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New Cell-Based Assay Indicates Dependence of Antioxidant Biological Activity on the Origin of Reactive Oxygen Species

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ABSTRACT: The mobility of the Ty1 transposon in *Saccharomyces cerevisiae* was found to vary proportionally with the level of ROS generated in cells, which provides the possibility to determine antioxidant activity by changes in a cellular process instead of using chemical reactions. The study of propolis, royal jelly, and honey with the newly developed Ty1antiROS test reveals an inverse exponential dependence of antioxidant activity on increased concentrations. This dependence can be transformed to proportional by changing the source of ROS: instead of cell-produced to applied as hydrogen peroxide. The different test responses are not due to excess of added hydrogen peroxide, as evidenced by the exponential dependence found by usage of *yap1*Δ tester cells accumulating cell-generated ROS. Results indicate that the activity of antioxidants to oxidative radicals depends on the origin of ROS, and this activity is elevated for cell-generated ROS compared to ROS added as reagents in the assay.

KEYWORDS: *Ty1antiROS test, Ty1 transposition, exponential, proportional test response*

INTRODUCTION

Reactive oxygen species (ROS) including superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (HO^-), and singlet oxygen ($^1O_2$) are oxygen-derived products, and their accumulation in cells can lead to oxidative stress associated with chronic infections, cardio-vascular disease, cancer, Alzheimer's disease, and age-related functional decline.[1,2] Cells possess protective systems of different antioxidants including enzymes, glutathione, and vitamins. Fruits, vegetables, whole grains, and honey bee products contain a variety of compounds with antioxidant activity, and epidemiological studies evidenced that their regular consumption is associated with a reduced risk of developing cancer and cardiovascular disease.[3]

In order to be biologically meaningful, antioxidant compounds must be able to either (1) help protect an organism along the body surface (external or internal surfaces), (2) enter into the bloodstream and help reduce the level of free radical damage, or (3) enter into living cells and protect the cellular interior from free radical damage. Recently, it has been recognized that the mechanisms of action of antioxidants in living cells go beyond the activity to scavenge free radicals or to activate antioxidative enzymes[4] and can have effects on regulation of gene expression, carcinogenesis, modulation of enzyme activity and signal transduction pathways, and stimulation of the immune system.[5]

The increasing interest in the role of free radicals in the pathogenesis of human diseases and the benefits of consumption of foods with antioxidant properties has led to an increased necessity to develop new techniques and testing systems to measure antioxidants in vitro and in vivo. Considering the biological effect of antioxidants, these methods have to take into account the main characteristics and complex nature of antioxidants' action. The first major problem that has to be considered in this field is that free radicals are extremely reactive and consequently short-lived. Any oxidative radical produced in the cell reacts at or close to its source of formation,[6] making unreliable the estimation of antioxidants in cellular lysates or extracts. Another question that need to be addressed in determining the biological effects of antioxidants is their bioavailability and metabolism.[5,7] In some cases the original antioxidant compounds may be metabolized such that original chemical structures never actually reach the target, whereas their metabolites may do so. It becomes crucial to have detailed knowledge on exactly which compound can accumulate in the target places and how active is it. Consequently, antioxidant activity needs to be assayed by methods taking into account the ability of antioxidants to penetrate cells and their availability on target subcellular structures in active form necessary to neutralize oxidative radicals shortly after their generation. Few of the existing tests for determination of antioxidant activity reach these criteria.

In vitro assays for measuring antioxidant activity are based on chemical reactions resulting from direct interactions between antioxidants and oxidative radicals.[4,7] Linear dose dependence between antioxidant concentration and activity has been found in the test tube assays. Most in vitro tests are designed to measure one specific ROS, and none of the existing assays truly reflect the total antioxidant capacity of a particular sample. For this purpose, the usage of batteries of in vitro tests has been recommended.[7] Although these chemical in vitro antioxidant assays are easy, fast, cheap, and suitable for measuring antioxidant properties of food and dietary supplements, they are conducted under nonphysiological conditions and the obtained results cannot be extrapolated to the in vivo situation.

An alternative is given by in vivo tests with animal models and human studies, which are of extreme importance because they detect the effect an antioxidant can have on a whole organism. Antioxidant-containing food is consumed during

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these trails, and the antioxidant status is studied in blood or tissue samples using in vitro assays.[8,9] Assessment of the relative contribution of individual antioxidants to the total antioxidant capacity requires separate specific assays. The significant advantages of in vivo assays are that they estimate not only the penetration of antioxidants into cells but also the passage of antioxidants through the intestinal tract and their circulation in blood vessels in an active form. Unfortunately, the in vivo assays are time-consuming, very expensive, and not applicable to study a large number of samples. Therefore, there is a need for cell-based test systems to allow trustworthy and relevant antioxidant research prior to animal studies and human clinical trials.

Cell-based tests represent assays in which an in vitro reaction between oxidative radicals and antioxidants is carried out in live cells. Different cellular models have been published that made use of various cells in culture[5,10,11] or human blood cells,[12] Basically, host cells are saturated with the antioxidant-containing sample by incubation in the corresponding cell-culture media and washed, and the intracellular ROS level is increased by addition of H_2O_2. Hydrogen peroxide readily crosses cell membranes through the aquaporins,[13] and numerous studies[14] evidenced a dose-dependent increase of ROS following exposure of cells to exogeneously added H_2O_2. In the different cell-based assays concentrations of H_2O_2 between 1 mM[11] and 167 mM[12] were used to achive the necessary initial level of ROS in cells. Some of these concentrations are unphysiological and toxic for cells,[15] and their impact on the results obtained will be considered in the Discussion section. The antioxidant in the test product neutralizes part of the H_2O_2, and the remaining peroxides can be measured. Where it was studied, a linear dependence of antioxidant activity on concentration was found, indicating stoichiometric interactions between oxidants and antioxidants in chemical reactions.[11,12] The main advantages of cell-based assays are that they directly measure the penetration of the studied antioxidant into cells and the ability of the original compound or its metabolites to neutralize oxidative radicals inside the living cells. In a recent publication[12] the comparison of in vitro and cell-based antioxidant methods clearly showed the advantages of cell-based assays as a more relevant way to study biological systems.

In this article we demonstrate that the mobility of the Ty1 transposon in *S. cerevisiae* cells is activated proportionally to the level of ROS generated in the cells, thus providing the possibility to measure antioxidant activity by estimation of the decrease of the Ty1 transposition rate. Contrary to all other in vitro and most cell-based assays using chemical reactions, the newly developed Ty1antiROS test determines quantitatively the activity of antioxidants using a cellular process, the Ty1 transposition. We used the Ty1antiROS test to study antioxidant properties of honey bee products and found that the activity of antioxidants to oxidative radicals depends on the origin of the ROS. This activity is clearly elevated for cell-generated ROS compared to ROS added as reagents in the assay.

MATERIALS AND METHODS

Materials and Chemicals. Water solutions of honey bee products (origin Bulgaria) were used since it has been shown that the water-soluble fractions contain all antioxidant compounds and exhibit higher antioxidant activity compared to ethanol extracts.[16] Commercially available honey, propolis, and royal jelly were obtained from the Bulgarian Association of Honey Producers. Honey and royal jelly were dissolved in sterile water, while propolis was first treated with dimethylsulfoxide for 10 min and then diluted with 9 parts of the initial volume of sterile water. Stock solutions were filter sterilized and kept at +4 °C until used.

All carcinogens, including hexavalent chromium (as CrO_3), disodium hydrogen arsenate (Na_2HAsO_4), *N*-acetyl-L-cysteine, and hydrogen peroxide, were from Sigma Aldrich (Germany). The S9 metabolite mix was from Microbiological Associates (Rockville, MD, USA). The components for the nutritional media used to cultivate yeast cells were from Difco Chem. Co. (USA).

Strains and Cultivation Procedures. The *Saccharomyces cerevisiae* 551 strain with genotype *MATα ura3-167 his3Δ200:TymHI-S3AI sec53 rho*$^+$ (National Bank for Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria, Cat. No. 8719) was used as a tester strain in the Ty1antiROS assay. This strain is a derivative of *S. cerevisiae* DG1141[17] and has a Ty1 element marked with the indicator gene *HIS3AI*, which allows the determination of Ty1 transposition in the genome as a whole. The *SEC53* gene in *S. cerevisiae* 551 has been replaced with a *sec53* mutation, which destroys the barrier function of the cell wall, and evidence for a generally increased permeability of 551 cells was presented.[18] The *rho*$^-$ mutants of *S. cerevisiae* 551 were obtained by ethidium bromide treatment.[19] The isogenic strain *S. cerevisiae* 551 *yap1*Δ has a disrupted *YAP1* gene and was obtained by integrative transformation of 551 cells with the *yap1::hisG-URA3-hisG* cassette.[20]

Strains were cultivated at 30 °C on a rotary water bath shaker in YEPD liquid rich medium (1% bacto yeast extract, 2% bactopeptone, 2% dextrose, pH = 6.8) to exponential phase of growth corresponding to (5−7) × 10^7 cells/mL and then used in the experiments. The media for cultivation of yeast cells have been made according to published protocols.[19]

Determination of Ty1 Transposition Rate. The Ty1 transposition test was performed with the *S. cerevisiae* 551 strain as described[18] and used to study the dependence between activation of Ty1 transposition and increase of ROS level. Briefly, experimental cells treated for 30 min with carcinogen to induce the mobility of Ty1 transposon were collected, suspended in fresh YEPD medium, and cultivated at 20 °C for 16 h to complete the initiated transposition events. When 551 *rho*$^-$ mutants were used as tester cells, the treatment with carcinogen was omitted and 0.5 mM H_2O_2 (final concentration) was added for a period of 30 min. Appropriate dilutions of cells were plated to determine survivals (on YEPD) and number of His$^+$ transposants (on SC-His). Ten SC-His plates for each concentration of carcinogen were plated with suspension aliquots corresponding to a total of about 10^8 cells surviving the procedure. The number of His$^+$ transposants counted on the SC-His plates was in the range 100−600 in samples trated with different concentrations of carcinogen and about 20 in the control untreated samples, indicating the background level of spontaneous Ty1 transposition. Each transposition event of the marked Ty1 in the tester strains gives rise to one histidine prototrophic colony on selective medium, and the number of His$^+$ transposants is a quantitative measure for the frequency of transposition of the marked Ty1 transposon.[17] Median transposition rates were determined, and results are presented as fold increase of Ty1 transposition rate related to the control sample taken as a fold increase of 1.0. When metabolic activation was needed to convert precarcinogens in their active forms, the S9 mix was added to samples 60 min before treatment with genotoxins.

Quantitative Assay for Superoxide Anions. We used an assay for superoxide anion determination as adapted for *S. cerevisiae* cells.[21] The assay is based on reduction of the tetrazolium dye XTT (2,3-bis(2-methoxynitro-5-sulfophenyl)-5-[(phenylaminocarbonyl]-2*H*-tetrazolium hydroxide). XTT is taken up only by living cells, where it is reduced by $O_2^{\bullet-}$ to water-soluble orange-colored formazans. A molar extinction coefficient of 2.16 × 10^4 M^{-1} s^{-1} for XTT at 470 nm has been estimated, which allows determination of the quantity of $O_2^{\bullet-}$ per one live cell. The superoxide anions assay was performed immediately after the treatment with carcinogens and before the cultivation of cells at 20 °C in the transposition test. When necessary,

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ROS were scavenged by addition of *N*-acetyl-L-cysteine (60 mM final concentration) to the cell suspension 60 min before the treatment with carcinogen. Results obtained are presented as pM $O_2^{\bullet-}$/cell ± SD.

Ty1antiROS Test for Determination of Antioxidant Activity. The Ty1antiROS assay is based on the inhibition by antioxidants of the Ty1 transposition process induced proportionally to the level of ROS generated by treatment of tester cells with carcinogens. A typical protocol of the Ty1antiROS test is given below.

S. cerevisiae 551 tester cells were grown at 30 °C in YEPD liquid medium to a density corresponding to (5−7) × 10^7 cells/mL.

The culture is divided into 4 mL aliquots, and growing cells are treated for 60 min at 30 °C with increasing concentrations of the substance studied for antioxidant activity. Water-insoluble substances are dissolved in dimethylsulfoxide or ethanol and used in volumes not exceeding a final concentration of 5% of the assay volume. The control samples are treated with the same volume of the solvent.

Cells are washed by centrifugation, suspended in the same volume of fresh YEPD medium containing the inducer of Ty1 transposition, and cultivated for 30 min at 30 °C in a water bath shaker. Hexavalent chromium (CrVI) at a final concentration of 5 mM is the preferred inducer of Ty1 transposition because it is a direct carcinogen, has a high ROS generating capacity (e.g., is a powerful inducer of Ty1 mobility), and gives a good reproducibility of results within run and between different days. Very similar results have been obtained by usage of other inducers of Ty1 transposition, such as 12 mM methylmethansulfonate (MMS) or 0.16 mM benzo(a)pyrene (B(a)P).

Cells are collected by centrifugation, suspended in the same volume of fresh YEPD medium, and cultivated at 20 °C to determine the Ty1 transposition rate (see "Determination of Ty1 Transposition Rate").

Data obtained for Ty1 transposition rates are plotted against the concentrations of the studied antioxidant, and the IC_{50} value is calculated. The IC_{50} value is the amount (mkg/mL, mg/mL for extracts or μM/mL, mM/mL for purified substances) inhibiting 50% of the Ty1 transposition rate in the control sample.

The IC_{50} value measurement by this protocol is relevant for final concentrations of CrVI, MMS, and B(a)P at levels of 5, 12, and 0.16 mM, respectively. These concentrations of carcinogens induced a fold increase of Ty1 transposition rate equal to 10.0 ± 1.0. When usage of other carcinogens or concentrations is required, the obtained IC_{50} values change according to the Ty1 fold increase in the control samples. In such cases results are related to the IC_{50} values obtained at a Ty1 fold increase of 10.0 using the formula

$$IC_{50} = \frac{10}{A} \times IC^{A}_{50}$$

where "*A*" is the Ty1 fold increase in the control sample of the experiment with a Ty1 fold increase difference of 10, "IC^{A}_{50}" is the corresponding IC_{50} value, obtained in the same experiment, and "IC_{50}" is the calculated value, related to a Ty1 fold increase equal to 10.0.

Statistical Analysis. All results were presented as mean ± SD from 4 to 10 independent experiments. Comparisons between two means were performed using unpaired Student's *t* tests. The 0.05 probability level was chosen as the point of statistical significance throughout.

RESULTS

Proportional Dependence between Activation of Ty1 Transposition and Increase of ROS Level in *S. cerevisiae*. Previously, it was found that ROS have an independent and key role in the induction of Ty1 transposition.[20] Here we extended this study by determination of the dependence between ROS level and Ty1 transposition rate to cells treated with different concentrations of carcinogens. Table 1 and Figure 1 show the results obtained with B(a)P, CrVI, and MMS. The increase of the carcinogen's concentrations was associated with an increase of both superoxide anion level and Ty1 transposition rate. A linear dependence exists between enhanced $O_2^{\bullet-}$ and Ty1 transposition. The similarity of $O_2^{\bullet-}$ levels necessary to achieve a certain fold increase of Ty1 transposition indicates that a specific activation of Ty1 transposition rate is due to a specific level of ROS and is not dependent on the chemical structure of the carcinogens. The different carcinogens are not equally powerful ROS generators. For instance, a 10-fold increase of Ty1 transposition rate was achieved at $O_2^{\bullet-}$ levels in the range 0.9−1.0 pM/cell generated by 0.16 mM B(a)P, 5 mM CrVI, or 12 mM MMS. Results similar to those shown in Table 1 have also been obtained with other carcinogens, such as dichloromethane, tetrahydrofuran, and arsenic (data not shown), indicating that the proportional dependence between the activation of Ty1 transposition and the increase of ROS level is a property common to *S. cerevisiae* cells treated with different carcinogens. The finding that Ty1 transposition rate is proportional to the level of ROS generated in *S. cerevisiae*

Table 1. Activation of Ty1 Transposition Is Proportional to Superoxide Level

carcinogen[a] (mM)	superoxide anions[b] (pM/cell)	Ty1 transposition rate[b] (fold increase)
control	0.05 ± 0.01	1.00
B(a)P		
0.08	0.48 ± 0.04	5.2 ± 0.4
0.12	0.90 ± 0.08	8.1 ± 1.0
0.16	1.02 ± 0.13	10.5 ± 1.1
0.24	1.59 ± 0.14	18.2 ± 1.4
0.32	2.45 ± 0.19	25.6 ± 1.9
CrVI		
2	0.56 ± 0.04	5.5 ± 0.5
4	0.80 ± 0.09	7.9 ± 0.9
5	0.94 ± 0.11	10.2 ± 1.2
8	1.55 ± 0.15	16.0 ± 1.9
10	2.20 ± 0.22	23.3 ± 2.5
MMS		
4	0.40 ± 0.04	3.2 ± 0.6
8	0.76 ± 0.07	7.7 ± 0.8
12	1.08 ± 0.14	10.3 ± 1.5
24	2.11 ± 0.22	22.1 ± 1.9
32	2.92 ± 0.31	30.0 ± 2.9

[a]B(a)P, benzo(a)pyrene; CrVI, hexavalent cromium; MMS, methylmethanesulfonate. Experiments with B(a)P were conducted in the presence of S9 mix. [b]Mean ± SD values of 5 experiments ($p < 0.005$).

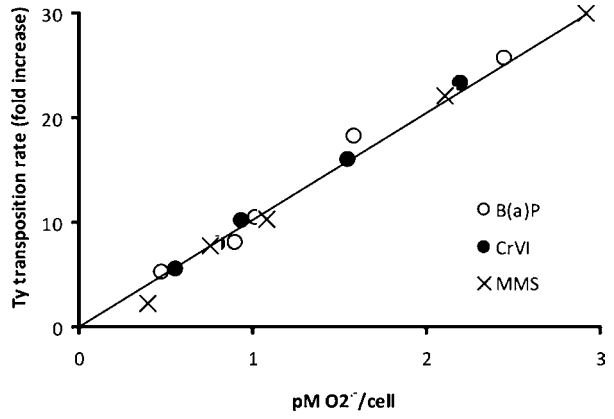

Figure 1. Proportional dependence between Ty1 transposition rate and superoxide anion level. Ty1 transposition rate and $O_2^{\bullet-}$ level were determined in *S. cerevisiae* 551 cells treated for 30 min with increasing concentrations (see Table 1) of hexavalent chromium (−●−), methylmethansulfonate (−×−), or benzo(a)pyrene (−○−).

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cells was used to develop the Ty1antiROS method for determination of antioxidant activity based on the inhibition of the Ty1 transposition process by the studied antioxidant (see Materials and Methods).

Antioxidant Capacity of Honey Bee Products Determined with the Ty1antiROS Test. Antioxidant activity has been found in all honey bee products.[11,22] Most studies have been performed by using propolis (in vitro and cell-based tests) and honey (in vitro and in vivo tests). Royal jelly has been rarely studied, and a weak antioxidant activity was reported using in vitro methods,[23] while subsequent studies with a cell-based assay[11] showed the absence of antioxidant activity. The antioxidant capacities of honey bee products, especially of honey, have been recently reviewed.[24,25]

We studied propolis, royal jelly, and honey with the Ty1antiROS test, and the results obtained for propolis are shown in Figure 2. *S. cerevisiae* cells were treated with increased concentrations of propolis, ROS were generated by treatment with CrVI, and the rate of Ty1 transposition was determined. The inhibition of Ty1 transposition rate with an increase in the concentrations of propolis follows a curve (Figure 2a), which can be transformed into a straight line by changing the scale for Ty1 transposition rate to a logaritmic one (Figure 2b). The Ty1 transposition decreased log linearly in the range 0−2 mkg/mL propolis. Higher concentrations of propolis possessed a total inhibiting activity of the Ty1 transposition process, indicating a complete scavenging of ROS in the cells. This was confirmed by measurements of $O_2^{\bullet-}$: While control (0 mkg/mL propolis) samples showed 1.050 ± 0.110 pM $O_2^{\bullet-}$/cell, the samples treated with 2 mkg/mL propolis showed only 0.035 ± 0.011 pM $O_2^{\bullet-}$/cell, which is similar to the basal level of 0.048 ± 0.005 pM $O_2^{\bullet-}$/cell in *S. cerevisiae*. The study of royal jelly and honey in the Ty1antiROS test also gave a dependence of activity on concentration manifested by an exponentially declining curve (data not shown), and the calculated IC_{50} values showed the following rank order for antioxidant activity: propolis > royal jelly > honey (Table 2). Almost equal IC_{50} values were found when MMS was used, instead of CrVI, demonstrating that the usage of different carcinogens as ROS generators did not influence the results obtained with the Ty1antiROS assay.

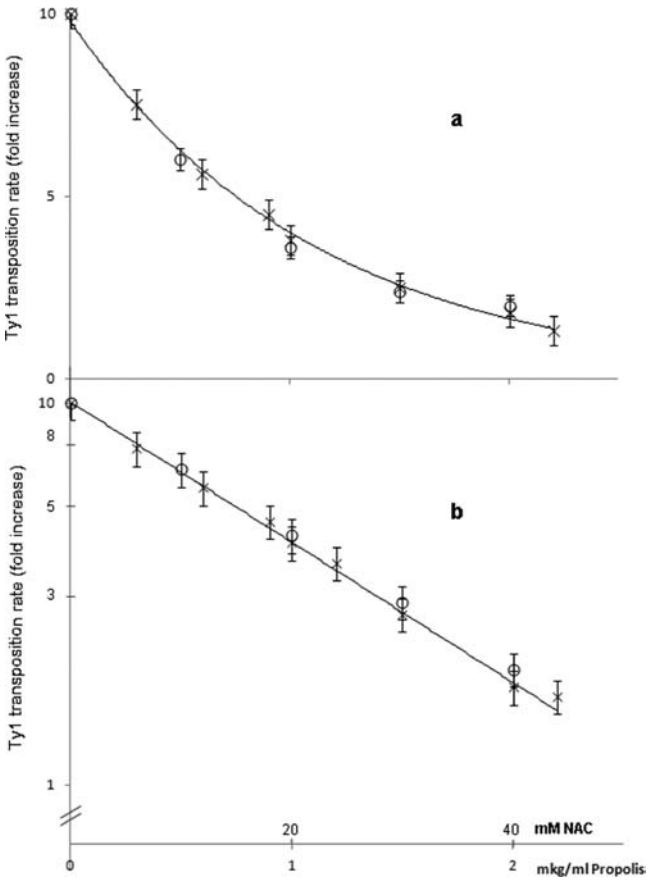

Figure 2. Ty1antiROS test of propolis. *S. cerevisiae* 551 cells were treated with increasing concentrations of propolis (−×−) or NAC (−○−), ROS level was induced with CrVI, and Ty1 transposition rate (fold increase) was determined. The linear dependence curve of Ty1 fold increase on concentration of propolis or NAC (a) is transformed to a straight line (b) after changing the scale for Ty1 fold increase to a logarithmic one. Calculated IC_{50} values are 0.7 mkg/mL for propolis and 14 mM for NAC. Means ± SD from 8 experiments ($p < 0.05$).

Table 2. IC_{50} Values of Honey Bee Products in Ty1antiROS Test[a]

product	IC_{50} (mkg/mL)	
	CrVI	MMS
propolis	0.7 ± 0.1	0.7 ± 0.2
royal jelly	10.0 ± 1.3	9.5 ± 2.0
honey	250.0 ± 15.0	255.0 ± 22.2

[a]CrVI (5 mM) or MMS (16 mM) was used as ROS generator in the Ty1antiROS test. Mean ± SD values of 10 experiments ($p < 0.005$).

The exponentially decreased rate of Ty1 transposition to increased concentrations suggested higher antioxidant activity in the product studied by the Ty1antiROS test compared to the proportional dependence that has been found with most of the other cell-based assays. This higher activity may be due to the complex nature of the studied honey bee products, which are extremely rich in sugars, vitamins, proteins, and other bioactive molecules. The control experiment however made with the superoxide anion scavenger *N*-acetyl-L-cysteine (used instead of propolis) showed also a log linear dependence (Figure 2) with a calculated IC_{50} of 14 mM. This result evidenced that the observed exponential decrease of Ty1 transposition is due to the antioxidant activity of the studied honey bee products.

Together, the results obtained in the Ty1antiROS test proved an inverse exponential dependence of antioxidant activity on concentration, which suggests high intracellular activities of antioxidants when studied with the Ty1antiROS test. This high activity may be due to the cellular mechanism of the Ty1antiROS test, which has been studied in some detail.

Cellular Mechanism of the Ty1antiROS Assay. The main difference between the Ty1antiROS test and the other cell-based assays is the way ROS levels are increased in tester cells. In our assay ROS production is induced in tester cells, while in most other tests H_2O_2, added to the test system as an assay reagent, penetrates tester cells and increases intracellular ROS level. We have studied the different responses that antioxidants may have to the ROS produced by the tested cells and to exogeneously added H_2O_2.

More than 95% of ROS in *S. cerevisiae* cells are produced by leakage of electrons along the mitochondrial oxidative phosphorylation chain. The *rho*$^-$ mutants, representing large deletions of mitochondrial DNA genes involved in oxidative phosphorylation, cannot generate oxidative radicals, and *rho*$^-$

cells are devoid of ROS.[26] We isolated *rho*$^-$ mutants of the *S. cerevisiae*551 strain and used them as testers in the Ty1antiROS assay. Since *rho*$^-$ cells cannot produce ROS, the treatment with carcinogen in the assay was omitted and H_2O_2 was added to supply the missing ROS in tester cells. In preliminary experiments we found that treatment of *S. cerevisiae* 551 *rho*$^-$ cells with 0.5 mM H_2O_2 induced a 10-fold increase of Ty1 transposition rate, which is similar to the values found by treatment with CrVI (5 mM) or MMS (12 mM). We conducted the Ty1antiROS test to measure antioxidant activity of propolis and royal jelly using *S. cerevisiae* 551 *rho*$^-$ cells as testers and raised the level of ROS by addition of H_2O_2 at a final concentration of 0.5 mM instead of inducing ROS generation by treatment with a carcinogen. The results obtained (Figure 3) showed a gradual decline of Ty1

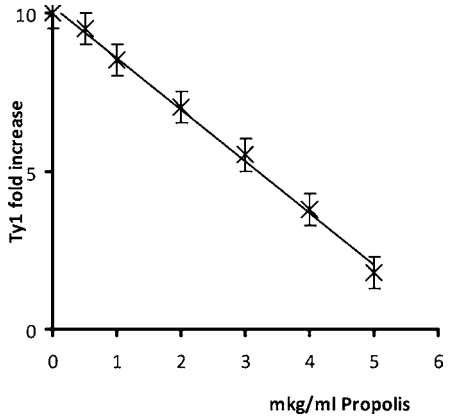

Figure 3. Ty1antiROS test of propolis with *S. cerevisiae* 551 rho$^-$ tester strain. Cells were treated with increasing concentrations of propolis, hydrogen peroxide (0.5 mM final concentration) was added, and Ty1 transposition rate (fold increase) was determined. Calculated IC_{50} = 3.3 mkg/mL propolis. Means ± SD of 5 experiments ($p < 0.05$).

transposition rate with increasing concentration of the honey bee product. The antioxidant activity was proportional to the concentration of studied products and is manifested with a straight line.

The calculated IC_{50} values were 3.30 ± 0.78 mkg/mL for propolis and 55.6 ± 3.5 mkg/mL for royal jelly, which are about 5-fold higher than the IC_{50} values obtained for the same samples in the Ty1antiROS assay conducted with cell-generated ROS (Table 2). This result indicated that in the Ty1antiROS test the change of ROS origin from cell-produced to added as a reagent is associated with a change in the activity of the studied antioxidants toward a less effective neutralization of oxidative radicals and consequently to higher IC_{50} values.

During the adaptation of the Ty1antiROS assay to the *rho*$^-$ tester cells we noticed that the addition of H_2O_2 at a final concentration of 0.5 mM necessary to achieve a 10-fold increase of Ty1 transposition rate corresponded to an intracellular $O_2^{\bullet-}$ level of 3.421 ± 0.822 pM/cell. This $O_2^{\bullet-}$ value is significantly higher compared to $O_2^{\bullet-}$ levels of about 1.00 pM/cell generated after treatment of tester *rho*$^+$ cells with carcinogens (Table 1) in order to induce a 10-fold increase of Ty1 transposition rate. We considered the possibility that an intracellular ROS excess in the case of H_2O_2 addition may contribute to the Ty1antiROS test response. To study this possibility, we created conditions in which tester cells accumulate high amounts of ROS that are cell-generated.

S. cerevisiae has been shown to have distinct protective oxidative stress responses to superoxides and peroxides.[27] The *YAP1* gene encodes a transcription factor that binds to AP-1 sites in promoters of target genes involved in the defense response against H_2O_2.[28] Mutants with deletions for the *YAP1* gene (*yap1*Δ) accumulate ROS in the cells due to the absence of an active detoxifying system. We used a strain with a deleted *YAP1* gene (*S. cerevisiae* 551 *yap1*Δ) as tester in the Ty1antiROS assay to study the activity of antioxidants from propolis in cells with accumulated cell-generated ROS. Since *S. cerevisiae* 551 *yap1*Δ showed increased sensitivity to treatment with ROS generators, MMS at a low concentration of 4 mM was used to induce high-level ROS production in the Ty1antiROS test. The *S. cerevisiae* 551 *yap1*Δ cells treated for 30 min with MMS (4 mM) increased Ty1 mobility 27-fold, and propolis applied at increasing concentrations inhibited the Ty1 transposition rate in these cells with a log dependence on concentration (Figure 4), as found in the test performed with

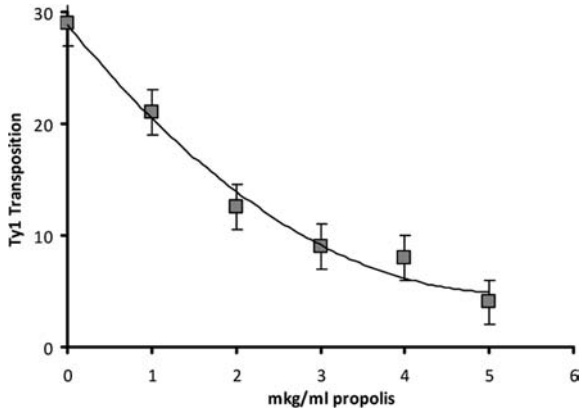

Figure 4. Ty1antiROS test of propolis with *S. cerevisiae* 551 *yap1*Δ strain. *S. cerevisiae* 551 *yap1*Δ cells were treated with increasing concentrations of propolis, the ROS level was induced with MMS (4 mM final concentration), and Ty1 transposition rate (fold increase) was determined. Means ± SD of 4 experiments ($p < 0.05$).

the original tester strain *S. cerevisiae* 551 (Figure 2). These results suggested that the excess of ROS is not the major reason for the different Ty1antiROS test responses if oxidative radicals are produced in tester cells. When ROS are cell-generated, antioxidants are more effective in neutralizing oxidative radicals, which is demonstrated by an exponential dependence of antioxidant activity on concentration. The effectiveness of antioxidants seems lower if ROS are accumulated in tester cells following an exposure to extracellularly added H_2O_2.

■ DISCUSSION

One of the major reasons for the dramatic expansion of antioxidant research since the mid 1990s was the accumulation of data evidencing a causal link between oxidative stress and carcinogenesis.[2,29] It was found that carcinogens are powerful generators of ROS in different cells,[30,31] including *S. cerevisiae*.[32,33] One of the effects of carcinogen-increased ROS level in *S. cerevisiae* is the activation of Ty1 transposon mobility,[20] and in this communication we provide evidence for a proportional dependence between the carcinogen-induced ROS level and the Ty1 transposition rate. This observation promoted the development of a new yeast cell-based assay for quantitative measurement of antioxidant activity. The Ty1an-

tiROS test is based on the reduction by antioxidants of the Ty1 transposition rate induced by treatment of tester cells with a carcinogen. The study of several antioxidant's concentrations gives a dependence of antioxidant activity on concentration and the possibility to calculate the IC_{50} value for the studied antioxidant. The IC_{50} value corresponds to a 50% inhibition of ROS level measured in the Ty1antiROS test as a 50% inhibition of the Ty1 transposition rate. The principal difference between the Ty1antiROS test and the other cell-based assays is that the antioxidant activity is determined using a cellular process and not a chemical reaction. The Ty1 transposition is quantitatively measured by the number of colonies on selective medium related to the total number of survived cells, assuring that antioxidant activity is determined within live cells. This characteristic supposed that the measurement of antioxidant activity with the Ty1antiROS test means determination of the effect antioxidants have only on oxidative radicals that are physiologically active and able to trigger cellular processes, such as the Ty1 transposition process. In most of the other cell-based assays the antioxidant activity is determined on all available oxidative species in tester cells irrespective of their physiological state and by means of chemical reactions.

The study of several honey bee products with the Ty1antiROS test reveals an inverse exponential dependence of antioxidant activity on concentration, which can be transformed to a proportional dependence by changing the origin of ROS from cell-generated to externally applied as an assay's reagent. An exponential dependence designates a higher antioxidant activity compared to a proportional dependence, and IC_{50} values differ about 5-fold between the two kinds of determinations. The only parameter that has been changed between the two trials is the origin of ROS, and the different responses of the Ty1antiROS test most likely are due to the different ROS: cell-generated or added as a chemical. The simplest explanation of this observation would be that in the cell antioxidants differentiate the two kinds of ROS, being more effective for cell-generated oxidative species. However, data for the existence of different interactions in vivo between antioxidants and oxidative radicals are not currently available, and until firm data are obtained, this explanation remains completely hypothetical.

Linear[34] and nonlinear[35] but not exponential responses of antioxidant activity to antioxidant concentration have been found with other cell-based tests in the study of different natural products including honey bee products. A proportional dependence of antioxidant activity on the concentration of honey bee products was also found with the Ty1antiROS assay when tester cells were depleted of ROS (*rho*$^-$ tester, Figure 3) and oxidative radicals were added in the form of H_2O_2. These assay conditions are very similar to those in other tests where extracellular addition of H_2O_2 is used to achieve an intracellular ROS level high enough to study antioxidant activity of different foods or components.[11,12] The concentrations of H_2O_2 required for sufficient initial ROS level in the different cell-based tests are high, unphysiological, and toxic for cells.[15] The high levels of H_2O_2 cause rapid autoinactivation of catalase by conversion the active enzyme−H_2O_2 complex I to the inactive complex II,[36,37] followed by insufficient decay of H_2O_2. Some of the oxidative radicals such as $O_2^{\bullet-}$ cannot cross cell membranes, remain inside cells,[38] and accumulate ROS. Contrary to what is widely believed, recent studies[39] show that in *S. cerevisiae* cells H_2O_2 does not freely defuse across biomembranes and the permeability of the plasma membrane to peroxides is limited. In such a case the removal of H_2O_2 introdiced into cells by addition to the assay mix is decreased and peroxides will also accumulate in cells. As a result, artificially high levels of intracellular ROS due to the extracellular addition of H_2O_2 and not to cell-generated ROS have to be detoxified by the studied antioxidants, which results in higher IC_{50} values, about 5-fold higher in the case of the Ty1antiROS test, performed with a *rho*$^-$ tester strain. The error in determination of antioxidant activity would be even greater in assays using cell cultures. It has been found that antioxidants, mainly polyphenolic compounds, interact with commonly used cell-culture media components to generate high levels of H_2O_2, which account for some of the reported effects.[10] Since honey bee products contain polyphenolic compounds as main antioxidants,[22,25] we checked if the propolis, royal jelly, and honey studied here exert pro-oxidant effects on the nutritional media used to cultivate *S. cerevisiae* cells. We were unable to find any oxidation using 3-fold higher concentrations than those used in the Ty1antiROS assay (data not shown). This observation confirms previous studies[40] showing that quercetin does not promote the production of H_2O_2 when added to media for cultivation of yeast cells. Different activity of antioxidants on oxidative radicals was found in in vitro studies using cyclic voltammetry to investigate antioxidant/oxidant interactions.[41] It has been shown that the immediate and frequent interactions characteristic for the short-lived reagents can be changed to slow and infrequent interactions when reagents showing higher stability are used. Although the results obtained in vitro do not always correspond to the in vivo situation, it is relevant to mention that cell-generated ROS are extremely unstable and short-lived, while H_2O_2 is a relatively stable chemical.[6]

All these data fit well with the results obtained in the present study and suggest the following cellular mechanism of the Ty1antiROS test. The cell-generated ROS are biologically active and induce the Ty1 transposition proportionally to their concentration. The antioxidants in honey bee products taken up in advance scavenge ROS involved in triggering the Ty1 transposition process, showing a high effectiveness against physiologically active ROS, as evidenced by the exponential dependence of antioxidant activity on concentrations. When the ROS level is elevated in tester cells with compromised mitochondrial function (*rho*$^-$ mutants) by addition of H_2O_2, the oxidative radicals accumulate inside the cells at a high level due to low permeability of the plasma membrane and inactivation of catalase responsible for H_2O_2 decay. These ROS are more stable and not very active as inducers of Ty1 transposition since 5-fold higher levels are required to activate the Ty1 transposition at rates found after induction of ROS production in cells. The neutralization of these ROS levels requires higher amounts of antioxidants because their interaction with oxidants may be slower, not so frequent, and therefore less effective. As a consequence, the dependence of antioxidant activity on concentration is changed to proportional and the calculated IC_{50} values are higher.

Considering the shortcomings and advantages of the Ty1antiROS test, it is obvious that this assay cannot compete with most of the other in vitro or cell-based assays because it is very time-consuming. Although the Ty1antiROS test is easy to perform and does not require special laboratory equipment or training of personnel, results with this test are obtained in 6 days and the test is not automated as a high-throughput assay. The main advantage of the Ty1antiROS test consists in

measuring antioxidant activity by following the changes of a cellular process, the Ty1 transposition, instead of using chemical reactions. Transposones are ubiquitous, from *E.coli* to humans, and changes in their transposition are regulated by hundreds of gene functions involved in the main cellular processes.[42] In the Ty1antiROS test the oxidative species are produced by tester cells and the interaction with antioxidant takes place in live cells. The assay shows good sensitivity, as evidenced by its ability to detect antioxidant activity in royal jelly (Table 2), which is often impossible with some other cell-based assays.[11] These characteristics make the Ty1antiROS test a new highly sensitive cell-based assay that gives results very close to the in vivo situation and suitable to study the cellular mechanisms of interactions between oxidative radicals and antioxidants. The advantages of utilizing yeast cells as a model to screen in vivo for natural antioxidants have been demonstrated previously[40] in a study showing that quercetin protects cells from H_2O_2 stress by a mechanism independent of its metal-chelating properties and the induction of antioxidant defense systems.

The Ty1antiROS test is not the first assay measuring antioxidant activity by utilizing a cellular function. Recently, several bacterial cell-based systems for intracellular antioxidant activity screening using green fluorescence protein have been developed,[43,44] and the methods of assessment of antioxidant capacity were critically reviewed.[45] Although the question of the cellular mechanism of oxidant/antioxidant interactions was not addressed in these studies, some of the data shown in the tables indicate an exponential dependence between antioxidant concentration and function similar to that found with the Ty1antiROS test. A significant advantage in the field was the development of a high-throughput reporter gene assay to test the influence of natural compounds on promoter activities of rat catalase and human gluthathione peroxidase and superoxide dismutase in V79 cells.[46] The usage of such assays will allow easy and fast study of oxidant/antioxidant interactions in higher eukaryotic cells.

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Notes

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