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ARTICLES

Determination of Antioxidant Capacity Using the Biological System Bacteriophage P22/Bacterium *Salmonella typhimurium*

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Bacteriophage/bacterium systems have been employed in the past in assays for virucidal activity. A novel application of one such system is proposed here for the in vivo determination of antioxidant capacity. It was shown that an antioxidant such as gallic acid can effectively protect against oxidative damage brought about by H_2O_2—but only within a narrow range of concentrations (i.e., from 250 to 500 mg L^{-1}); ascorbic acid, on the other hand, did not exhibit any protective effect against H_2O_2. Finally, neither ascorbic nor gallic acid demonstrated a virucidal effect. The P22/*Salmonella typhimurium* model system thus proved to be useful in the assessment of antioxidant capacity in vivo, at least using those two alternative model antioxidants.

KEYWORDS: Ascorbic acid; gallic acid; in vivo assessment

INTRODUCTION

Assays for antioxidant (or free radical scavenging) activity have actual and potential applications in many fields; one such field is food science and technology, to monitor the ability of a compound as preservative upon addition or to ascertain its active contribution to health upon ingestion.

High-throughput methods have indeed been developed to assist in extensive and rapid screening for members of the aforementioned family of compounds; these methods represent typically a tradeoff between expediency and accuracy. However, those currently available—while greatly contributing to the efficiency of the overall process—are far from highly reliable; examples of such methods include $ABTS^{\bullet+}$ and deoxyribose/DNA degradation (*1, 2*), for first- and second-level screening approaches, respectively. Current screening methods based on attenuation of oxidative effects upon DNA take advantage of preservation of the integrity of this molecule (and its moieties), when exposed to free radical mediated chemical oxidation: such an integrity is assessed via electrophoresis, but the oxidative regimens required to produce detectable effects are much more drastic than those likely to be encountered on the cell level (*3, 4*).

Lytic and temperate bacteriophage populations can be progressively inactivated by a number of environmental stresses, and the associated extent of damage is dependent on the intensity and duration of said exposure. This degree of inactivation can be revealed by simply (and comparatively) assaying the viable phage population prior to and following exposure to stress. However, the molecular nature of the aforementioned damage depends on its specific nature: therefore, exposing identical phage populations to distinct chemical and radiation stresses will lead to populations that exhibit different molecular lesions (*5, 6*).

The nucleic acid of a phage is the primary site of damage when a number of environmental stresses are imposed upon it (*7, 8*); this is the case of exposure to UV (254 nm) radiation or, alternatively, to chemical action by free radicals (*9*). However, the DNA damage produced following low-intensity exposure to free radicals in progressive inactivation scenarios is, in structural terms, so slight that it will hardly be detectable by current chemical or biochemical analytical methods. On the other hand, the intensity of the oxidation regimen required to generate measurable biological inactivation is much closer to those levels that threaten human cells than the regimens required to degrade DNA to electrophoretically detectable levels (*10*). As a consequence, phage inactivation represents a simple but more sensitive approach to measure the effects of chemical oxidation on the biological integrity of plain DNA; this is why temperate and lytic phages have been used to monitor DNA damage (and repair) since the 1960s.

In this paper, a novel method aimed at screening for scavenger activity in aqueous samples will be presented and discussed; it

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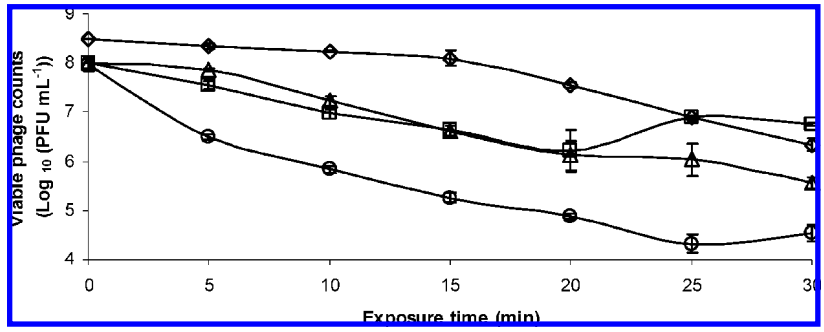

Figure 1. Inactivation curve of bacteriophage P22 (average ± standard deviation) in the presence of (◇) 122, (□) 172, (△) 228, and (○) 250 mM H_2O_2.

is based on attenuation of the oxidative inactivation of the P22 temperate bacteriophage of *Salmonella typhimurium*.

MATERIALS AND METHODS

Bacterium and Phage Preparation. The stock of phage P22 and *S. typhimurium* (ref 19585 P1) was prepared according to ATCC (American Type Culture Collection) indications.

To test the infection capacity of the virus upon the host bacterium, dilutions of the former up to 10^{-12} were performed (in duplicate) in tryptone soy broth (TSB, from LAB M, U.K.); 100 μL of each dilution was then mixed with 300 μL of *S. typhimurium* culture, harvested in the exponential phase, that is, 1% (v/v) inoculum of an overnight culture in TSB at 37 °C, into fresh TSB, and incubated at 37 °C for 2.5 h. Infection was allowed to proceed for 10 min in a water bath at 37 °C; afterward, 100 μL was spread onto predried plates of tryptone soy agar (TSA, from Biokar Diagnostic, France) and incubated at 37 °C for 24 h. After incubation, the plaques were counted, and the titer of the viable phage was accordingly calculated (and expressed in plaque-forming units per unit volume, PFU mL^{-1}).

Titration of Hydrogen Peroxide. The concentration of H_2O_2 used as oxidant was confirmed by titration with 0.1 M $KMnO_4$, using a mixture of 25 mL of H_2O, 10 mL of H_2O_2, and 6 mL of H_2SO_4, based on the classical redox reaction

$$2KMnO_4\,(aq) + 5H_2O_2\,(aq) + 3H_2SO_4\,(aq) \rightarrow 2MnSO_4\,(aq) + 5O_2\,(g) + 8H_2O\,(l) + K_2SO_4\,(aq)$$

Determination of Oxidant Effect upon Phage. One milliliter of P22 (diluted up to 10^{-5}) was added to 9 mL of 100, 150, 200, or 250 mM of a 33% (v/v) H_2O_2 solution (Panreac, Spain); the mixture was left to react for 30 min, at room temperature (in duplicate). Aliquots of 0.1 mL were collected every 5 min for a whole period of 30 min afterward, and serial decimal dilutions were made up to 10^{-8}. To quench the reaction, 50 μL of 500 units mL^{-1} catalase solution was added (Sigma, Germany).

For the infection stage, 0.1 mL of each phage dilution and at each time sampled was added to 0.3 mL of *S. typhimurium* in the exponential phase (as described previously) and incubated at 37 °C for 10 min. Then, 100 μL was spread onto predried TSA plates (in duplicate) and incubated at 37 °C for 24 h. The plaques were then counted, and an inactivation curve [labeled OP (oxidation of phage)] was consequently obtained (**Figure 1**).

Determination of Antioxidant Effect upon Phage with Oxidant. The antioxidants selected were gallic acid at 0.25, 0.50, and 1 g L^{-1} (Sigma, Germany) and ascorbic acid (99%) at 0.125, 0.250, 0.5, and 1 g L^{-1} (Sigma).

To assess the antioxidant effect of either standard compound upon the phage when in the presence of oxidant, 9 mL of 250 mM H_2O_2 was mixed with 1 mL of phage (in triplicate), which had previously been in contact with 1.5 mL of standard solution, and was left to react at room temperature for 20 min. (It was assured that the phage was mixed with the antioxidant first and only then with the oxidant.) Aliquots of 100 μL were collected every 5 min, and the reaction was quenched with 50 μL of catalase solution. Dilutions were carried out up to 10^{-8}; to each one, 300 μL of *S. typhimurium* solution in the exponential phase was added and incubated at 37 °C for 10 min. After infection, 100 μL of each standard was spread onto predried TSA plates (in duplicate) and incubated at 37 °C for 24 h. The plaques were then counted, and a curve [labeled SPO (oxidation of phage in the presence of antioxidant standard)] was consequently obtained (**Figure 2**).

Determination of Antioxidant Effect upon Phage without Oxidant. For both antioxidant model compounds, controls were duly tested. For that purpose, 9 mL of 250 mM H_2O_2 was replaced by Ringer solution, mixed with 1 mL of phage solution and 1.5 mL of standard antioxidant solution, and left to react at room temperature for 20 min. Aliquots of 100 μL were collected every 10 min, and the reaction was quenched with 50 μL of catalase solution. This suspension was then mixed with 300 μL of *S. typhimurium* solution in the exponential phase and incubated at 37 °C for 10 min. After infection, 100 μL of each mixture was spread onto predried TSA plates (in duplicate) and incubated at 37 °C for 24 h. The plaques were then counted, and a curve [labeled SP (standard antioxidant effect upon phage)] was consequently obtained (**Figure 3**).

In all experiments, a curve of phage inactivation with 250 mM H_2O_2 and the effect of the standard on the phage were carried out as controls.

Statistical Analyses. Nonparametric tests were applied to each experimental data set. Friedman and Wilcoxon tests were used to ascertain the effect of each parameter on the observed results. Kruskal−Wallis tests were used to check whether, at each time and for either standard compound, the concentration had an influence. Mann−Whitney tests were applied to analyze all possible differences, in terms of concentration of standard. These analyses were conducted using SPSS v. 16.0.0 (Chicago, IL).

RESULTS AND DISCUSSION

The major goal of this study was to assess the possibility of using a progressive phage inactivation method (via oxidation) as the starting point to set up a method to determine antioxidant activity under conditions that approach in vivo levels. This is why a system of inactivation that yields a significant, yet convenient, level of oxidative inactivation was sought. The limit of detection of the phage counts was that regularly considered for bacteria using spread plate methods; the units were taken as a datum point only from the dilution assay in which the counts ranged between 30 and 300.

The concentration of H_2O_2 was set in the range 100−250 mM, to produce inactivations of ca. 3 log cycles over a 30 min period. From inspection of **Figure 1**, one concludes that only the highest concentration tested was able to reduce 3 log (PFU mL^{-1}) during that period of exposure; such an exposure period could even be shortened to 20 min, with essentially similar results. Therefore, using 250 mM H_2O_2 and an exposure period of 20 min, the effects of the two model antioxidants, ascorbic and gallic acids, could be evaluated.

The aforementioned two compounds were chosen because they are often found in food matrices and also because of the

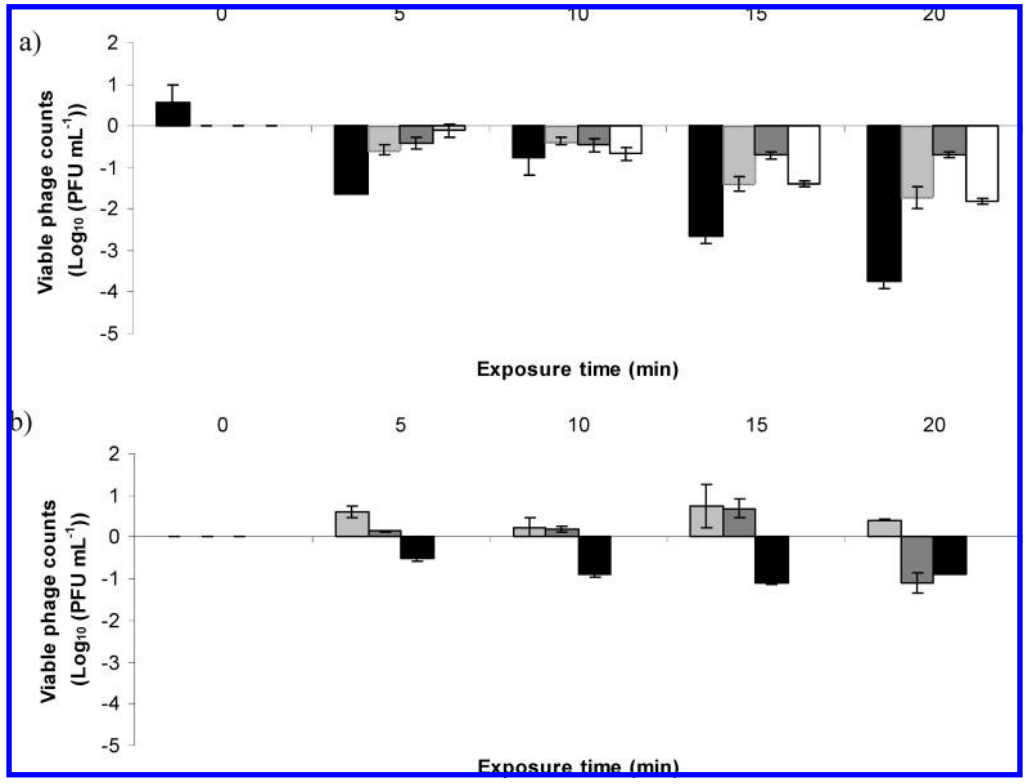

Figure 2. Evolution of the difference (average ± standard deviation) between the effect of antioxidant standard solutions, (**a**) ascorbic acid and (**b**) gallic acid, in the presence of oxidant (H_2O_2, 250 mM), and inactivation by the isolated oxidant, on the bacteriophage P22 viability, at various antioxidant concentrations: (black bars) 1000, (light gray bars) 500, (dark gray bars) 250, and (white bars) 125 mg L^{-1} (for gallic acid, the concentration of 125 mg L^{-1} was not tested because 250 mg L^{-1} was already inefficient).

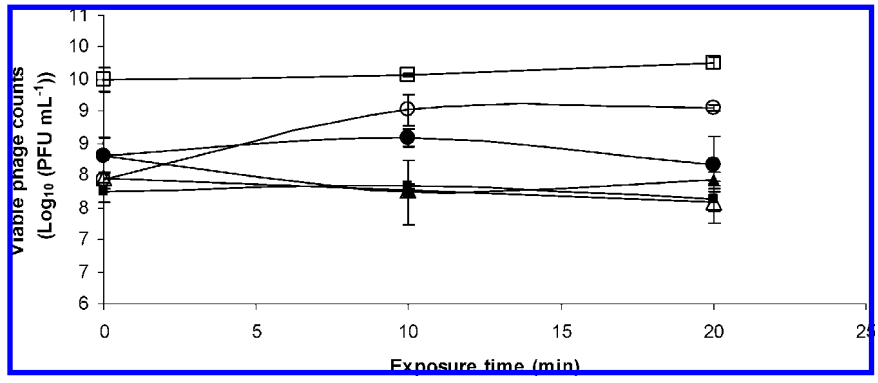

Figure 3. Evolution of the effect (average ± standard deviation) of antioxidant standard solutions, (□, △, ○) ascorbic acid and (■, ▲, ●) gallic acid, on the bacteriophage P22 viability, at various antioxidant concentrations: (□, ■) 1000, (△, ▲) 500, and (○, ●) 250 mg L^{-1}.

ease of assaying, as no interferences are anticipated. In addition, gallic acid is the most common standard for the assessment of antioxidant capacity, especially of phenolic compounds. On the other hand, the range of concentrations chosen encompassed both the normal levels encountered in plain foods (especially in the case of ascorbic acid) and the expected levels encountered in extracts (especially in the case of gallic acid). To permit adequate comparison, the difference between the concentration of viable PFU obtained with the oxidant in the presence of standard solution and that obtained with only the oxidant was calculated every 5 min: whenever this difference was positive, inhibition occurred, whereas a negative value indicated enhancement of the underlying oxidant effect. From an inspection of **Figure 2a**, one concludes that ascorbic acid did not exhibit any protective role; instead, it even increased the effect brought about by H_2O_2, especially at high concentrations. On the other hand, gallic acid (see **Figure 2b**) produced some degree of protection from inhibition by H_2O_2. The results observed pertaining to ascorbic acid can be associated with its high reactivity, as it can even be oxidized by atmospheric oxygen, hence becoming unavailable to protect the chemical structures under consideration. When present at high concentration, both acids could act as oxidants and, consequently, entertained pro-oxidant effects.

From inspection of **Figure 3**, one realizes that the effect of antioxidant concentration upon P22 is not fully consistent, especially in the case of ascorbic acid; the difference in viable counts actually goes up to 2 log units, which is close to that depicted in **Figure 2**. These apparent inconsistencies may be accounted for by different starting values of viable phage counts (in experiments that were run independently on distinct days). The aim underlying these data was to demonstrate that none of the antioxidant standards, at every concentration, caused damage (i.e., decrease in viable numbers) to the phage. In other words, antioxidant compounds considered solely (i.e., in the absence of an oxidant) did not cause damage to the phage.

One might argue the potential problems arising from permeability of, and absorption across, the bacterial cell wall, as this might impinge upon the efficacy being tested. Note that the oxidant/antioxidant system acts upon the phage, so the limitation associated with permeability would lie on the virus capsule, and no hampered crossing of said coating protein is likely on bacteria. On the other hand, the excess of oxidant was taken care of via addition of catalase. Finally, bacteria were employed to permit visualization of the damage on the phages themselves, in terms of decrease in PFU.

The high concentration of hydrogen peroxide tested (or needed) would be of concern, in terms of the biological relevance of the method, if it were to be extrapolated. The system selected is intrinsically more resistant than others commonly used; however, the experimental conditions were optimized so as to achieve a method that responds to both oxidants and antioxidants in a short period of time (20−30 min). Hence, this method is not to be extrapolated directly to eucaryotic cells; instead, it is intended for use as a complementary tool, to expand the study of the performance of antioxidant compounds in vivo. In terms of complexity, one might rank the proposed system between a test on a DNA strand and a test on a whole eucaryotic cell.

Other species might have been assessed to ascertain how antioxidants modulate their effects, so a measure of DNA damage would be in order. However, the proposed method was not tailored to look at DNA as such, but as a complementary tool to detect whether damage would be present and could eventually be prevented. Studies focusing directly on DNA damage have been reported elsewhere (*1*) and involved thymus DNA deliberately exposed to an antioxidant system, so that point was not further pursued here.

From a statistical point of view, the data did not obey the normality assumption, so nonparametric tests had to be applied. The Friedman test showed that time influenced the results ($p < 0.001$), whereas the Wilcoxon test allowed three different groups to be pinpointed: 0 and 5 min; 10 and 15 min; and 20 min. Afterward, one proceeded to ascertain whether, at each time, the various concentrations considered (irrespective of the standard at stake) had an influence on the results produced. The Kruskal−Wallis test was accordingly applied, and one thus concluded that concentration had a significant impact only at 5 min ($p < 0.022$). The Mann−Whitney test permitted one, in turn, to derive that 1000 mg L^{-1} was the concentration (for either standard) that accounted for the differences observed at 5 min. The Mann−Whitney test was applied once again to all times and indicated that, except for time 0, the standard under consideration had an influence. Because the concentration of 1000 mg L^{-1} led to a unique behavior, the Mann−Whitney was then applied to the whole time frame to finally prove that there was a difference only at 5 min.

Therefore, the nature of the standard, rather than its concentration, appeared to have a major effect on the experimental results. However, the existence of a concentration dependence cannot be fully ruled out: an unequivocal tendency for an increase in inhibition without increase in gallic acid concentration was indeed observed between 250 and 500 mg L^{-1}: the concentration of 250 mg L^{-1} appeared to be inefficient, when the time of exposure to H_2O_2 was above 15 min. Nevertheless, 250 mM H_2O_2 is considerably high compared with the usual test range in biological systems, which suggests that this specific system is particularly resistant to oxidation.

Because some antioxidant solutions may exhibit inhibitory properties even in the absence of an oxidant, our study included also an evaluation of whether the solution by itself had any negative effect upon the phage. From the corresponding data (**Figure 3**), one can easily conclude that the solutions per se could not inactivate the phage. Because neither ascorbic nor gallic acid is virucidal (at least at the concentrations studied), the inactivation effect observed was thus attributable only to H_2O_2.

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LITERATURE CITED

(1) Gião, M. S.; González-Sanjosé, M. L.; Muñiz, P.; Rivero-Pérez, M. D.; Kosinska, M.; Pintado, M. E.; Malcata, F. X. Protection of deoxyribose and DNA from degradation, by aqueous extracts of several wild plants. *J. Sci. Food Agric.* **2008**, *88*, 633–640.

(2) Gião, M. S.; González-Sanjosé, M. L.; Rivero-Pérez, M. D.; Pereira, C. I.; Pintado, M. E.; Malcata, F. X. Infusions of Portuguese medicinal plants: dependence of final antioxidant capacity and phenolic content on extraction features. *J. Sci. Food Agric.* **2007**, *87*, 2638–2647.

(3) Halliwell, B.; Aruoma, O. I. DNA damage by oxygen-derived species. *FEBS Lett.* **1991**, *281*, 9–19.

(4) Jornot, L.; Petersen, H.; Junod, A. F. Hydrogen peroxide-induced DNA damage is independent of nuclear calcium but dependent on redox-active ions. *Biochem. J.* **1998**, *335*, 85–94.

(5) Hanlon, G. W. Bacteriophages: an appraisal of their role in the treatment of bacterial infections. *Int. J. Antimicrob. Agents* **2007**, *30*, 118–128.

(6) Susskind, M.; Botstein, D. Molecular genetics of bacteriophage P22. *Microbiol. Rev.* **1978**, *42*, 385–413.

(7) Platt, R.; Reynolds, D. L.; Phillips, G. J. Development of a novel method of lytic phage delivery by use of a bacteriophage P22 site-specific recombination system. *FEMS Microbiol. Lett.* **2003**, *223*, 259–265.

(8) Siqueira, R. S.; Dodd, C. E. R.; Rees, C. E. D. Evaluation of the natural virucidal activity of teas for use in the phage amplification assay. *Int. J. Food Microbiol.* **2006**, *111*, 259–262.

(9) Demple, B.; Halbroook, J. Inducible repair of oxidative DNA damage in *Escherichia coli*. *Nature* **1983**, *304*, 466–468.

(10) Shen, L.; Ji, H. F.; Zhang, H. Y. How to understand the dichotomy of antioxidants. *Biochem. Biophys. Res. Commun.* **2007**, *362*, 543–545.

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