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Application of growth tests employing a Δ*sod1* mutant of *Saccharomyces cerevisiae* to study the antioxidant activity of berry fruit extracts

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ABSTRACT

The aim of the study was to evaluate the content of major bioactive compounds (BACs) and the antioxidant properties of aqueous berry extracts using $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ biochemical assays and a novel bioassay based on the growth of a Δ*sod1* mutant of *Saccharomyces cerevisiae* on osmotic shock plates (OSPs). The extracts were prepared from raspberry ('Cascada Delight', 'Glen Fyne' and 'Octavia') cultivars, blackberry ('Navaho') cultivar, and wild blackberry, as well as a raspberry-blackberry hybrid. Concentrations of phenolic compounds, anthocyanins and ascorbic acid differed significantly in the fruit extracts, but were highest in the raspberry-blackberry hybrid. The antioxidant activity against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ among the berry cultivars and hybrid was highly dependent on the anthocyanin content. The Δ*sod1* yeast mutant growth test confirmed the antioxidant properties of all the extracts, with the variant based on counting of single colonies proving more sensitive than the spot test.

1. Introduction

Fruits, especially berries, have long been known to exert beneficial effects on the human body. They are currently used in production of functional food, because they can prevent health problems such as cardiovascular and neurodegenerative diseases, diabetes, cancer and obesity (Bowen-Forbes, Zhang, & Nair, 2010; Novotny, Baer, Khoo, Gebauer, & Charron, 2015; Tavares et al., 2013). Their health-promoting properties result in part from high content of bioactive compounds (BAC), many of which exhibit antioxidant properties (Manganaris, Goulas, Vicente, & Terry, 2014).

Various methods have been used to evaluate the antioxidant activity of natural compounds in foods. Commonly used biochemical assays based on the free radicals 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) and 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) scavenging method are not always adequate for testing the antioxidant capacity of food components following ingestion, i.e. *in vivo*. For this reason antioxidant properties are increasingly assessed using model organisms, such as *Saccharomyces cerevisiae* yeast. These are single-celled eukaryotes whose structure and biological and physiological processes are similar to those of cells of higher eukaryotes. Research using yeast can be an intermediate stage between biochemical *in vitro* studies and animal and human studies.

The aim of the study was to evaluate the antioxidant properties of berry extracts using biochemical assays with $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ and a novel biological assay based on growth of a Δ*sod1* yeast mutant on hypertonic medium. Currently, various strains of yeast (wild-type and mutants) are used to determine antioxidant properties of food samples. Usually they are impaired by means of a pro-oxidant substance. Restoration of physiological or biochemical parameters to their previous state (prior to treatment with the pro-oxidant) in the presence of BACs is indicative of their antioxidant properties (Frassinetti, Della Croce, Caltavuturo, & Longo, 2012; Golla & Bhimathati, 2014; Höferl et al., 2014; Piovezan-Borges, Valério-Júnior, Gonçalves, Mielniczki-Pereira, & Valduga, 2016). However, antioxidant substances present in the samples may interact not only with the yeast but also with components of the medium or directly with the pro-oxidant, neutralizing its effect.

In this study, instead of a pro-oxidant, a hypertonic environment was used. The test used cells of the Δ*sod1* mutant of *S. cerevisiae*, which

Abbreviations: BAC, Bioactive compounds; OSPs, Osmotic shock plates; $ABTS^{\bullet+}$, 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid; $DPPH^{\bullet}$, 2,2-diphenyl-1-picrylhydrazyl

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lacks activity of cytoplasmic superoxide dismutase (Cu,ZnSOD), an enzyme which removes the superoxide anion radical from the cytoplasmic space. Literature data show that Δ*sod1* mutants are hypersensitive in comparison with wild-type cells, not only to chemical pro-oxidants but also to hypertonic stress (Koziol, Zagulski, Bilinski, & Bartosz, 2005; Lewinska, Bilinski, & Bartosz, 2004; Sadowska-Bartosz, Pączka, Małoń, & Bartosz, 2013; Wallace, Bailey, Fukuto, Valentine, & Gralla, 2005). The agent inducing hypertonic stress is usually sodium chloride in a high concentration (about 0.8 M), which besides the typical effects of severe osmotic stress secondarily generates symptoms of oxidative stress (Zyracka et al., 2005; Koziol et al., 2005).

The hypersensitivity of Δ*sod1* yeast to both pro-oxidants and osmotic stress is manifested as a complete inhibition or slowing down of their growth. Growth defects can be eliminated by adding certain antioxidants to the medium for the Δ*sod1* mutant cells, such as ascorbate, cysteine, glutathione, or dithiothreitol (Koziol et al., 2005; Lewinska et al., 2004; Zyracka et al., 2005). Restoration of growth to these cells can thus be used to detect these antioxidants and/or determine their effective concentration in complex environmental or food samples.

The study was conducted on three cultivars of raspberry ('Cascada Delight', 'Glen Fyne' and 'Octavia'), one cultivar of blackberry ('Navaho'), wild blackberry, and a raspberry x blackberry hybrid. By analysing the chemical and antioxidant properties of the fruit from the parent varieties and the hybrid, we can evaluate the effect of the genetic background on these parameters.

2. Materials and methods

2.1. Chemical compounds

$ABTS^{+\bullet}$, $DPPH^{\bullet}$, potassium persulfate 99%, the Folin-Ciocalteu's phenol reagent, L-glutathione reduced (GSH) ≥98.0%, L-cysteine 97%, sodium salicylate 99%, coumarin ≥98%, kaempferol ≥97.0%, trans-cinnamic acid ≥99%, 4-hydroxybenzoic acid ≥99%, FG; *p*-coumaric acid ≥98.0% (HPLC), caffeic acid ≥98.0% (HPLC), trans-ferulic acid 99%, syringic acid ≥95%, gallic acid 97% were purchased from Sigma-Aldrich (Poznan, Poland). Butylhydroxytoluene, (2,6-di (tert-butyl)-p-cresol) (BHT), L-ascorbic acid, benzoic acid were from Institute of Industrial Organic Chemistry (Warszawa, Poland). All solvents used for L-ascorbic acid estimation were HPLC grade (Merck) and reference standards were obtained from Sigma-Aldrich (Poznan, Poland). Other reagents were of analytical grade. Components of culture media were from BD Difco (Becton Dickinson and Company, Spark, USA) except for glucose (POCh, Gliwice, Poland).

2.2. Yeast strains and growth conditions

The strains used were wild-type SP4 MAT α leu1 arg4 (Bilinski, Lukaszkiewicz, & Sledziewski, 1978), and Δ*sod1* mutant, isogenic to SP4, MAT α leu1 arg4 sod1:natMX (Koziol et al., 2005). Yeast were grown in standard liquid YPD medium (1% yeast extract, 1% yeast bacto-peptone, and 2% glucose) on a rotary shaker at 150 rpm to achieve a logarithmic culture with a density of about 1–5 x 10^7 cells/mL.

2.3. Preparation of extracts

The berry fruits for analysis were obtained from the Felin Experimental Farm, University of Life Sciences in Lublin, Poland. The fruits were harvested in 2016 at full maturity in the amount of 1 kg. They showed no signs of infection or mechanical damage. The extracts were prepared using water. These conditions primarily favour acquisition of hydrophilic antioxidants, which unlike hydrophobic compounds are essential for protection against the effects of oxidative stress in yeast (Uemura, 2012). For most of the analyses, the extracts were prepared by grinding 10 g of fruit and 50 ml of distilled water in a mortar. The extract was then vacuum-filtered on Whatman No. 1 filter paper and sterilized with 0.45 μm pore size syringe filters. The extracts were numbered as follows: 1 - *Rubus idaeus* L cv. Glen Fyne, 2 - *Rubus idaeus* L cv. Octavia, 3 - *Rubus idaeus* L cv. Cascada Delight, 4 - *Rubus fructicosus* L. cv. Navaho, 5 - *Rubus fructicosus* L. (native population), 6 - *Rubus idaeus* L. x *Rubus fructicosus* L. (hybrid).

2.4. Estimation of total phenols

The amount of total phenolic compounds in the fruit extracts was determined by a modification of Singleton and Rossi's method (1965). A 75 μL volume of Folin-Ciocalteu reagent (1:1) was added to 150 μL of the sample or an appropriate solvent. After 5 min, 60 μL of 7.5% sodium carbonate was added. Absorbance was measured at 740 nm after 30 min of dark incubation. Total phenol content was calculated based on a gallic acid standard curve and expressed as μg/mL of gallic acid equivalents (GAE).

2.5. Estimation of anthocyanins

Total anthocyanin content was measured by the pH differential absorbance method, described by Cheng and Breen (1991). Briefly, absorbance of the extract was measured at 510 and 700 nm in buffers at pH 1.0 (hydrochloric acid – potassium chloride, 0.2 M) and 4.5 (acetate acid sodium acetate, 1 M). Anthocyanin content was calculated using a molar extinction coefficient of 29,600 (cyanidin-3-glucoside) and absorbance of $A = [(A510 - A700)pH\ 1.0 - (A510 - A700)pH\ 4.5]$. Results were expressed as μg of cyanidin-3-glucoside equivalents in 1 mL of fruit extract.

2.6. Estimation of L-ascorbic acid

The fruits (10 g) were extracted twice for 30 min with 2.5 ml 4.0% (m/V) L-cysteine and 10.0 ml water by sonification. All aqueous extracts were combined and diluted with water to 25 ml. The samples were analysed using high performance liquid chromatography. Analyses were done with a LaChrom-Merck HPLC system with a photodiode array detector (DAD L–7450), and all separations were on a Lichrospher 100 RP18 column (250.0 × 4.0 mm, 5.0 μm; Merck). The mobile phase consisted of 0.0272 g $L-1$ KH_2PO_4 adjusted to pH 2.40 with H_3PO_4, applied in isocratic elution for 30 min. The flow rate was adjusted to 1.0 mL/min. The detection wavelength was set to DAD at λ = 254.0 nm 20.0 μL samples were injected. All separations were performed at 24.0 °C. Peaks were assigned by spiking the samples with standard compounds and comparing the UV spectra and retention times (ascorbic acid 5.66 min). Calibration curves were obtained from 5 concentrations of each external standard ($0.01 - 1.40$ mg/mL). The regression coefficient (R2) of the calibration curve for ascorbic acid (Y = 85.231 × = 18.787). The RSD values for the repeatability (n = 4) of standard solution were 0.40% (0.01 mg/mL ascorbic acid). The limits of quantitation (LOQ) and detection (LOD) of ascorbic acid were 0.16 and 0.04 mg/L, respectively.

2.7. $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activity

$DPPH^{\bullet}$ radical scavenging was analysed according to Brand-Williams, Cuvelier, and Berset (1995). Each 100 μL of fruit extract (0.78, 1.56, 3.12, 6.25 and 12.5 mg/mL) was mixed with 100 μL of 25 mM $DPPH^{\bullet}$ solution in 96% ethanol. Following 30 min incubation at room temperature the absorbance of the sample was measured at λ = 515 nm using 96% ethanol as a blank sample. Scavenging of $ABTS^{\bullet+}$ was evaluated by a modification of the procedure described by Re et al. (1999). The solution was diluted to reach the absorbance at λ = 734 nm around 1.0. Each extract (10 μL) at concentration 6.25, 12.5, 25, 50 and 100 mg/ml was mixed with 250 μL $ABTS^{\bullet+}$ solution and incubated for 10 min. The decrease in $ABTS^{\bullet+}$ absorbance (A_{734nm})