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The influence of selenium addition during germination of *Brassica* seeds on health-promoting potential of sprouts

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Abstract

The correlation among selenium uptake, the content of bioactive compounds in sprouts, and biological activities triggered in cultured human cells by sprout extracts was investigated. Seeds of *Brassica* crops and rye were treated with SeO_2 water solution. The selenium levels in sprouts increased from 1.0–4.1 to 53.3–382 μg/g dw with no influence on plant physiology according to the indices used. Neither the composition of glucosinolates (GL) in *Brassica* sprouts nor the myrosinase activity nor the composition of GL breakdown lipophilic products were significantly affected. In all *Brassica* sprouts, conversion to health-promoting isothiocyanates (ITC) and indoles corresponded to only 1% of total GLs. Low ITC concentration may explain observed lack of induction of glutathione S-transferases (GST) and quinone oxidoreductase (NQO) detoxifying enzymes in HT29 cells exposed to sprout extracts. The insignificant impact on cell growth and genome function suggests that *Brassica* sprouts may be safe vehicle of selenium to combat its dietary deficiency.

Keywords

Brassica sprouts, glucosinolate degradation products, myrosinase, phase II enzymes, selenium fortification

Introduction

Selenium is an essential micronutrient whose biological role results from the presence of selenocysteine in at least three groups of proteins: the iodothyronine deiodinases responsible for the peripheral deiodination of thyroxin (Bianco, 2002), thioredoxin reductases (Arner, 2009), and glutathione peroxidases (Savaskan, 2007). The later two cooperate in stabilizing the cellular thioredox state and, therefore, potentially affect a number of processes which depend on signalling pathways involving reactive oxygen species (ROS) such as apopotosis, proliferation, or differentiation (Hernández-García, 2010). Glutathione peroxidases restoring the reduced glutathione (GSH) pool are particularly important cytoprotective enzymes since this major cellular antioxidant not only prevents tissue oxidative damage but also takes part in phase II detoxification of xenobiotics including carcinogens (Hayes, 2005). Apart from carcinogenic risk reduction, also anti-inflammatory and antiviral properties of selenium are known (Duntas, 2009; Garner, 2001; Rayman, 2012). Moreover, selenium directly counteracts the toxicity of heavy metals, such as arsenic, cadmium, mercury, and copper by forming complexes which are subject of further metabolism facilitating their release from the organism (Chen et al., 2007; Feroci, 2005; Li, 2012). The multitude of protective mechanisms relying on selenium guaranteed this element the crucial role in redox homeostasis, maintaining the integrity of intracellular organelles, regulation of cell survival, and the overall wellbeing of the whole organism.

The importance of selenium for human health has been suggested in a number of studies, although because this mineral has a narrow safety margin, the controversies surrounding supplementation still remain and epidemiological data are far from conclusive. Proper intake of selenium was shown to be associated with the reduced risk of prostate, skin, colorectal, liver, mammary, and lung cancers (Clark, 1998; Lener et al., 2013; Reid et al., 2002; Yoon et al., 2001; Zeng & Combs, 2008; Złowocka-Perłowska et al., 2012). Other studies revealed correlation between appropriate selenium level in plasma and diminished type 2 diabetes incidence (Park et al., 2012). Moreover, an increased occurrence of neurological disorders was observed in patients with inadequate nutritional selenium supply (Steinbrenner & Sies, 2013). However, the potential to prevent cardiovascular ailments was not proven (Flores-Mateo et al., 2006; Rees et al., 2013). Selenium has also been connected with male reproductive performance (Ursini et al., 1999). Daily supplementation of this mineral (200 μg) in combination with vitamin E (400 units) was shown to improve sperm motility and morphology in more than half of a studied group of men (Moslemi & Tavanbakhsh, 2011). As selenium is required for the proper function of the immune system, this microelement has been proposed to be a key nutrient in counteracting the development of virulence; even Se-dependent inhibition of HIV progression to AIDS was demonstrated in some studies (Baum et al., 1997; Rayman, 2000; Stone et al., 2010). Finally, the analysis of published data revealed a significant relationship between age-related disease risk and selenoprotein function,

which led McCann and Ames to the conclusion that even modest selenium deficiency or/and genetic dysfunction of these proteins is a causal factor in conditions such as cancer, heart disease, or failing immune system (McCann & Ames, 2011). All these findings suggest that ensuring appropriate dietary intake of selenium is of pivotal social importance.

Although selenium is widely distributed in the environment, its content in the food chain may vary as it is affected by the elemental composition of soil on which crops are grown. Selenium concentration in soils in different regions varies extremely, ranging between 0.1 μg/kg and 1000 mg/kg. The lowest levels have been recorded in New Zealand, the Balkans, and Sichuan Province in China. In contrast, other provinces in China, Venezuela, and the USA have the highest selenium content in soil (Rayman, 2005; Yang et al., 1983). The USDA recommended dietary allowance for selenium in the USA is 55 μg/d for both men and women, while WHO recommends the intake of, respectively, 40 and 30 μg/d for men and women. European daily intake recommended by European Commission Directorate General for Health & Consumers is 65 μg/d. Therefore, the optimal human intake of this micronutrient is yet to be established. Actual intake of selenium varies throughout the world, typical intakes range from 11 μg/d in New Zealand (Duffield et al., 1999) to 177 μg/d in Japan (Yoneyama et al., 2008). In European countries, the mean selenium intake level is as low as 30–40 μg/d (Rayman, 2008) and has been falling since the EU imposed restrictions on wheat imports from the USA, where selenium levels in soil are high.

Clearly dietary selenium deficiencies are not infrequent around the world and may cause gradual deterioration of health. In this paper, the Se-enriched sprouts of *Brassica* plants are tested as a possible source of readily bioavailable inorganic and organic forms of selenium in the diet. Importantly, the use of brassicas for this purpose may bring about additional benefits due to the presence of organosulfur compounds – glucosinolates (GLs) – secondary metabolites characteristic for this plant family. Products of myrosinase catalyzed degradation of GLs, isothiocyanates (ITC), in particular, are well known for their chemopreventive potential (Dinkova-Kostova & Kostov, 2012; Fimognari et al., 2012; Latté et al., 2011). ITC are believed to act as anticarcinogens by decreasing the activation of carcinogens through the inhibition of phase I enzymes, while increasing their detoxification by the induction of phase II enzymes. GLs degradation products were also shown to inhibit growth, as well as to stimulate the apoptosis of tumor cells (Śmiechowska et al., 2008; Surh & Na, 2008). ITC also influences cellular redox status by their demonstrated ability to react with the SH group of cysteine in GSH and antioxidant proteins including thioredoxin, which in turn may serve as a trigger of gene expression mediated by the antioxidant response element (ARE). Among ARE inducible genes are those coding protective enzymes and transcription factors associated with cell proliferation and cell death (Dinkova-Kostova & Kostov, 2012). In addition, it has been proposed that alternative mechanisms of cancer chemoprevention by ITC and selenium, e.g. epigenetic mechanisms, both individually and synergistically, may regulate the selenoproteins responsible for the removal of damaging reactive molecules implicated in the progression and development of cancer (Barrera et al., 2012). Moreover, selenium can be incorporated instead of sulfur into *Brassica* bioactive phytochemicals. For example, synthetic Se-sulforaphane (4-methylthiobutyl isoselenocyanate) was demonstrated to exhibit an enhanced anticarcinogenic potential compared to its sulfur-containing counterpart (Emmert et al., 2010; Irion, 1999; Matich et al., 2012; Sharma et al., 2009).

The aim of current research was to examine the influence of selenium addition during seed germination on the growth and condition of sprouts, as well as the content of bioactive compounds in the obtained plant material. To determine whether inorganic selenium (SeO_2) can be transformed by plants into organic forms with better bioavailability, the increase in the total concentration of this element in the obtained sprouts was compared with that of selenoamino acids. Also measured was myrosinase activity in the sprouts and the yield of GL conversion to desirable degradation products. The biological properties of sprouts were assessed by evaluating their ability to induce activity of phase II enzymes: glutathione S-transferases (GST) and quinone oxidoreductase (NQO1) in human colon cancer HT29 cells exposed to sprout extracts. Based on these results, the conclusions are drawn with regard to the possibility of using brassica sprouts as a selenium carrier recommended for dietary supplementation.

Experimental

Chemicals and biochemicals

Seeds used in this research were produced by PNOS (Ożarowice Mazowieckie, Poland). The following chemicals: HPLC grade acetonitrile and methanol, 2-propanol, imidazole ACS, acetic acid (glacial), formic acid, nitric acid, and Folin–Ciocalteu reagent were purchased from Merck (Darmstadt, Germany). Glucotropaeolin (GTL) was obtained from AppliChem (Germany), NaCl and NaOH, $(K)PO_4$ were from P.P.H Standard (Lublin, Poland). Standard indolic compounds: I3ACN, I3AA were purchased from Merck (Darmstadt, Germany); DIM and I3C from Sigma (Schnelldorf, Germany). Reagents used to perform electrophoresis were also purchased from Sigma: 30% acrylamide mix, tris-HCl, $(NH_4)_2S_2O_8$, tetramethylethylenediamine (TEMED), sodium dodecyl sulfate (SDS), glycerol, bromophenol blue, Coomasie brilliant blue, and 2-mercaptoethanol. The same company was a source of other chemicals used in this study: 3-butenenitrile, 4-pentenenitrile, 3-phenylpropanenitrile, allyl ITC, 3-(methylthio)propyl ITC, benzonitrile, MeSeCys, HCl (37%), 1,2-benzenedithiol, ABTS, DPPH, CDNB, NADPH (2′-phosphate reduced tetrasodium salt hydrate), DCPIP, $CuSO_4$, potassium sodium tartrate, Na_2CO_3, myrosinase from *Sinapsis alba*, sulfatase isolated from *Helix Pomatia* H1 (22 400 units/g solid), and DEAE Sephadex A-25 anion-exchange resin. SeMet, SeCys, and 4-(methylthio)butyl ITC were purchased from Santa Cruz Biotechnology (Dallas, TX). Methylene chloride (CH_2Cl_2) was from Carl Roth GmbH (Karlsruhe, Germany), Na_2SO_4 from VWR International GmbH (Darmstadt, Germany), 3-butenyl ITC from TCI Deutschland GmbH (Berlin, Germany).

Plant material

Seeds of chosen *Brassica* plants: white cabbage (*Brassica oleracea* var. *capitata* f. *alba*), broccoli (*Brassica oleracea* L. var. *italica*), mustard (*Sinapsis alba*), and crop rye (*Secale cereale*) were germinated in germination plates. The seeds were sprinkled twice a day with 600 mL of spring water containing SeO_2 (10 mg/L) or pure spring water as a control. The germination was carried out in the phytothron with controlled temperature (25 °C) and photoperiod (16 h of light, 8 h in the dark). After 7 d of germination, sprouts were harvested and rinsed with spring water to remove contamination by inorganic Se adsorbed on surface. Finally, the sprouts were freeze-dried and stored at −20 °C until investigation.

Determination of Se content

The atomic absorption spectrometer (SavantAA Zeeman, GBC) was used to quantify the total level of selenium in the sprouts. The lyophilized sprouts (0.8 g) were mineralized in 65% nitric acid for

1.5 h using a microwave assisted digestion system (Microwave 3000, Anton Paar, Microwave Digestion System, Matthews, NC). The solutions of mineralized samples were analyzed by AAS. The selenium content was calculated using calibration line and was expressed as μg Se per g dw of sprouts.

Content of selenoamino acids

Sprouts' lyophilizates (0.2 g) were mixed with 3 mL of 6 N HCl with 0.5% phenol addition. Samples were left for 24 h at 105 °C to allow protein hydrolysis. Then extracts were filtered by passing through syringe filters (Chromafil xtra MV-45/25 0.45 μm for aqueous solutions) and analyzed using a LC-DAD-ESI-MS system (Agilent Technologies, Walnut Creek, CA) with a Grace Altima HP AQ RP-C18 column (150 × 4.6 mm, 3 μm). The mobile phase contained 1% (v/v) formic acid in water (A) and 1% (v/v) formic acid in methanol (B). Chromatographic resolution was performed at 30 °C with 1 mL/min flow rate and composition of 30% B in phase A. Data acquisition was performed in a selected ion monitoring (SIM) mode using the interface parameters: capillary voltage, 3000 V; fragmentor, 70–80 V; drying gas temperature, 350 °C; gas flow (N_2), 12 L/min; nebulizer pressure, 35 psig. The instrument was operated in positive ion mode $[M + H]^+$. Characteristic fragmentation of the selenoamino acids ions was observed: 184 (MeSeCys), 198 (SeMet), and 335 (Se-cystine). Levels of selenoamino acids were calculated based on the peak areas, in comparison with calibration curves generated with the use of commercial standards.

Electrophoretic protein profiling

Sprout extracts used for SDS-PAGE electrophoresis were prepared by homogenizing 50 mg of lyophilized plant material with 1 mL of 0.5 M NaCl, 20 mM potassium phosphate (pH 7.4). The homogenates were centrifuged (5000 rpm, 10 min, 4 °C, Eppendorf Centrifuge 5415 R, Hamburg, Germany) to remove debris. In collected supernatants, the protein content was measured by Lowry's method. The samples were then diluted with water to obtain protein concentration of 4.0 mg/mL. Then, 120 μL of samples were combined with 30 μL of buffer containing per 100 μL: 23 μL of 1 M Tris-HCl (pH 6.8), 45.5 μL of 50% glycerol solution, 18 μL of SDS solution (concentration of 10%), 9 μL of 1% solution of bromophenol blue, and 4.5 μL of 2-mercaptoethanol. After heating at 90 °C for 10 min, the 20 μL aliquots of protein preps were loaded onto PAGE gel. Additionally, protein molecular weight markers (range 6.5–200 kDa, Sigma) and purified myrosinase solution (Sigma) were loaded for comparison. To perform electrophoresis, the 10% separating acrylamide gel and 5% stacking acrylamide gel were used according to modified Laemmli's method (Kusznierewicz et al., 2008). The Biorad PowerPac Basic apparatus (Hercules, CA) was employed for electrophoresis, the separation of proteins was carried out at 80 V for 20 min, then voltage was increased to 120 V, and the process continued for 70 min. The gels were submerged for 2 h in 0.25% Coomassie Brilliant Blue dissolved in methanol:water:glacial acetic solution (5:5:1 v/v), then destained overnight in stain free methanol:water:glacial acetic solution (2:8:1 v/v).

GL analysis

In order to determine the content of GLs, the ISO 9167-1 method with modifications described by Kusznierewicz was used (Kusznierewicz et al., 2013). For this purpose, 100 mg of each freeze-dried sprout sample was extracted twice with 3 mL of 70% boiling methanol. During first extraction, 0.2 mL of water solution of 5 mM GTL was added to each sample as an internal standard for the quantitative analysis. The extracted GLs were purified on 1 mL column filled with 0.5 mL of DEAE-Sephadex A-25 anion-exchange resin. The column was pre-washed with 2 mL of 6 M imidazole formate, then twice with 1 mL of water, and loaded with 6 mL of each extract. Next, 0.2 mL of sulfatase solution (1.67 mg/mL) was added and desulfatation reaction was carried out overnight at room temperature. Desulfo-GLs (ds-GLs) were eluted from the column with deionised water (2 × 0.75 mL) and analyzed by a LC-DAD-ESI-MS system (Agilent Technologies) using a Grace Altima HP AQ RP-C18 column (150 × 4.6 mm, 3 μm). The mobile phase contained water (A) and acetonitrile/water (20:80, v/v, B). Chromatographic resolution was performed at 30 °C with 1 mL/min flow rate and the following gradient program: linear gradient rinsing from 5% B to 100% B within 10 min and then isocratic separation with 100% B for 15 min. The injection volume of samples was 30 μL. The chromatographic peaks were first detected by DAD (Agilent 1200 series) at 229 nm, then the identity of individual ds-GLs was confirmed by API-ESI-MS (Agilent 6130 Quadrupole LC/MS). MS parameters were as follows: capillary voltage, 3000 V; fragmentor voltage, 120 V; drying gas temperature, 350 °C; gas flow (N_2), 12 L/min; nebulizer pressure, 35 psig. The instrument was operated both in positive and negative ion modes, scanning from *m/z* 100 to 800.

Determination of ITC content

The content of ITC was determined by modified Zhang's et al. method (Zhang et al., 1996). Lyophilized sprouts (0.2 g) were mixed with 5 mL of 0.01 M sodium phosphate buffer (pH 7.4) and incubated for 3 h at 37 °C to enable myrosinase catalyzed conversion of parent aliphatic and aromatic GLs into ITC; the unstable indolic ITC hydrolyzed spontaneously to indoles. The samples were subsequently centrifuged (4000 rpm, 10 min, 4 °C, Thermo Scientific Heraeus Megafuge 16, Waltham, MA) and the collected supernatants were passed through a Bakerbond SPE C_{18} 500 mg cartridge (J. T. Baker, Center Valley, PA) preconditioned with 6 mL of methanol and 6 mL of 10% methanol in water (v/v). The retained ITC (together with indolic breakdown products) were then eluted with 1 mL of methanol and the eluates were centrifuged again to remove particulates (13 000 rpm, 15 min, 4 °C). The concentrated ITC (0.1 mL of the eluate) were submitted to cyclocondesation reaction in a mixture containing 0.5 mL of 2-propanol, 0.5 mL of 0.1 M potassium phosphate buffer (pH 8.5), and 0.1 mL of 60 mM 1,2-benzenedithiol dissolved in 2-propanol. The mixture was incubated for 1 h at 65 °C. The product of reaction, 1,3-benzenedithiol-2-thione, was analyzed by reverse phase HPLC using Agilent 1200 system coupled with a DAD detector. The reaction mixture (30 μL) was injected onto a 150 mm × 4.6 mm, 3.5 μm Zorbax Eclipse XDB-C8 column. The mobile phase used was 4.8% (v/v) formic acid in water (A) and methanol (B) with a flow rate of 1 mL/min. The gradient changed as follows: 60% B to 100% B within 12 min, then 100% B for 3 min. Chromatograms were traced at 365 nm. For quantification of the reaction product, a standard line was constructed using a series of 1,3-benzenedithiol-2-thione solutions with a concentration range of 0.01–10 mM.

Determination of indole GL hydrolysis products

The eluates from SPE obtained as described for ITC were also used to determine the indole content in the sprout samples. The HPLC-DAD-FLD system (Agilent Technologies 1200 Series) equipped with a Zorbax Eclipse XDB-C8 column (the same as for ITC) was applied for the analysis of indolic compounds. A linear gradient of acetonitrile in water changing from 10% to 100% within 30 min and the mobile phase flow rate set at 1 mL/min ensured the satisfactory resolution of indoles. The injection volume of eluates was 20 μL. Fluorescence emission at 360 nm

(excitation at 280 nm) enabled monitoring of indolic analytes. The calibration curves used for their quantification were generated by the integration of the areas of fluorescence peaks determined during analysis of serial dilutions of I3C, I3ACN, I3AA, and DIM.

Determination of other GL breakdown products

For the analysis of enzymatically formed breakdown products of the GLs, the method of Hanschen et al. (2012) was adapted. Lyophilized sprouts (0.03 g) were dispersed in 0.75 mL of 0.01 M sodium phosphate buffer solution (pH 7.4) and incubated for 3 h at 37 °C to allow complete GL hydrolysis. Afterwards, 2 mL of methylene chloride and 100 µL benzonitrile (2 mM, in methylene chloride) were added as an internal standard. After shaking the tubes for 20 s and centrifugation for 5 min (4000 rpm), the methylene chloride layer was removed and filtered through a small column of anhydrous sodium sulfate to remove residual water. The aqueous layer was re-extracted with 2 mL of methylene chloride. Dried extracts were combined, concentrated under nitrogen gas flow to 300 µL, and transferred into a vial. Analysis of the samples was done by gas chromatography-mass spectrometry detection (GC-MS) using an Agilent 6890A Series GC System (Agilent Technologies) with a Gerstel Multi Purpose Sampler MPS2 (Gerstel GmbH & Co. KG, Mülheim, Germany) and an Agilent 5973 Network MSD (Wilmington, DE). The GC was equipped with an Optima 5 MS column (Macherey-Nagel, 30 m × 0.25 mm × 0.25 µm film). After splitless injection of 1 µL of the sample at 190 °C, analytes were separated, using helium as a carrier gas (1.8 mL/min), and a temperature gradient starting at 35 °C (3 min) and raising up to 50 °C with 9 °C/min. After holding this temperature for 7 min, the temperature increased to 100 °C (9 °C/min, held for 3 min), then to 223 °C (3 °C/min), to 230 °C (9 °C/min, held for 1 min), and finally with 35 °C/min to 310 °C. The temperature of the transfer line was 310 °C, and the ion source of the MSD was set to 230 °C. Mass spectra were acquired in the EI mode (70 eV) and the full scan mode (TIC) was used for qualitation and quantitation (*m/z* 30–350). The analytes were identified by comparing mass spectra and retention times with those of authentic standards and with literature data (Kjaer, 1963; Spencer & Daxenbichler, 1980). The content of analytes was calculated using benzonitrile as an internal standard and the response factor (R_F) of each compound relative to benzonitrile. The R_F was experimentally determined for allyl ITC ($R_F = 1.70$), 3-butenenitrile ($R_F = 3.70$), 4-pentenenitrile ($R_F = 2.45$), 3-(methylthio)propyl ITC ($R_F = 1.07$), 2-phenylethyl ITC ($R_F = 0.68$), and 3-phenylpropanenitrile ($R_F = 0.82$); 3-butenyl ITC ($R_F = 1.06$), 4-(methylthio)butyl ITC ($R_F = 0.76$), and for I3ACN ($R_F = 0.84$). For those compounds, that were commercially not available, the RF of the chemically most similar compound was used: for the epithionitriles of allyl and 3-butenyl GL the RF of the corresponding ITC was used, diastereometric 1-cyano-2-hydroxy-3,4-epithiobutane and 5-vinyl-1,3-oxazolidine-2-thione were calculated with the RF of 3-butenyl ITC, and 3-hydroxypentenenitrile was calculated with the RF of 4-pentenenitrile. The corresponding nitriles of 3-(methylthio)propyl GL and 4-(methylthio)butyl GL were calculated with the R_F of the analogous ITC. For breakdown products of 4-hydroxybenzyl GL, the R_F of the internal standard benzonitrile was used and 4-methoxy-3-indoleacetonitrile was quantified with the R_F of I3ACN ($R_F = 0.84$).

Determination of myrosinase activity by pH-stat method

The determination of myrosinase activity was based on Piekarska et al. (2013). The samples of lyophilized sprouts (0.5 g) were added to 15 mL of 80 mM NaCl (pH adjusted to 6.5 with 1 mM HCl) and homogenized using a Heidolph SilentCrusher M homogenizer (Suwanee, GA) (6500 rpm for 5 min with 12FG stainless steel generator) to ensure the complete liberation of the enzyme. During homogenization, the samples were kept on ice to maintain low temperature. Initial hydrolysis of endogenous GLs was carried out by stirring the reaction mixture at 37 °C with monitoring pH value. When stabilization of mixture pH at the level of 6.5 was achieved (usually within 1 h), 0.15 mL of 0.5 M GTL was added and its hydrolysis was carried out at 37 °C. Acidification of the reaction mixture caused by the release of H^+ was counterbalanced by the addition of 1 mM NaOH. To maintain constant pH at a 6.5 level over the whole reaction period, a T70 titrator (Mettler Toledo, Columbus, OH) with pH-stating option was employed. Typically, GTL hydrolysis was monitored for 1 h. Myrosinase activity was calculated based on the consumption of 1 mM NaOH and is given as µmoles of hydrolyzed GTL per min per 1 g of dry weight (U/g dw of sprouts).

Determination of antioxidant potential

Standard methods employing ABTS or DPPH radicals or FCR reagent were used for the colorimetric determination of the total antioxidant activity of methanol extracts of the sprouts (30 mg/mL). In each case, a Trolox solution was used to generate the standard line (concentration range 0–1.5 mmol/L). The stock solutions of derivatization reagents were diluted before measurements as follows: DPPH was diluted with methanol until the absorbance reached 1.0 ± 0.02 at $\lambda = 515$ nm; the ABTS radical cation solution was diluted with methanol to display the absorbance of 0.7 ± 0.02 at 734 nm; the commercial FCR preparation was diluted with water (1:9 v/v). All determinations were carried out in 48 well plates at room temperature, and the absorbance was measured using a Tecan Infinite M200 spectrophotometer (Tecan Group Ltd, San Jose, CA) as described before (Kusznierewicz et al., 2012). Results are expressed as Trolox equivalents [µmol TE/g dw].

Determination of activity of glutathione S-transferases (GST)

The water extracts obtained from lyophilized sprouts (0.1 g/3 mL) were sterilized by passing through Millex-GP 0.22 µm filtres (polyethersulfone, gamma sterilized) and added to cultures of human colon cancer HT29 cells to reach a concentration of 10% (v/v) in the medium. The cells were exposed to sprout extracts for 3, 6, or 24 h at 37 °C. Each cell culture with extract addition was repeated twice. After removing the medium, the cells were rinsed with cold PBS and detached with a cell scraper. The suspension was centrifuged (4000 rpm, 10 min, 4 °C, Thermo Scientific Heraeus Megafuge 16R). Cell pellets were resuspended in a portion of fresh PBS and again centrifuged (4000 rpm, 10 min, 4 °C). This step was repeated three times. After washing, the cells were resuspended in 250 µL of distilled sterile water, left for 10 min to swell, then combined with 250 µL of KCl-phosphate buffer (0.1 M (K)PO_4, 0.2 mM EDTA, 2.3% (w/v) KCl, pH 7.7) and homogenized in a Dounce homogenizer (30 strokes). Finally, the homogenates were centrifuged (4000 rpm, 15 min, 4 °C, Eppendorf Centrifuge 5415 R) to remove cell debris. The cytosolic fractions (supernatants) were aliquoted and stored at −80 °C until use. Before enzymatic activity measurements, the protein content was determined in the cytosols by modified Lowry's method. Determination of activity of GSTs was based on Habig's method as described elsewhere (Kusznierewicz et al., 2007). Briefly, 940 µL of 1 mM CDNB (dissolved in 2% ethanol and then added to 0.1 M phosphate buffer, pH 6.5, heated to 55 °C) was transferred to a spectrophotometric cuvette, followed by 50 µL of 20 mM GSH. To determine the background reaction

rate corresponding to non-enzymatic conjugation of GSH with CDNB, the spectrophotometric measurement was carried out for 3–4 min at 340 nm at 37 °C. Then, 10 µL of cytosolic fraction was added to the cuvette and absorbance measurements were continued for about 10 min to determine the rate of reaction catalyzed by GSTs present in the cells. For each sample, two independent measurements were carried out. The GST activity was expressed as nanomoles of conjugated CDNB per min per mg of cytosolic protein.

Determination of NAD(P)H:quinone oxidoreductase (NQO1) activity

The activity of NQO1 was determined in cytosolic fractions of HT29 cells obtained as described in Determination of activity of GST section using the method described elsewhere (Kusznierewicz et al., 2007). Briefly, the reaction mixture consisted of 100 µL of 0.1 M KCl-phosphate buffer (0.1 M (K)PO_4, 0.2 mM EDTA, 2.3% (w/v) KCl, pH 7.7), 30 µL of 1.2 mM DCPIP solution, and 800 µL of water. The absorbance of this solution was measured at 600 nm at 37 °C for few minutes. Then, 30 µL of freshly prepared 6 mM NADPH and 30 µL of cytosolic fraction were added to the cuvette and measurements continued. Control samples contained 30 µL of buffer instead of cytosolic fraction. The NQO activity was expressed as nanomoles of reduced DCPIP per min per mg of cytosolic protein. For each sample, two independent measurements were carried out.

Statistical analysis

All determinations were performed as two (enzymatic activities) or three independent measurements and the data are expressed as means ± standard deviation (SD). The statistical analyses were performed by a two-tailed Student's *t*-test using the Prism 4.0 software package (GraphPad Software, Inc., San Diego, CA). The level of statistical significance was set at $p \leq 0.05$.

Results and discussion

Content of selenium and selenoamino acids

Some *Brassica* vegetables have been designated as selenium accumulators because of their ability to absorb relatively large amounts of inorganic forms of selenium. At least part of this mineral is utilized by plants for the biosynthesis of selenoamino acids, which are then converted into numerous phytochemicals (Irion, 1999). Therefore, the edible parts of *Brassica* seemed a vehicle of choice to combat selenium deficiencies in humans. In our research, sprouts of cabbage, broccoli, and mustard were tested; also rye was included as a typical reference source of selenium in diet. During 7 d of germination, half of the seeds were sprinkled with spring water containing SeO_2 (10 mg/L). The selenium concentration applied during germination did not appear to inhibit plant growth and no differences in biomass production or any other signs of toxicity were noticed (data not shown). In seeds germinated without supplementation, the selenium content in sprouts ranged from 1.0 to 4.1 µg/g dw (Table 1). Selenium levels increased in all the sprouts treated with Se-enriched water, even up to 400 µg/g dw in the case of broccoli (over 150-fold). The content determined in both categories of rye sprouts was the lowest (Table 1).

The observed levels of selenium in sprouts were in the same order of magnitude as published before, although it is known that the absorption of this mineral depends on the valence of inorganic selenium. For example, *Brassica juncea* accumulated 58–109 µg Se/g dw in shoots and 200–424 µg Se/g dw in roots after 10-d treatment with Na_2SeO_3 (selenite) in 5 mg/L concentration. However, when plants were treated with Na_2SeO_4 (selenate) in the same concentration, the selenium content reached 1003–1331 µg Se/g dw and 65–134 µg Se/g dw in shoots and roots, respectively (Kahakachchi et al., 2004). This is because the translocation of selenium from roots to shoots depends on the form of selenium supplied; selenate is transported to plant shoots more easily than selenite. Pedrero et al. (2007) determined selenium levels in broccoli to reach 27 µg Se/g fw for plants grown for 40 d in a hydroponic culture of Na_2SeO_3 solution (1 mg/L). Similar results (32.9 µg Se/g fw) were obtained by Ogra for Japanese radish (*Raphanus sativus*) grown on soil enriched with 5 mg/kg Na_2SeO_3 addition (Ogra et al., 2007). In contrast, non-brassica plants accumulated much less selenium during various enriching treatments, e.g. pumpkin (*Cucurbia pepo*) – 1.1 µg Se/g dw (Smrekolj et al., 2005), dill (*Anethum gravealnes*) – 10.3 µg Se/g dw (Cankur et al., 2006), green onion (*Allium fistulosum*) – 30.3 µg Se/g dw (Shah et al., 2004).

Table 1. Selenium content in sprouts sprinkled with SeO_2-enriched water (10 mg/L) during seed germination.

	Selenium content (µg/g dw)[a]	
Plant	control	Se enriched
Mustard	3.6 ± 1.1	138 ± 12
Broccoli	2.60 ± 0.82	400 ± 29
White cabbage	4.1 ± 1.6	382 ± 17
Rye	1.00 ± 0.46	53.3 ± 6.3

[a]The values are means ± SD of three independent determinations.

Inorganic forms of selenium absorbed by plants may be then transformed into various organic forms. Because selenium and sulfur share the same uptake and assimilation pathways in plants, selenium can be incorporated into any organo-sulfur compound (Ramos et al., 2011; Zhu et al., 2010). Most common are selenomethionine (SeMet), selenocysteine (SeCys), and the methylated form of the latter: methylselenocysteine (MeSeCys). Both SeMet and SeCys are biogenic analogues of sulfur amino acids methionine (Met) and cysteine (Cys) with an atom of selenium replacing the atom of sulfur. However, only SeCys represents a natural, genetically encoded protein component (Johansson et al., 2005). Selenium speciation varies among plant species and the chemical form to which plants are exposed (Grant et al., 2004; Kapolna & Fodor, 2006; Kapolna et al., 2007; Wang et al., 1996; Ximenez-Embun et al., 2004). For example, in Indian mustard (*Brassica juncea*), the main selenium species in plants fed with selenate was still inorganic selenate, whereas in plants fed with selenite, SeMet and selenomethionine Se-oxide hydrate (SeOMet) dominated (Kahakachchi et al., 2004). In turn, Se-methylselenocysteine (SeMeSeCys) was a major selenium compound in Se-enriched garlic (*Allium sativum*), onion (*Allium cepa*), leek (*Allium ampeloprasum*), and broccoli (*Brassica oleracea* var. *italica*), accounting for approximately half of the total selenium absorbed (Beilstein et al., 1991). In contrast, SeMet is the predominant selenium form in most grains, such as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and rye (*Secale cereale*), accounting for 60–80% of the total selenium (Stadlober et al., 2001; Zhu et al., 2010).

In our study, only SeMet and MeSeCys were detected in sprouts. In Se-enriched *Brassica* sprouts, the content of these two amino acids increased by 27% in broccoli, by 34% in cabbage, and as much as 90% in mustard compared with their regular counterparts. It follows that most of the selenium absorbed was not converted into an amino acid form. Probably, protective mechanism against toxicity of this element prevailed during 7 d of sprouting. This could be changed by proloned cultivation since the amount of SeMet grows with time, e.g. in the case of broccoli

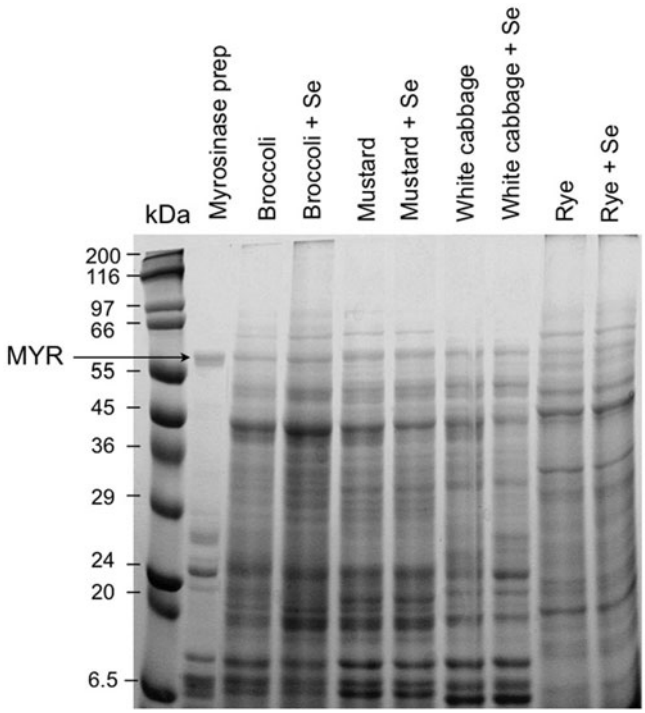

Figure 1. Protein profiles obtained for sprouts by SDS-PAGE electrophoresis. Extracts were loaded at 20 µL per lane (corresponding to 0.08 mg of protein) and proteins were detected by standard Coomassie blue staining after electrophoresis. First lane corresponds to molecular standards (200–6.5 kDa), second lane to commercial prep of myrosinase.

sprouts this increase was from 0.98 mg/kg fw after 10 d to 6 mg/kg fw after 40 d of Se-enrichment (Pedrero et al., 2007).

Protein profiling

Since exposure of seeds to SeO_2 during germination resulted in a somewhat altered composition of selenoamino acids, it could also impair the synthesis of proteins. To check this possibility, we examined the total content, as well as the composition of proteins isolated from sprouts. Of special interest was myrosinase (β-D-thioglucosidase glucohydrolase, EC 3.2.1.147), the enzyme characteristic for *Brassica* plants, constituting the most important element of their defence system. The protein content of each sprout pair germinated without or with Se-enrichment was similar and, respectively, amounted to 13.13 and 13.02 mg/mL for mustard, 8.85 and 9.12 mg/mL for cabbage, 7.38 and 7.52 mg/mL for broccoli, and 4.28 and 4.13 mg/mL for rye. The basically unchanged total protein biosynthesis, additionally confirms that applied selenium enrichment during germination had no influence on growth and the state of the obtained sprouts. As can be seen in Figure 1, comparison of SDS PAGE gel patterns did not reveal noticeable effects of Se addition. Although, as expected, rye displayed a different protein pattern than the brassicas.

Degradation of GLs

Brassica plants are known to synthesize GLs, a class of organosulfur secondary metabolites containing a β-D-thioglucose group linked to a sulfonated aldoxime moiety and a variable side chain derived from amino acids. The first step of GL hydrolysis, releasing pest repellents, is catalyzed by myrosinase. This leads to the formation of thiohydroximate-*O*-sulfonate, an intermediate that can be further converted into a variety of products, whose structures depend on the parent GLs, hydrolysis conditions, the presence of ferrous ions, and additional protein factors. The degradation products (Figure 2) include ITC, indoles, nitriles, epithionitriles, and thiocyanates (Finiguerra et al., 2001; Mithen, 2001). Of these, the greatest health benefits are associated with ITC and indoles (Johnson, 2002; Śmiechowska et al., 2008). This means that not only the GL content but also the pathway of their degradation might influence chemopreventive effects of sprouts, apart from the selenium supplementation.

As shown in Figure 3, the amount of GLs was not considerably affected by selenium addition during seed germination. Also the composition of GLs was not changed; sinigrin, progoitrin, glucoibervirin, glucoiberin, gluconapin, and neoglucobrassicin were detected in cabbage sprouts; mainly gluconapin and glucobrassicanapin were found in broccoli sprouts and sinalbin was dominant in mustard sprouts. GL levels were similar in regular and Se-enriched pairs of *Brassica* sprouts; as expected, no GLs were detected in rye sprouts. These findings are in agreement with the results reported for broccoli sprouts exposed to different forms of inorganic selenium (selenite and selenate) (Ávila et al., 2013). Also other researchers have not found significant GL level differences in mature broccoli florets after selenate treatment (Hsu et al., 2011; Ramos et al., 2011). In contrast, Charron et al. (2001) reported decreased GL concentration in broccoli grown in the presence of selenate, 70% lower than in non-enriched florets. According to Finley, a high concentration of selenium may suppress GL breakdown in plants. In his research, the amount of GL in broccoli, mainly glucoraphanin (GRH), was only slightly reduced after Se-fertilization. However, the content of sulforaphane, ITC obtained from GRH, fell by 85% in Se-enriched broccoli as compared with regular broccoli (Finley et al., 2005). In our case, GL conversion into ITC and indoles (Table 2), as well as myrosinase activity responsible for GL hydrolysis (Figure 4), was not affected by the selenium concentration in sprouts. Although all GLs were hydrolyzed, only 1% was converted into ITC and indoles. Epithionitriles and nitriles were predominant products in the examined *Brassica* sprouts, regardless of selenium addition. Among other products, goitrin (5-vinyl-1,3-oxazolidine-2-thione) was detected in the cabbage sprouts, and 4-hydroxybenzyl alcohol and 4-hydroxybenzyl acetic acid in the mustard sprouts. The latter compound was probably derived from the corresponding nitrile in a reaction catalyzed by nitrilase, which occurs in mustard sprouts (Agerbirk et al., 2008). The GL conversion rate to ITC and indoles in the sprouts was very low, especially in comparison with 96.5% rate observed in Daikon sprouts (De Nicola et al., 2013). Also Hanlon & Barnes (2011) reported a higher conversion rate in radish sprouts (*Raphanus sativus*), ranging from 17% to 58% depending on the radish variety. As myrosinase activity in sprouts is higher than or comparable with that in mature vegetables (Piekarska et al., 2013), low GL conversion into ITC cannot be attributed to the lack of myrosinase activity. However, as mentioned above (Figure 2), the formation of GL breakdown products depends on the presence and activity of additional protein factors, e.g. ESP, NSP, and TFP, which may react with intermediate thiohydroximate-*O*-sulfonate. Generation of different compounds (nitriles, epithionitriles, and thiocyanates) supported by these proteins can explain why the GL hydrolysis produced such a low amount of ITC and indoles in extracts obtained from the lyophilisates of the examined sprouts. Also ITC and unstable indoles might be converted into products not identified in analyses dedicated to the detection of the former (Hanlon & Barnes, 2011).

Antioxidant properties

Another group of phytochemicals that could affect the healthiness of Se-enriched sprouts are antioxidants. They are protective agents preventing the oxidative damage of proteins, nucleic acid, and lipids induced by reactive oxygen, nitrogen, and chloric

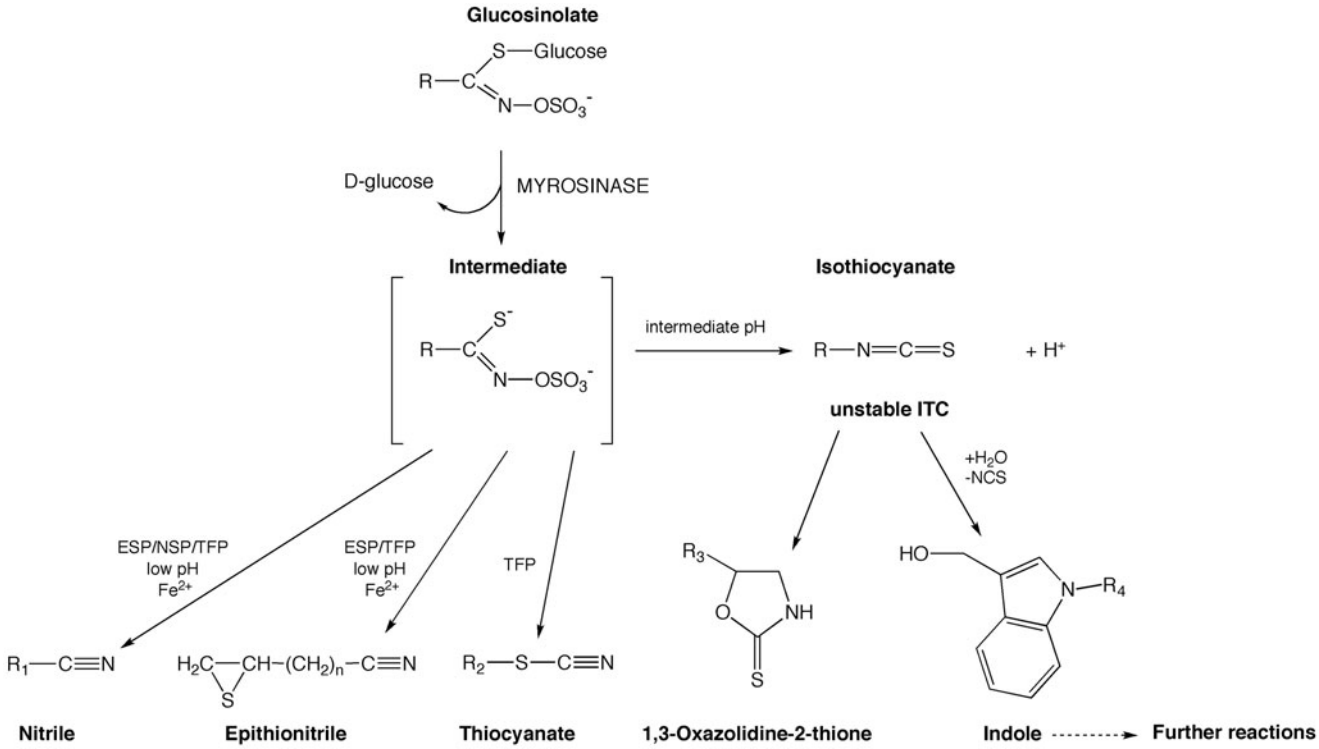

Figure 2. The scheme of glucosinolate enzymatic hydrolysis by myrosinase depending on the environmental and protein factors. The acronyms refer to: ESP, epithiospecifier protein; NSP, nitrile specifier protein; TFP, thiocyanate-forming protein.

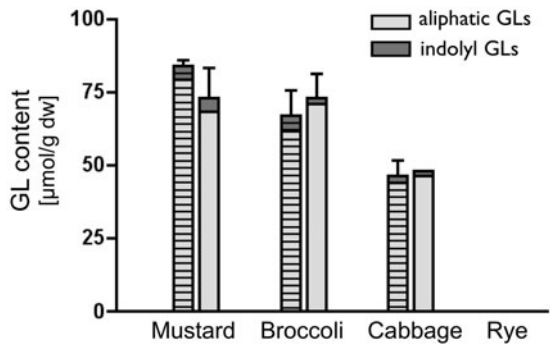

Figure 3. Content of aliphatic and indolyl glucosinolates in *Brassica* sprouts germinated with (dashed bars) or without selenium addition. The results are means of three independent determinations; SD refers to sum of GLs.

species (Halliwell, 2006). In numerous studies, plant components with low reduction potential were shown to retard the progress of chronic diseases, as well as processes associated with ageing. *Brassica* plants do not exhibit particularly strong antioxidative potential (Kusznierewicz et al., 2007, 2010), nonetheless they contain antioxidant compounds, like ascorbic acid, tocopherols, phenolics, and carotenoids (Davey et al., 2000; Singh et al., 2007; Volden et al., 2009; Zhang & Hamauzu, 2004). To examine the impact of SeO_2 treatment during germination on antioxidant activity of sprouts, standard ABTS, DPPH, or FC tests were performed for methanol extracts from lyophilized plant material. As shown in Figure 5, no clear correlation between Se-treated and non-treated plant samples were observed. Only antioxidant activity determined for Se-enriched mustard sprouts seemed repeatedly higher in all tests than for corresponding regular sprouts. The increased total antioxidant activity was reported in a number of Se-enriched plants, such as broccoli (Ramos et al., 2011), garden cress (Frias et al., 2010), and radish (Hanlon & Barnes, 2011). Enhanced antioxidant activity is one of the plants' protective mechanisms induced by UV radiation (Wilson et al., 2001). The amount of UV exposure during brassica growth may explain the antioxidant activity differences between sprouts and mature plants after Se-enrichment.

Influence on phase II enzymes in cultured cells

Undoubtedly, the most important additional benefit of the use of *Brassica* sprouts as a selenium carrier would be the presence of GLs and especially their conversion into ITC. The latter have been widely investigated for their ability to trigger a number of health promoting effects (Johnson, 2002; Śmiechowska et al., 2008), most importantly inhibition of tumorigenesis (Kim et al., 2011; Peng et al., 2008; Sehrawat & Singh, 2011), anti-inflammatory properties (Dey et al., 2010) and prevention of heart diseases (Piao et al., 2010; Wu et al., 2004). Several mechanisms have been proposed as being responsible for the anticarcinogenic benefits of ITC. Among the best recognized are inhibition of phase I enzymes, e.g. certain isoforms of cytochrome P450 (Kalpana Deepa et al., 2011); induction of phase II enzymes (e.g. quinone reductase, GST, UDP-glucuronosyl transferases) responsible for the detoxification of potential carcinogens (Ernst et al., 2011; Fahey et al., 1997; Smith et al., 2003); reduction of mitosis rate and stimulation of apoptosis in human tumor cells (Dinkova-Kostova & Kostov, 2012; Surh et al., 2008). This explains why the rate of GL conversion into ITC must be considered if the health benefits connected with brassica consumption are to be achieved. What is more, selenium can be incorporated into the GL structure, preferentially into the side chain (Matich et al., 2012). Se-GLs may undergo myrosinase catalyzed hydrolysis resulting in selenium-containing products such as Se-ITC. Synthetic Se-sulforaphane and several phenylalkyl-isoselenocyanates were reported to be more potent inhibitors of cancer cell growth and tumor development than their sulfur analogues (Emmert et al., 2010; Matich et al., 2012;

Table 2. Concentration of different glucosinolate breakdown products in *Brassica* sprouts germinated with or without selenium addition.

Sprouts	µmol/ g dw[b]						Conversion of GLs (%)
	GLs total	ITC	Indoles	Nitriles	Epithionitriles	Other products[a]	
Mustard + Se	83.94 ± 2.07	0.11 ± 0.03	0.05 ± 0.02	1.19 ± 0.06	n.d.	1.58 ± 0.14	2.75
Mustard	72.87 ± 8.49	0.09 ± 0.04	0.03 ± 0.01	0.81 ± 0.29	n.d.	1.02 ± 0.08	3.50
Broccoli + Se	67.11 ± 7.45	0.31 ± 0.01	0.33 ± 0.02	3.27 ± 0.11	7.86 ± 0.28	n.d.	18.82
Broccoli	72.95 ± 8.34	0.39 ± 0.04	0.31 ± 0.03	4.18 ± 0.22	8.97 ± 0.14	n.d.	17.51
Cabbage + Se	46.57 ± 5.16	0.35 ± 0.01	0.07 ± 0.01	5.76 ± 0.71	2.98 ± 0.35	2.36 ± 0.15	25.58
Cabbage	48.19 ± 0.02	0.25 ± 0.01	0.10 ± 0.02	7.10 ± 0.47	2.57 ± 0.26	2.12 ± 0.31	25.19

n.d., not detected.
[a]Other products refer to 4-hydroxybenzyl alcohol, 4-hydroxybenzyl acetic acid (mustard), and goitrin (cabbage).
[b]The values are means ± SD of three independent determinations.

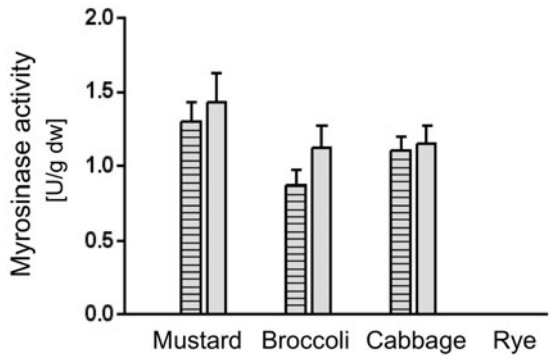

Figure 4. Myrosinase activity determined by pH-stat method in lyophilized *Brassica* sprouts germinated with (dashed bars) or without selenium addition. The results are means ± SD of two independent determinations.

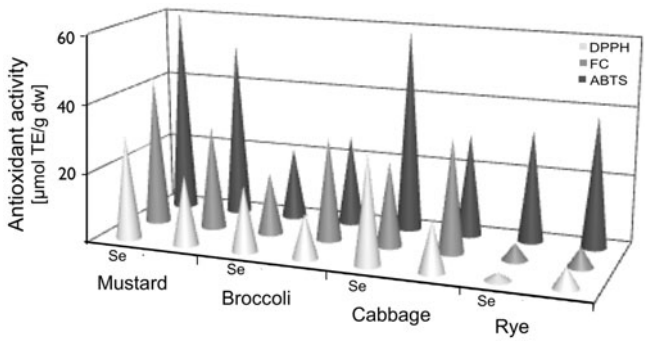

Figure 5. Antioxidant properties of regular and Se-enriched sprouts determined by DPPH, FC, and ABTS tests. The results are means of three independent determinations carried out for methanol extracts obtained from lyophilizates; SD was in the range of ± 10%.

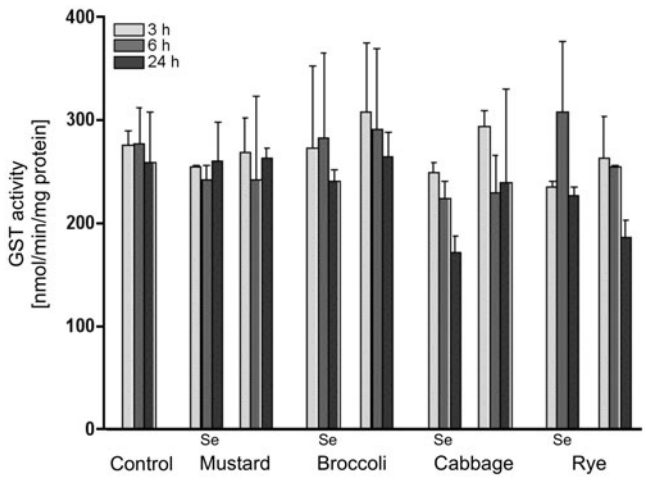

Figure 6. Activity of glutathione S-transferases in HT29 cells treated with water extracts (10% v/v in cultured medium) from regular and Se-enriched sprouts. The results are means of three independent determinations ± SD.

Sharma et al., 2009). Although, in the case of the studied sprouts, the rate of conversion into ITC was not high, we investigated their biological activity as it could be potentially influenced also by other degradation products of GLs.

As already mentioned, one of the most important anticarcinogenic functions of ITC is the stimulation of the expression of detoxification enzymes, resulting in a more efficient xenobiotic transformation and release from the organism (Latté et al., 2011). This mechanism is initiated by the reaction of ITC with GSH and its depletion (Kensler & Wakabayashi, 2010), which triggers the nuclear factor Nrf2-dependent signalization pathway controlling expression of cytoprotective genes with the ARE sequence in their promoter regions. Our previous studies showed that 10% (v/v) addition of fresh white cabbage juice to culture medium, significantly increased activity of Nrf2-dependent enzymes such as GST and NQO1 in HepG2 and HT29 cells (Kusznierewicz et al., 2007, 2010). In the current study, the influence of extracts obtained from lyophilized, Se-enriched *Brassica* sprouts on GST (Figure 6) and NQO1 (Figure 7) activity in HT29 cells was investigated. No significant differences in any of the two enzyme activities were observed in cells exposed to sprout extracts, either between Se-enriched and regular sprouts or when compared with control. The fact that, in contrast to earlier studies carried out for mature plants, the sprouts used in current research did not stimulate GST or NQO1 activity can be explained by the very low rate of GL degradation to bioactive ITC and indoles (Table 2). Our observations thus confirm the primary role of these two groups of components in cancer chemoprevention. It is also important to note that our results suggest that the induction of phase II enzymes is not caused by other GL degradation products, such as nitriles or epithionitriles, which were predominantly found in the studied sprout extracts.

The lack of stimulation of cytoprotective mechanisms in HT29 cells was disappointing. Though, in the case of selenium supplementation this neutral biological impact of sprouts can be perceived as an advantage. Above all, no significant differences in cytotoxicity toward HT29 cells (here a model of the human alimentary tract) were seen after the addition of extracts obtained from regular and Se-enriched cabbage sprouts (data not shown). Furthermore, at the relevant concentration and time of exposure, i.e., 6-h incubation of cells with 10% of extracts, only about 20% decrease of cell growth was observed in relation to the non-treated cells. Assuming that the fasting volume of gut fluids

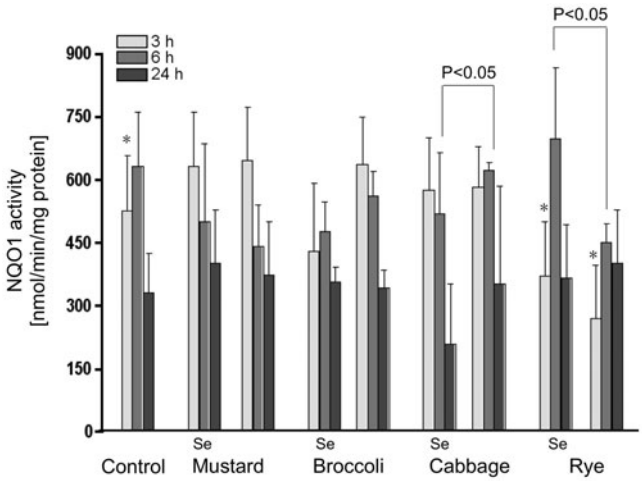

Figure 7. Activity of quinone oxidoreductase (NQO1) in HT29 cells treated with water extracts (10% v/v in cultured medium) from regular and Se-enriched sprouts. The results are means of two independent determinations ± SD. Significantly different values determined by two-tailed Student's *t*-test were marked as $p < 0.05$ between samples or (*) compared with the control.

is 115 mL (ca. 100 mL intestine, 15 mL colon according to Schiller et al., 2005), 30 µg of selenium supplementary intake, corresponding to 1 g of broccoli or cabbage sprouts and 2.78 g of mustard sprouts, the 'sprout concentration' would not even reach 2.5% v/v. Therefore, the observed low cytotoxicity toward human cultured cells, which probably resulted from low ITC and indole content in the cabbage sprout extracts used, suggests no risk of toxicity also in humans.

Conclusions

We have demonstrated that the germination of seeds watered with SeO_2 addition at a concentration of 10 mg/mL is an efficient way of enriching *Brassica* sprouts with selenium without visible impact on plant physiology, according to the indices used. The selenium uptake had no considerable influence on the content of bioactive compounds, including antioxidants and GLs. Neither the composition of GLs in *Brassica* sprouts, nor the enzymatic activity of myrosinase responsible for GL hydrolysis, nor the composition of detected GL breakdown products were significantly affected. In all the studied *Brassica* sprouts, the conversion to health-promoting ITC and indoles was very low. The formation of nitriles, epithionitriles, and thiocyanates, as it turned out less biologically relevant breakdown products, dominated. Low ITC concentration explains the observed lack of induction of GST and NQO1 activities in human colon cancer HT29 cells treated with sprout extracts, regardless of Se-enrichment. The observed neutral effects of selenium on the physiology of *Brassica* sprouts and their neutral biological effects in human cells suggest that Se-enriched *Brassica* sprouts may be recommended as a good and safe means for combating selenium deficiencies in the diet.

Our studies have also allowed for an unforeseen and very important conclusion. GL degradation products such as nitriles, epithionitriles, and thiocyanates do not seem to trigger detoxification mechanisms responsible for the anticarcinogenic properties of *Brassica* vegetables.

Declaration of interest

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