excellent signal-to-noise (S/N) ratio. In over a year of screening, the S/N was consistently greater than 500:1.

Assay Versatility and General Applicability

The Malachite Green assay was applicable to a variety of phosphatases and substrates. Table II shows the results of experiments using a tyrosine phosphatase (cCD45), a serine

Table II. Phosphatase Selectivity and Substrate Utility[a]

	Sp Act (U/mg)				
	Phosphopeptides			Phospho-amino acids	
Phosphatase	pY	pS	pNpp	pY	pS
CD45	148	0	81	100	0
Alkaline phosphatase	2.5	1.6	11.4	9.1	10.2
PP-1	0.7	17.9	10.3	0	0

[a] Phosphopeptides were used at a final concentration of 0.5 m*M*; pNpp and phospho-amino acids, at a final concentration of 5 m*M*. Substrates and enzymes were prewarmed to 30°C, and the reaction was initiated by the addition of enzyme, then allowed to proceed for 10 min before terminating with the Malachite Green/water mixture. Units are μmol PO_4/min. pY, phosphotyrosine; pS, phosphoserine.

phosphatase (PP-1), and a nonspecific phosphatase (alkaline phosphatase), each assayed with five substrates. cCD45 hydrolyzed phosphotyrosine, a phosphotyrosine-containing peptide, and the nonspecific phosphatase substrate, pNpp, but showed a complete lack of activity with phosphoserine and a phosphoserine containing peptide. As expected, the serine/threonine phosphatase PP-1 showed a strong preference for the phosphoserine-containing peptide, while the nonspecific phosphatase, alkaline phosphatase, hydrolyzed all substrates. PP-1 also worked well with pNpp but, for unknown reasons, was inactive with the phospho-amino acids. These results suggested that the Malachite Green assay was equally applicable to a variety of substrates utilized by tyrosine specific, serine/threonine specific, and nonspecific phosphatases. In addition to the phosphatases in Table II, we have used the Malachite Green assay with several other phosphatases (calcineurin, acid phosphatase, T-cell PTPase, PTP1c, PTP1b, LAR, and PP-2A) without alteration, except for the required buffer.

The nonspecific phosphatase substrate, pNpp, is most often assayed by determining the absorbance at 405 nm of one of the reaction products, *p*-nitrophenol. The Malachite Green assay can be used with pNpp to measure the other reaction product, phosphate. In a direct comparison experiment, cCD45 was serially diluted in a 96-well plate and the reaction initiated by the addition of pNpp to a final concentration of 5 m*M*. After 10 min at 30°C, the reaction in companion wells was terminated by the addition of either the Malachite Green reagent or NaOH and the plate sequentually read at the two appropriate wavelengths. Comparing the last reliable absorbance value (0.1 AU) for each, phosphate measurements using Malachite Green were approximately 10-fold more sensitive for accessing phosphatase activity using pNpp as a substrate than $A_{405\ nm}$ measurements of *p*-nitrophenol (data not shown).

Evaluation of Enzyme Characteristics

The Malachite Green assay was used to help characterize phosphatases and their inhibitors. For kinetic experiments substrate, or substrate plus inhibitor, was added to the wells of the plate in a total of 20 μL and the plate placed in a 30°C heating block for 10 min. The reaction was initiated by the addition of 20 μL of enzyme, which had been prewarmed to 30°C, and allowed to proceed for 5–20 min to keep substrate conversion to ≤10%. Kinetic experiments were terminated with the single addition of the Malachite Green/water mixture to ensure precise timing of the reaction. Results of a representative kinetic experiment using cCD45 phosphatase and the *fyn* phosphotyrosine peptide are shown in Fig. 3. The Malachite Green assay has worked well for kinetic experiments using both the phosphotyrosine peptide and pNpp as substrates. The only difficulty encountered in the kinetic assays was when a 25-amino acid phosphotyrosine peptide was used at concentrations above 1 m*M*. With high concentrations of large peptides or proteins a precipitate forms, most likely due to the denaturing effect of the sulfuric acid in the Malachite Green reagent. Phosphopeptides of 14 or fewer amino acids were routinely used at concentrations up to 2 m*M* without difficulty. The assay was readily amenable to determining K_i values and evaluating whether inhibitors were competitive or noncompetitive and/or time dependent.

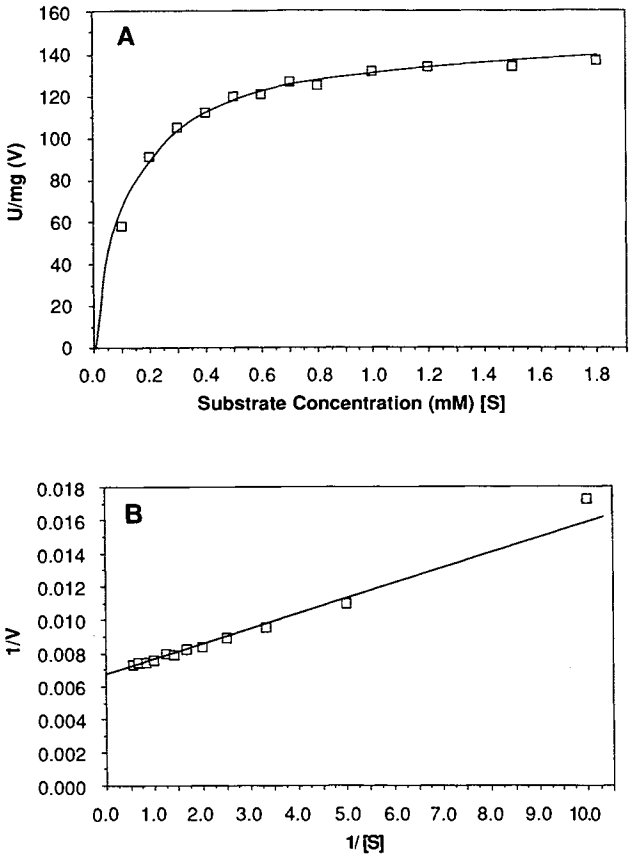

Fig. 3. Enzyme kinetics using the Malachite Green assay. The *fyn* phosphopeptide substrate was added to the plate in CD45 assay buffer and prewarmed to 30°C for 10 min. The reaction was initiated by prewarmed cCD45 in assay buffer and the reaction carried out for 10 min before terminating with the Malachite Green/water mixture. (A) Michaelis–Menten plot; (b) Lineweaver–Burk plot.

DISCUSSION

We have developed a sensitive, high-volume, 96-well plate assay for phosphatase inhibitor screening using Malachite Green. Due to the limited number of reagent additions, small volumes, and microtiter plate format, the assay was readily adaptable to robotic manipulation. The simplicity of the assay allows large numbers of samples to be assayed and the data analyzed in a single day. The Malachite Green assay was applicable to all classes of phosphatases and a variety of substrates. We have had considerable experience with this assay for screening diverse chemical files and all types of natural products and found the assay to be suitably robust, reproducible, and sensitive for all aspects of this work. The reproducibility of the assay with various phosphatases, substrates, and inhibitors has been excellent, with an interassay variability over the various protocols averaging 3% (range, 0–10%).

Pursuit of inhibitors uncovered in mass screening efforts has shown the Malachite Green Assay to be very versatile. Since the assay is applicable to all phosphatases, we have used it to determine the selectivity of CD45 inhibitors versus other tyrosine phosphatases, alkaline phosphatase, acid phosphatase, and calcineurin. The Malachite Green assay has performed admirably in the different buffer systems and

at the pH values required for these phosphatases. The assay has also proven suitable for kinetic analysis of CD45 and other phosphatases, as well as for the determination of the class and mechanism of action of inhibitors. These types of analyses can be easily and quickly performed in a single 96-well microtiter plate.

The only drawback of the assay found to date was that high concentrations of larger phosphopeptides and proteins tended to precipitate out of solution following the addition of the Malachite Green reagent. No problems were encountered with phosphopeptides of 14 or fewer amino acids, when used at concentrations up to 2 m*M*, or with the phospho-amino acids at any concentration tested. The rarity of protein precipitation we have experienced with the assay has obviated any need to include a protein removal or stabilization step as described by Geladopoulos *et al.* (17). Precipitates also formed using pNpp as a substrate when more than 1 mmol substrate was converted to product. By this point, however, the absorbance readings were already off-scale. Furthermore, measuring the release of phosphate from this substrate with the Malachite Green assay affords greater sensitivity than A_{405} measurements of *p*-nitrophenol requiring less product formation. The increased sensitivity observed with the Malachite Green assay using pNpp as a substrate was most likely due to the higher extinction coefficient for the product of this reaction. Under the conditions of the assay, an extinction coefficient of 90,900 M^{-1} cm^{-1} was obtained for Malachite Green, while the extinction coefficient of *p*-nitrophenol (in equivalent NaOH used for reaction termination) was 15,300 M^{-1} cm^{-1}.

Specific inhibitors of phosphatases may have considerable therapeutic and commercial value. We have developed a robust, high-volume, and sensitive screening assay for the CD45 PTPase. This assay is applicable to phosphatases of all classes and a variety of substrates. Since the Malachite Green assay uses a standard ELISA plate reader, this assay should be readily accessible to all laboratories and facilitate the search for specific inhibitors of therapeutically important phosphatases.

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