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THE ANTIOXIDATIVE PROPERTIES OF WHITE CABBAGE (*BRASSICA OLERACEA* VAR. *CAPITATA F. ALBA*) FRESH AND SUBMITTED TO CULINARY PROCESSING

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ABSTRACT

White cabbage, fresh or fermented belongs to major dietary ingredients in Central Europe. Here we concentrated on antioxidative properties of cabbage since such activities are believed to prevent undesirable effects in human organism. These properties were assessed for juices in cell-free system by antioxidant capacity, in HT29 cells by protection against DNA oxidation, and stimulation of GSTs and DNA repair. Although cabbage does not possess high antioxidative potential compared with other plantborne foods, it may provide a very effective antioxidative barrier especially if culinary processing caused the release of antioxidants. All juices increased GST activities to various degree regardless of cabbage origin or year of cultivation and stimulated DNA repair enzymes. Cabbage juices also prevented DNA damage when applied to HT29 cells concomitantly with H_2O_2; however, no protective effect was seen after prolonged (24 h) incubation with these juices prior to ROS exposure. Interestingly, in this tumor cell line, both cabbage juices displayed moderate inhibitory effect on cell growth and induced DNA fragmentation readily detectable by comet assay.

PRACTICAL APPLICATIONS

The presented research demonstrated different antioxidative activities for cabbage juices, that is, the actual food component, at dietarily relevant concentrations, which allows one to draw practical conclusions. Technologically, the direct ability to scavenge ROS, especially its increase upon heat processing, suggests that cabbage may protect other food components against undesirable oxidative changes. In another study, we confirmed that animal fats low in natural antioxidants, therefore very liable to thermooxidative spoilage, are effectively protected by cabbage phytocomplex. Biologically, the ability to induce in human cells a variety of protective mechanisms at low concentrations implies that the consumption of this vegetable should be recommended. However, our results also demonstrated dangers of overdosing. The exposure to cabbage juices prior to ROS strongly sensitized cells to oxidative stress and in this context, the use of concentrated extracts from cruciferous vegetables, offered by several producers as dietary supplements, seems to require more careful consideration.

INTRODUCTION

Reactive oxygen species (ROS) are widely believed to be involved in the etiology of many diseases as indicated by markers of oxidative stress in such clinical conditions as atherosclerosis, diabetes, neurological disorders (Moskovitz *et al.* 2002; Ferrari and Torres 2003), cancer (Hussain *et al.* 2003; Valko *et al.* 2004), as well as in the aging processes (Sohal *et al.* 2002). ROS are generated in human body by normal physiological processes, most of all during aerobic metabolism. Their production considerably increases during inflammatory responses and is also stimulated by ingested or inhaled xenobiotics. ROS are highly reactive to biological molecules and can damage DNA, proteins, carbohydrates as well as lipids. Apart from chemical reactivity, it was demonstrated in numerous studies that ROS are able to modulate gene expression, cell growth and signal transduction pathways (Menon and Goswami 2007). Mutations as an aftereffect of oxidative DNA damage, impairment of signaling pathways and modification of key cancer-related proteins lead to deregulation of cellular homeostasis and drive carcinogenesis; therefore, protection against oxidative stress has become a key issue in cancer chemoprevention (Hussain *et al.* 2003; Valko *et al.* 2004).

ROS overload can jeopardize cell function, but defense mechanisms exist that protect cells from oxidants and radicals. The endogenous antioxidant barrier is enhanced by dietary antioxidants present mainly in fruits and vegetables (Thompson *et al.* 2005). These antioxidants may be working directly as

ROS scavengers or indirectly by chelating metals or boosting organism's own defense system. Their various protective activities underlie the great interest in applying plant antioxidants in cancer prophylaxis (Ferrari and Torres 2003; Finley 2005; Seifried *et al.* 2007).

Studies on dietary protection against oxidative stress usually concentrate on fruits and vegetables that are rich sources of such antioxidants as flavonoids or carotenoids. We took different perspective and concentrated our interest not that much on plantborne foods with high antioxidative potential, but on white cabbage (*Brassica oleracea* var. *capitata* f. *alba*), a vegetable whose intake is potentially of great impact as it is consumed in many regions around the world and constitutes a major ingredient of Central European diet. Cabbage belongs to cruciferous vegetables shown to be more strongly associated with cancer protection than vegetable consumption in general (Finley 2005). The anticarcinogenic properties of crucifers are most frequently ascribed to glucosinolates (GLS) and products of their decomposition – isothiocyanates (ITC) and indoles, while the most studied mechanisms are modulation of phase I and II enzymes (Steinkellner *et al.* 2001; Pool-Zobel *et al.* 2005) and induction of apoptosis of tumor cells (Pappa *et al.* 2006; Mas *et al.* 2007). The antioxidative potential of these vegetables has drawn relatively little attention so far. However, crucifers, among them cabbage, also contain antioxidative compounds (Kusznierewicz *et al.* 2008), including flavonoids (Hertog *et al.* 1992) as well as vitamin C (Podsedek 2007). GLS (Barillari *et al.* 2005) and ITC (Fahey and Talalay 1999) were shown to display some antioxidative potential as well. Another, probably very important, mechanism by which ITC may influence cellular redox status results from the demonstrated ability of these compounds to react with SH group of cysteine in GSH (Zhang and Callaway 2002) and probably also in proteins including thioredoxin, which in turn may serve as a trigger of ARE-mediated gene expression. Among ARE inducible genes are those coding protective enzymes and transcription factors associated with cell proliferation and death (Pool-Zobel *et al.* 2005; Eberhardt and Jeffery 2006).

The research described here was aimed at determination of the antioxidative potential of white cabbage phytocomplex at several levels: as a ROS scavenger, modulator of endogenous antioxidative defense, as well as inducer of repair of oxidant-induced DNA damage. In contrast to reports demonstrating such activities for isolated compounds, our experiments were carried out for natural juices obtained from fresh or culinary processed vegetables and for concentrations to which cells of alimentary tract may be exposed after consumption of a typical meal containing cabbage. By this approach, we tried to consider two distinct matrices in parallel, namely, the food matrix in its natural complexity and the cellular matrix serving as a model of a human body after food consumption.

MATERIALS AND METHODS

Chemicals and Biochemicals

Agarose was purchased from Promega (Mannheim, Germany); GSH, ABTS, DAPI, OPT were from Sigma Chemicals Co. (Taufkirchen, Germany); 1-chloro-2,4-dinitrobenzene (CDNB) and Folin–Ciocalteu's reagent were from Fluka (Steinheim, Germany); tissue culture biochemicals were from Gibco BRL (Eggestein, Germany). The glass plates coated with 0.2 mm of silica gel 60 F254 were purchased from Merck (Darmstadt, Germany). Microscope slides used for comet assay were CometSlides® from TREVIGEN (Gaithersburg, MD). All other reagents were of appropriate grade.

Preparation of Cabbage Juices

White cabbage (*Brassica oleracea var. capitata f. alba*) from conventional cultivation was purchased in a wholesale shop supplying the area of Gdansk (Northern Poland) in vegetables, while cabbage from organic cultivation was obtained form registered ecological farm "FOHAT." After removal of outer leaves, cabbage heads were cut in a shredder into ~2-mm thick strips and divided into two parts, one of which was mixed with 20 g of NaCl per kg and submitted to spontaneous fermentation in a traditional way for 2 weeks. The portions of fresh cabbage and sauerkraut were stewed for 2 h at 100C under cover. From all cabbage products, juices were obtained in such a way as to ensure minimal influence on their chemical composition (low temperature, no organic solvents). The products were blended, juices squeezed out and spun down (4,000 rpm, 30 min, 4C and 14,000 rpm, 20 min, 4C) to remove particulates. For tissue culture experiments, sauerkraut juices were neutralized to pH 7 with 5M NaOH and sterilized by passing through Millex-GP filters (Millipore). The juices were kept frozen until used.

Cell Culture

Human colon tumor HT29 cells were cultured in McCoy's medium (Sigma Chemicals Co.) supplemented with 10% of fetal calf serum and antibiotics at 37C in 5% CO_2 atmosphere.

TLC Analyses

The FCJ and SJ were aliquoted as 4-mL portions into glass vials, sealed to avoid evaporation and placed in a thermoreactor TR 300 (Merck) set on 100C. The heating was carried out up to 12 h. Every 2 h, one sample of each juice was collected and cooled to room temperature. One milliliter of each sample collected was freeze dried and extracted with methanol (1 mL).

Methanolic extracts from cabbage were applied using glass capillary onto plates coated with silica gel 60 F_{254}, 0.25 mm (Merck). Mobile phase was a mixture of chloroform and methanol (9:1 v/v). After developing and drying, TLC plates were sprayed with 0.2% DPPH solution in methanol and examined 30 min after spraying. Active antioxidant compounds appeared as yellow spots against purple background (Cavin *et al.* 1998; Galvez *et al.* 2005).

Free Radical Scavenging Activity

Three free radicals were used to determine scavenging capacity of cabbage juices. $ABTS^+$ radical cation was generated by the interaction of 7 mmol/L ABTS and 2.45 mmol/L $K_2S_2O_8$. After 12 h, the dark green solution was diluted with methanol until the absorbance reached 0.7 at 734 nm (Huang *et al.* 2005). For measurements, 1 mL of the resulting solution was mixed with 10 µL of cabbage juices unprocessed or heated at 100C. The absorbance was read 6 min after mixing at 734 nm. In DPPH method, 3.9 mL of DPPH radical solution (25 mg/L) in methanol was mixed with cabbage juices (0.1 mL). The reaction progress was monitored at 515 nm until the absorbance became stable (Huang *et al.* 2005). Photochemiluminescence (PCL) method was used to determine superoxide radical scavenging. The luminol PCL assay was carried out for cabbage juices according to the procedure described by Popov and Lewin (1999) with the ACL kit (Analytikjena, Jena, Germany). A 2.3-mL portion of reagent 1 (methanol), 0.2 mL of reagent 2 (buffer solution), 25 µL of reagent 3 (photosensitizer) and 20 µL of standard (Trolox solution in reagent 1) or sample (cabbage juice with methanol) were mixed and measured in the Photochem apparatus. A light emission curve was recorded over 180 s, using inhibition as the parameter to evaluate antioxidant potential. The antioxidant capacity was then determined by using the integral under the curve (Yen and Duh 1994).

In the case of all methods, Trolox solutions (0–2 µmol/mL) were used to generate the standard line or calibration curve. Radical scavenging capacity was expressed as Trolox Equivalents Scavenging Capacity (TEAC) per mL of juice or per gram of dry matter.

Preparation of Cytosolic Fraction from HT29 Cells

Human colon tumor HT29 cells were seeded in culture flasks (2.5×10^6 cells per 25 cm^2 flask) 24 h prior to experiments. Each flask was used to prepare a separate cytosolic fraction in which GST and DNA repair activity and GSH content were determined. The cells were treated with the cabbage juices at different doses and various times. After treatment, the cells were rinsed with PBS, harvested, suspended in cold PBS and spun down. From this moment, all operations were carried out on ice. The final cell pellet was

suspended in 0.25 mL of water and left for 10 min to swell. Then, 0.25 mL of KCl-P buffer was added (0.1 M KH_2PO_4/K_2HPO_4, 0.2 mM EDTA, 2.3% [w/v] KCl). The cells were homogenized in Dounce homogenizer and centrifuged to remove cell debris. The cytosolic fractions were stored at −85C until used.

Determination of GST Activity and GSH Content

The activity of GSTs was determined by the method of Habig *et al.* (1974). The conjugation reaction was initiated by the addition of 50 µL of cytosolic fraction derived from HT29 cells treated with cabbage juices to a mixture consisting of 940 µL of CDNB (1 mM CDNB, 2% [w/v] EtOH, 0.1 M KH_2PO_4/K_2HPO_4, pH 6.5) and 10 µL of 100 mM GSH kept at 37C. The rate of formation of the CDNB conjugate with GSH was followed spectrophotometrically at 340 nm. The GST activities were calculated as nmoles of the conjugate formed per minute per milligram of cytosolic protein at 37C, using the extinction coefficient of 9.6 mM^{-1} cm^{-1}.

GSH content was determined fluorometrically with the use of OPT as described previously (Hissin and Hilf 1976). A portion of 0.25 mL of each cytosol was mixed with the equal amount of 10% TCA to precipitate proteins and spun down (5 min, 13,000 rpm, 4C). Then 0.1 mL of the supernatant was combined with 1.8 mL of 0.1 M sodium phosphate, 5 mM EDTA, pH 8 and OPT (0.1 mL 1 mg/mL) in MeOH. The reaction was carried out at room temperature for 25 min before fluorescence measurements.

Antigenotoxic Versus Growth Inhibition Effects in HT29 Cells Exposed to Oxidative Stress and/or Cabbage Juices

The influence of FCJ and SJ on genetic material integrity in cells exposed to hydrogen peroxide was evaluated by comet assay (Hertog *et al.* 1992; Wu *et al.* 2004). Cell growth inhibition was evaluated by cell counting with Coulter Counter. Two treatment schemes were used: juice and hydrogen peroxide added concomitantly or cells preincubated with juices then treated with hydrogen peroxide. Human colon tumor HT29 cells were seeded in 24-well plates at the density of 2×10^5 cells per well for the first scheme and at 1×10^5 cells per well for the second scheme, 24 h prior to the experiments. The used concentrations of juices were adjusted so as not to alter cell growth considerably.

In the first scheme, the cells were treated with 20% (v/v) or 40% (v/v) juices and/or 300 µM H_2O_2 for 30 min. The trypsinized cells were resuspended in 2 mL of fresh medium and 0.5 mL of suspension was taken for cell counting, while 0.75 mL was for comet assay. The remaining 0.75 mL was diluted to 2 mL with fresh medium, left in the same plate for the additional 48 h and then cell number in each well was determined. In the second scheme, the cells

were preincubated with 10% (v/v) juices for 24 h, then the medium was replaced and cells exposed to 300 μM of H_2O_2 for 30 min. After the treatment, the cells were trypsinized and resuspended in 2 mL of fresh medium and divided as described above.

For comet assay, the cells were spun down (14,000 rpm, 15 min, 4C), resuspended in 1 mL of cold PBS and spun down again. Rinsing with PBS was repeated three times. Finally, the cells were carefully suspended in 50 μL of LMP agarose (1% [w/v]) warmed up to 37C and 20 μL of mixtures were pipetted onto TREVIGEN CometSlides®. The prepared slides were placed on ice and left protected from light until LMP agarose solidified. The cells were lysed by submerging the slides in lysis buffer (0.1 M EDTA, 2.5 M NaCl, 10 mM Tris-HCl, pH 10) for 1 h at 4C. After lysis, the buffer was replaced with electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH 13.3) and DNA was unwound for 30 min at 4C in a light protected container. The microscope slides were placed in an electrophoresis apparatus Kucharczyk Kometa DNA and electrophoresed in a new portion of the cold electrophoresis solution at 25 V, 300 mA for 1 h. After electrophoresis, the slides were neutralized by rinsing three times with cold neutralization buffer (0.4 M Tris-HCl, pH 7.5) and finally with cold water. The slides were stained with 20 μL of 2 μg/mL DAPI and nuclei were analyzed under fluorescence microscope BX 60 Olympus. The analysis involved counting of 100 consecutive comets divided into five categories (0–4) (Avishai *et al.* 2003).

Induction of DNA Repair Enzymes

The assessment of induction of DNA repair enzymes in HT29 cells by cabbage juices was carried out by modified comet assay based on Collins *et al.* (2003). The cells were treated for 20 min with 100 μM H_2O_2 to induce reproducible DNA damage mirrored by comets belonging mainly to category 4 (about 90%). The hydrogen peroxide-treated cells were harvested, combined with LMP agarose and placed onto CometSlides® (TREVIGEN) as described above. The cells were lysed for 60 min in 0.1 M EDTA, 2.5 M NaCl, 10 mM Tris-HCl, pH 10. After the lysis, 30 μL of cytosolic fraction derived from HT29 cells exposed to cabbage juices was placed onto one of the slide positions while 30 μL of KCl-P buffer was added to the other reference position. The microscope slides were placed in light-protected container and transferred to 37C for 20 min to allow time for repair of oxidative DNA damage induced by H_2O_2. After this incubation, the slides were processed and analyzed by comet assay procedure described above.

Statistical Analysis

Means and standard deviations were calculated for three independent experiments; typically carried out in either triplicate or duplicate. A statistical

program GraphPad PRISM 4 and Statistica 7 were used for data analysis. Depending on the type of analyzed data, different statistical tests were applied as indicated in the captions for figures (linear regression, one-way analysis of variance with Tukey's or Dunnett's test, *t*-test and Kolmogorov–Smirnov's test).

RESULTS

The portions of cabbage used in this study were derived from northern part of Poland and were purchased over years 2003–2006 in the same wholesale shop, most probably cooperating with the same producers. Culinary processing, i.e., spontaneous fermentation and heating of cabbage and sauerkraut were carried out in a typical home-like way.

Two standard tests were used to assess the anti-oxidant properties of FCJ and SJ, namely ABTS assay and DPPH assay (Fig. 1). FCJ displayed relatively low antioxidant activity around 0.5 TEAC μmoles per mL of juice. Spontaneous fermentation consistently increased this activity three to fourfold. The measured values were comparable (with significance of difference depending on sample preparation) between the years with the exception of 2005 crops for which TEAC values were significantly different from others for all assays used ($P < 0.001$ or $P < 0.05$).

In the case of FCJ, also heating at 100C increased linearly the ability to scavenge radicals; interestingly, this increase was much more evident towards DPPH radical (around sevenfold over 12 h of heating) than towards superoxide radical (Fig. 2A). These observations were confirmed by the slopes of lines calculated from linear regression: $y = 0.157x + 0.377$ ($R^2 = 0.977$) and $y = 0.039x + 0.2807$ ($R^2 = 0.984$) for DPPH and ACL, respectively. The linearity of changes was confirmed by the test ($P < 0.001$). In contrast, SJ radical scavenging ability followed similar kinetics for the both radicals; in the first 2 h it dropped, probably a result of vitamin C decomposition, then remained basically at the same level until up to 12 h of heat treatment (Fig. 2B).

The rising antioxidant potency during thermal processing suggested the changes in the chemical composition of cabbage juices. These changes were monitored with the use of methanolic extracts obtained from lyophilized cabbage and sauerkraut juices, heated at 100C in sealed vials. As can be seen in Fig. 3, the increase of radical scavenging potency of FCJ upon heating was reflected by the appearance of fast migrating spots on TLC chromatograms of methanolic extracts visualized using DPPH solution, thus representing substances with antioxidant properties. Although heat treatment of SJ also resulted in the formation of new antioxidants, the decomposition of vitamin C apparently counteracted their impact.

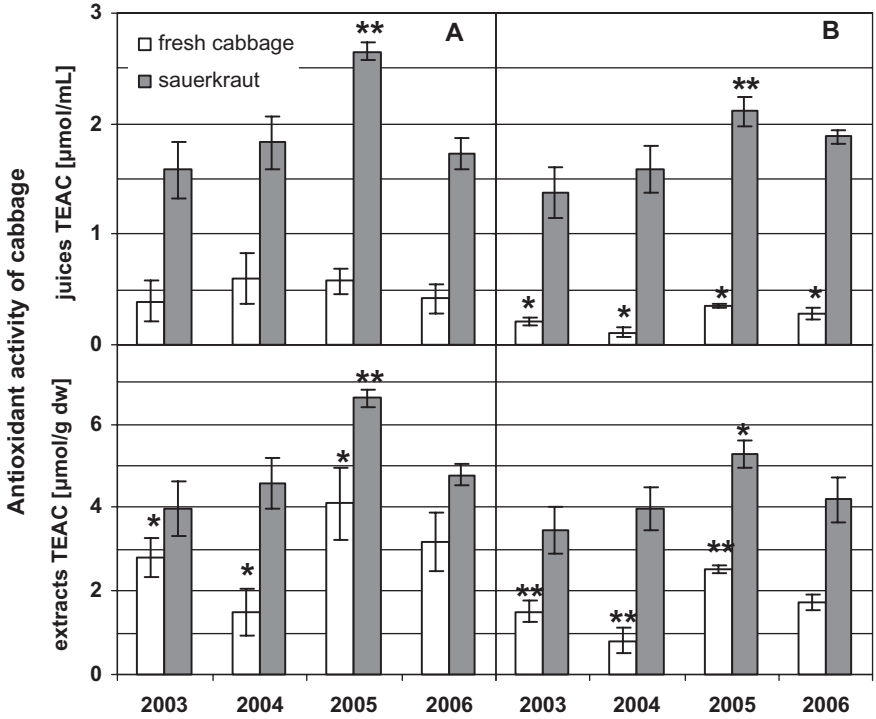


FIG. 1. THE COMPARISON OF ANTIOXIDATIVE PROPERTIES OF CABBAGES AND CORRESPONDING HOMEMADE SAUERKRAUTS DERIVED FROM CULTIVATIONS IN THE NORTHERN POLAND IN THE PERIODS 2003–2006

Total antioxidant activity was determined by ABTS assay – panel A or DPPH assay – panel B for juices or methanolic extracts obtained from fresh cabbage and sauerkraut. Results represent means ± standard deviation of three independent experiments. *, ** significantly different from other samples (analysis of variance with Tukey's multiple comparison test): $*P < 0.05$ and $**P < 0.001$.

In cells exposed to oxidative insult, similarly as in cell-free systems, antioxidative properties of plant phytochemicals may provide protection by scavenging ROS. However, in cellular system also, other mechanisms that are able to neutralize oxidants or detoxify toxic products of their reaction with cellular components may occur. Glucosinolates found in cruciferous vegetables, more precisely the products of their decomposition, are known for their ability to modulate gene expression; thus, the protection relying on boosting endogenous defenses seemed a very probable route of preventing oxidative stress also by cabbage phytocomplex. In the series of experiments described next, we tried to answer several questions. Firstly, will the induction of major detoxifying system consisting of GSTs and GSH demonstrated for

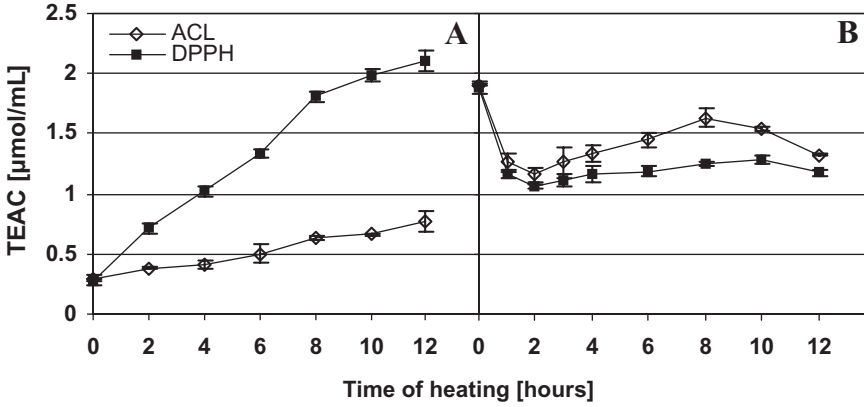


FIG. 2. THE INFLUENCE OF TIME OF HEATING ON ANTIOXIDANT ACTIVITY OF FRESH CABBAGE JUICE (2006) PANEL A AND SAUERKRAUT JUICE (2006) PANEL B AS DETERMINED BY ACL AND DPPH ASSAYS
The results represent means expressed as TEAC µmole/mL of three independent experiments.

isolated ITC be noticeable for natural food component such as FCJ or SJ at dietarily relevant doses? If so, how will this induction be affected by the culinary processing of cabbage? The concentrations of cabbage juices used in the experiments involving cultured cells were chosen, taking into account two factors. The typical meal containing cabbage may include from 10 to 80% v/v of this vegetable, hence the cells lining the alimentary tract might be exposed to the corresponding doses. In addition, based on determinations of cell growth inhibition by FCJ and SJ, carried out for each batch of cabbage by MTT (known also as MTS assay), the concentrations of juices were restricted to those not causing more than 50% of cell kill for a given time of treatment (data not shown).

Figure 4 presents results of measurements of the total GSTs and GSH levels in cytosolic fractions from HT29 cells exposed to different cabbage juices (crop 2002) for 3 h. The data are presented as percentage of the values obtained for cytosolic fraction from nontreated cells taken as 100%. As can be seen, 20 and 40% v/v FCJ was very effective in increasing the levels of both GSTs and GSH. However, the concentration 10% [v/v] seems not to be sufficient to ensure the consistent capability of GST induction; in relation to nontreated cells, no statistically significant changes were observed in cells treated with cabbage juices (data not shown). Raw SJ did not display such ability; however, after heating for 2 h at 100C, SJ also became a very effective inducer of the activities analyzed. As regards GSH measurements, the amounts determined varied strongly among replicate experiments and this was mirrored by the lack of statistical significance of difference compared with control

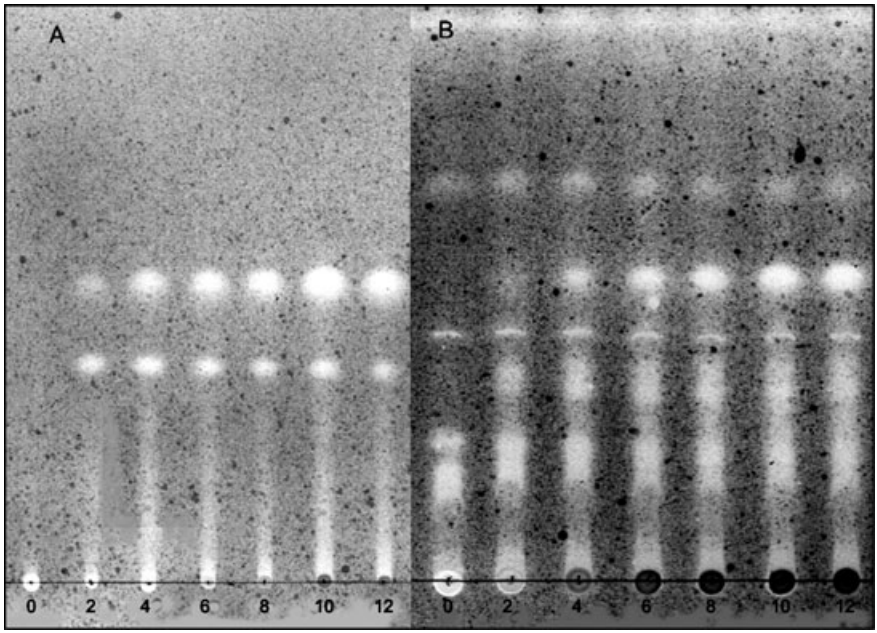


FIG. 3. TLC CHROMATOGRAMS OF THERMALLY TREATED FRESH CABBAGE JUICE (2006) (A) AND SAUERKRAUT JUICE (2006) (B) VISUALIZED BY 0.2% DPPH SOLUTION IN METHANOL
The numbers at the bottom (0–12) refer to the hours of heating.

cytosols. Overlapping processes of GSH synthesis induction and reaction of ITC with GSH may cause this variability, but closer examination of these phenomena was beyond the scope of the study.

The next series of experiments was designed to investigate the ability of FCJ and SJ to prevent DNA damage in HT29 cells exposed to oxidative insult (300 μM H_2O_2). The experimental scheme combined the evaluation of cytotoxic and genotoxic effects for two treatment schedules. In the first one, the cells were treated with H_2O_2 concomitantly with cabbage juices; thus, direct neutralization of ROS could be mainly considered as a protective mechanism. Our earlier studies showed that SJ diminished DNA degradation in human leukemia HL-60 cells exposed to H_2O_2 (Bartoszek *et al.* 2002). The other scheme involved the incubation of HT29 cells with FCJ or SJ for 24 h prior to oxidative insult, to boost the cells' own antioxidant defences.

In both schemes, the genotoxic effect was determined by comet assay, while growth inhibition was determined by cell number as described under Materials and Methods. HT29 cells turned out to be unexpectedly sensitive to comet assay procedure and about 50% of control cells exhibited some degree

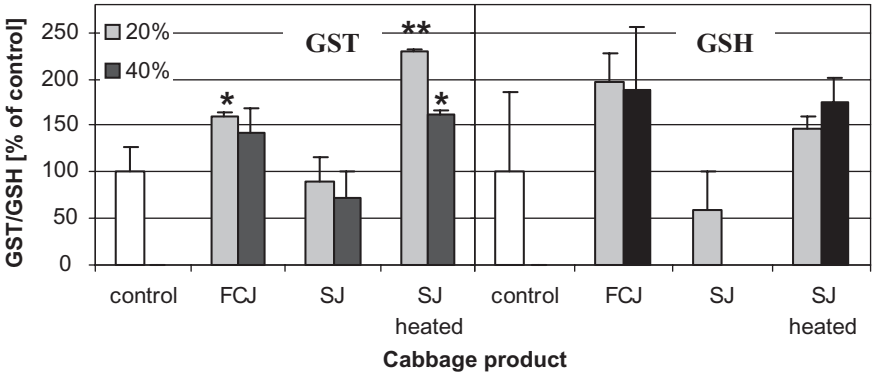
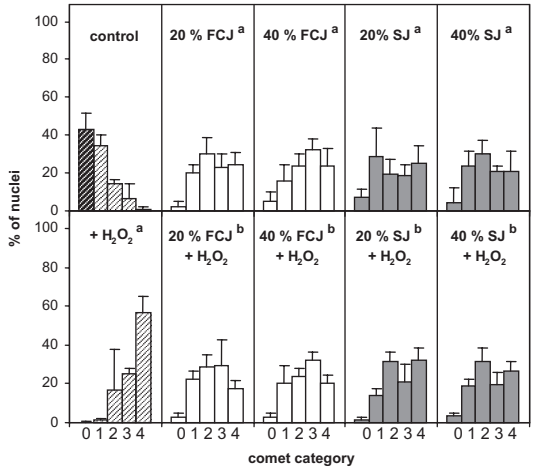


FIG. 4. THE GST ACTIVITY AND GSH CONTENT IN CYTOSOLIC FRACTIONS OBTAINED FROM HT29 CELLS TREATED FOR 3 H WITH CABBAGE JUICES INDICATED (2002 CROP)
The results are means ± standard deviation from triplicate activity measurements carried out for three independent cell incubations. The statistical significance of differences between control versus treated samples was determined by one-way analysis of variance with Dunnett's multiple comparison test: $*P < 0.05$ and $**P < 0.001$.

of DNA damage (Fig. 5A). However, the application of H_2O_2 increased the extent of DNA fragmentation strongly enough to make the assessment of protection possible. To our surprise, it turned out that both FCJ and SJ at concentrations used (20 and 40% v/v) and short incubation time amounting to 30 min in the first scheme, brought about the substantial level of DNA fragmentation (Fig. 5A). The statistically significant difference between control and juice-treated cells was confirmed by Kolmogorov–Smirnov's test ($P < 0.001$). This was mirrored by the decline in cell count right after treatment (t-test, $P < 0.05$), which was, however, no longer seen after 48-h incubation in fresh medium (Fig. 5B) (not statistically different from control). The combined treatment, cabbage juice and H_2O_2, caused neither increased level of DNA damage nor enhancement of growth inhibition compared with FCJ or SJ applied alone (no statistically significant difference compared with values obtained for cultures not treated with H_2O_2). The treatment with H_2O_2 alone inhibited cell growth by about 20% as assessed after 48-h incubation in fresh medium, but the combination of H_2O_2 and cabbage juice displayed no cytotoxicity after the same period of postincubation (not statistically different from control). Actually, it seemed that FCJ and SJ enabled mechanisms by which the cells quickly made up losses caused by oxidative insult.

In the experimental scheme involving preincubation of HT29 cells with cabbage juices followed by treatment with H_2O_2 (Fig. 6), we expected that the cells will tolerate better the exposure to ROS owing to the induction of endogenous antioxidative defense. It turned out that just contrary to our

A



B

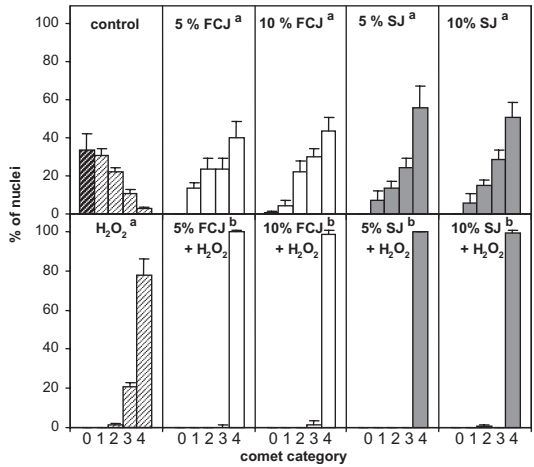
	No treatment (control)	Treatment conditions								
		20% FCJ	40% FCJ	20% SJ	40% SJ	300 μM H_2O_2	20% FCJ + H_2O_2	40% FCJ + H_2O_2	20% SJ + H_2O_2	40% SJ + H_2O_2
Just after treatment	100	86.5±7.8	88±4.2	77±3.6*	72±12.1**	73.3±2.9*	86.7±14.2	84.3±14.0	84±13.5	66.7±3.8**
	-	-	-	-	-	100	118.3±14.5	115± 14.3	114.6±13.8	91±4.8
48 h post-incubation in fresh medium	100	93±0.7	96±0.7	102±2.2	93.3±2.1	82 ±15.7*	103.3±2.5	103±4.4	97±7.6	94.7±8.1
	-	-	-	-	-	100	126±15.9	125.6±16.3	118.3±17.4	115.5±17.7

FIG. 5. GENOTOXIC EFFECT (PANEL A) AND CELL GROWTH INHIBITION INDUCED (PANEL B) BY H_2O_2 APPLIED TO HT29 CELLS FOR 30 MIN IN COMBINATION WITH EITHER FRESH CABBAGE JUICE (FCJ 2005) OR SAUERKRAUT JUICE (SJ 2005) AT CONCENTRATIONS INDICATED

After treatment, the cells were harvested by trypsinization, counted to determine cell number, then submitted to comet assay or 48-h postincubation in fresh medium. Growth inhibition determinations are given as % of control (Panel B) assuming the number of either nontreated cells or H_2O_2-treated cells as 100%. All results presented are means± standard deviation of three independent experiments carried out in duplicates. The statistical analysis of genotoxicity assessments (Panel A) was performed by Kolmogorov–Smirnov test: [a]statistically different from control cells ($P < 0.001$); [b]statistically different from H_2O_2-treated cells ($P < 0.001$). *, ** significantly different from nontreated control cultures (t-test: $^*P < 0.05$, $^{**}P < 0.01$).

expectations, 24-h pretreatment with both FCJ and SJ used at nontoxic concentrations (1 [not shown], 5 or 10% v/v) rendered cells more sensitive to H_2O_2 treatment. Also, the exposure to cabbage juices here increased DNA damage significantly (Fig. 6A) compared with nontreated cells ($P < 0.001$). However, in contrast to short incubation scheme, the combined treatment, i.e., cabbage juice followed by H_2O_2, caused significantly higher level of DNA

A



B

	No treatment (control)	Pre-treatment conditions +/- H_2O_2 treatment in fresh medium								
		5% FCJ	10% FCJ	5% SJ	10% SJ	300 µM H_2O_2	5%FCJ + H_2O_2	10% FCJ + H_2O_2	5% SJ + H_2O_2	10% SJ + H_2O_2
Just after treatment with H_2O_2	100	65±10.1	35.7±3.8	57.3±6.7	41±8.5	75.7±9.2	47±7.2	36±3.6	45.7±5.8	34.7±2.3
	-	-	-	-	-	100	62.1±11.7	47.6±9.9	60.5±10.9	45.8±9.5
48h post-incubation in fresh medium	100	72.3±0.6	33±4.5	68±1.0	33±5.2	40.3±11.7	27.6±13.6	15.3±2.9	26±11.3	14.7±2.3
	-	-	-	-	-	100	68.5±17.9	38±12.1	64.5±16.3	36.5±11.9

FIG. 6. GENOTOXIC EFFECT (PANEL A) AND CELL GROWTH INHIBITION INDUCED (PANEL B) BY H_2O_2 APPLIED FOR 30 MIN TO HT29 CELLS PRETREATED FOR 24 H WITH EITHER FRESH CABBAGE JUICE (FCJ 2005) OR SAUERKRAUT JUICE (SJ 2005) AT CONCENTRATIONS INDICATED

After treatment with H_2O_2, the cells were harvested by trypsinization, counted to determine cell numbers, then submitted to comet assay or 48-h postincubation in fresh medium. All results presented are means± standard deviation of three independent experiments carried out in duplicates. Growth inhibition determinations are given as % of control (Panel B) assuming the number of either nontreated cells or H_2O_2-treated cells as 100%. The inhibition of cell growth was statistically significant compared with control cells regardless of treatment (*t*-test, $P < 0.0001$). The statistical analysis of genotoxicity assessments (Panel A) was performed by Kolmogorov–Smirnov test: [a]statistically different from control cells ($P < 0.001$); [b]statistically different from H_2O_2-treated cells ($P < 0.025$).

fragmentation than H_2O_2 applied to nontreated cells ($P < 0.001$). These genotoxic effects were translated into increased growth inhibition (Fig. 6B), where cell numbers just after treatment and 48 h later were significantly decreased compared with control growth ($P < 0.0001$) as well as compared with cells exposed to H_2O_2 alone ($P = 0.002$ and $P = 0.009$ just after treatment and 48 h

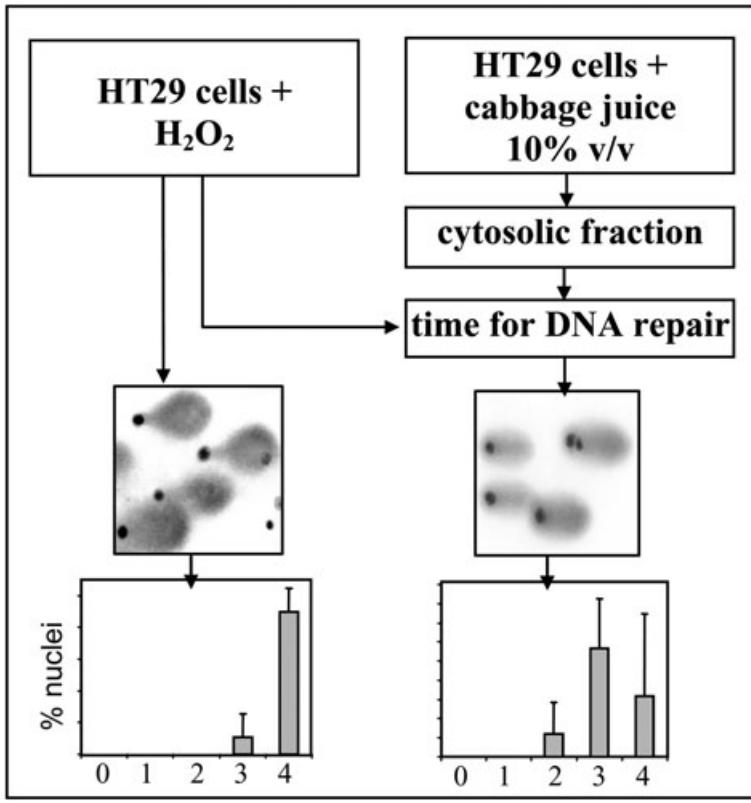


FIG. 7. THE EXPERIMENTAL SCHEME AND SAMPLE RESULTS OF DETERMINATIONS OF OXIDATIVE DNA DAMAGE REPAIR BY CYTOSOLIC FRACTIONS DERIVED FROM HT29 CELLS PRETREATED WITH CABBAGE JUICES

later, respectively). Thus, increased sensitivity towards ROS was seen at DNA damage level, as well as being mirrored by stronger cell growth inhibition (Fig. 6A,B, respectively).

Finally, we investigated whether cabbage phytocomplex can influence the efficiency of repair of ROS-induced DNA damage, which could explain the observed recovery of FCJ- or SJ-treated cells after oxidative insult in the short incubation protocol. The stimulation of mechanisms responsible for DNA repair was determined by modified comet assay according to experimental scheme depicted in Fig. 7. For these experiments, two populations of HT29 cells were used. One was treated with H_2O_2 to induce high level of DNA damage (~90% of comets from class 4), while the other population of cells was exposed to 10% v/v FCJ or SJ for different periods of time (3, 6 or 24 h). The

H_2O_2-treated cells were immobilized in LMP agarose and placed on CometSlides®, then incubated for 25 min with cytosolic fractions from control cells or cells exposed to cabbage juices to allow the repair of DNA damage; to reference slides, buffer was added instead. It was assumed that if cabbage juices displayed the ability to trigger expression of DNA repair enzymes, such *de novo* synthesized enzymes would appear initially in cytosolic fraction before reaching their target destination, i.e., nucleus. The DNA repair capacity of the cytosolic fractions was measured as the reversal of H_2O_2-induced DNA damage, that is, shortening of comet tails after comet assay in samples incubated with cytosolic fractions from cells either not exposed or exposed to FCJ or SJ compared with control cells incubated with the same volume of buffer. The statistical significance of distribution of nuclei among different comet categories was analyzed by Kolmogorov–Smirnov's test.

As shown in Fig. 8, both cabbage juices stimulated the activity of enzymes involved in repair of ROS-induced DNA damage quite effectively, though the kinetics was different for FCJ and SJ. The addition of control cytosolic fraction, that is, from HT29 cells not exposed to cabbage juices, to H_2O_2-treated cells caused no change in the level of DNA damage for all time points indicated ($P > 0.1$). In the case of cytosols derived from cells exposed to 10% v/v FCJ for 3 or 6 h, the statistically significant decrease of DNA fragmentation was seen compared with control cytosols ($P < 0.005$ and $P < 0.001$, respectively). However, after 24 h, the decline of activity of repair enzymes was observed, in the case of FCJ even below that in control cells ($P > 0.1$). Also, 10% v/v SJ exhibited the ability to stimulate mechanisms involved in DNA repair, which, however, required longer exposition times: 6 and 24 h. The repair of DNA lesions in H_2O_2-treated cells incubated with cytosols from HT29 cells exposed to 10% v/v SJ brought about statistically significant change in distribution of nuclei among comet categories ($P < 0.001$ and $P < 0.05$, for 6- and 24-h exposure, respectively).

DISCUSSION

Antioxidant dietary compounds are important for limiting damaging oxidative reactions in the cells, which may predispose to the development of major clinical conditions such as heart disease and cancer. The vegetables belonging to *Brassicae* are not especially rich source of substances with antioxidant or antiradical activity; therefore, they are rather not regarded as antioxidant foods. However, when considering the ability of a particular food item to prevent oxidative damage, even more important than amounts of antioxidants consumed may be other biological activities of phytochemicals that influence the impact of the exposure to oxidants. These biological

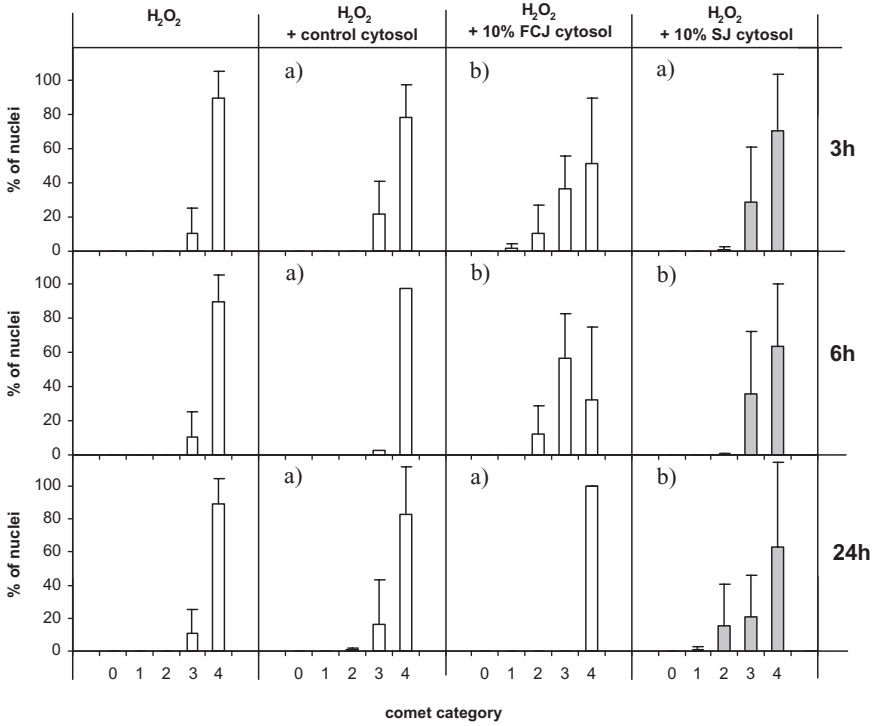


FIG. 8. THE INDUCTION OF DNA DAMAGE REPAIR IN HT29 CELLS BY FRESH CABBAGE OR SAUERKRAUT JUICE 2006

The HT29 cells were treated with H_2O_2, lysed on microscope slides and postincubated for 25 min with cytosolic fractions derived from control HT29 cells or exposed to 10% v/v juices for times indicated. The experimental design is well as the way explained in Fig. 7. Results are means ± standard deviation of triplicate determinations carried out for three independent experiments. Statistical analysis was carried out by Kolmogorov–Smirnov test: a) $P > 0.1$, not different from H_2O_2-treated cells (column H_2O_2); b) $P < 0.05$, statistically different from H_2O_2-treated cells postincubated with cytosols isolated from control cells (column H_2O_2 + control cytosols).

activities encompass the modulation of the activity/expression of enzymes detoxifying ROS (e.g., catalase) or products of oxidant-induced damage (e.g., GST/GSH conjugation of the products of lipid peroxidation) or repair of damages incurred (e.g., DNA repair enzymes). In this study, we demonstrated that the natural phytocomplex of white cabbage, both fresh and processed, at relevant doses regarding normal dietary consumption, seemed to enhance the antioxidative barrier at all these levels.

White cabbage, the subject of this study, has been ranked as 18 (14–24 depending on the assay) among 33 vegetables evaluated (Pellegrini *et al.*

2003) with the content of quercetin and kaempferol, the most common flavonoids, below the level of detection (Hertog *et al.* 1992). Nonetheless, because it is an ample dietary ingredient, when recalculated as TEAC mmoles per serving, it was graded similarly as carrot or tomato (Wu *et al.* 2004). Moreover, we have shown that antioxidant properties of fresh cabbage gradually increase upon heating for many hours, that is, during typical cooking procedure used while preparing meals, especially meat meals, containing this vegetable. It is an extremely important observation as it suggests that cabbage cooked with other food components particularly liable to oxidation such as fats, may prevent their deterioration. In the case of fats, this would diminish formation of lipid radicals and lipid-derived genotoxins, thus improving the quality of heat-processed food products. The very substantial ability of cabbage juice components to prevent thermooxidative changes of rapeseed oil and lard, the two types of fats which are most often cooked with this vegetable, has been recently demonstrated by our team (Tynek *et al.* 2008). Thus, naturally occurring cabbage phytocomplex can limit undesirable oxidative processes by direct ROS scavenging also after culinary processing such as fermentation or heating.

As mentioned before, one of the most important "indirect" antioxidative actions of cabbage phytochemicals would be the stimulation of the expression of genes involved in ameliorating undesirable effects of ROS. The induction of phase II enzymes by isolated compounds, extracts or ingested plants from *Brassicae* genus has been well documented *in vitro*, *in vivo* as well as in humans (extensively reviewed in IARC Handbooks of Cancer Prevention 2004), hence could be expected also for cabbage. Our research concentrated on GSTs that constitute a family of phase II enzymes involved in the detoxification of xenobiotics as well as toxic metabolites formed endogenously and GSH which is a major cellular antioxidant, but also the acceptor molecule of activated toxic substances, including those formed as a result of ROS attack on cellular biomolecules, in the conjugation reaction catalyzed by GSTs.

The certain levels of stimulation of activity of GSTs by FCJ and SJ were observed already after 3-h exposure of HT29 cells to these cabbage juices (Fig. 4). Higher concentrations (40% v/v) lowered GSTs activity in cells treated with FCJ as well as SJ raw or heated SJ. FCJ and SJ also modulated GSH level, but it was difficult to obtain reproducible results under experimental conditions applied (probably because of running in parallel reaction between GSH and ITC changing the concentration of the former).

These results confirmed that cabbage phytochemicals in their natural composition and at doses expected during normal daily consumption may prevent damage to biomolecules by removing ROS, and presumably toxic compounds generated by them, from cellular environment via stimulation of cell's own defense. The next question we asked was if nonetheless oxidative

damage occurred, and would its repair be augmented owing to exposure of cells to cabbage juices. Our experiments also gave positive answer to this question at least as regards ROS-induced DNA damage. Cytosolic fractions from HT29 cells exposed to FCJ or SJ exhibited increased ability to repair DNA damage induced by H_2O_2 compared with those from control cultures. The enhanced repair of DNA damage determined for cytosols derived from cells exposed to cabbage juices, especially FCJ, might have resulted from *de novo* synthesis of DNA repair enzymes as they reside in the nucleus, but without mRNA level measurements, it remains only an attractive hypothesis. In general, the increase of the mentioned activities preventing oxidative insult occurred to different extents for FCJ and SJ juices and followed different kinetics, which is not surprising as fresh and fermented cabbage vary in the chemical composition and this must be reflected by their biological properties.

The ability to induce GSTs and to increase GSH level in cells could be anticipated based on data found in literature. These abilities were demonstrated for sulforaphane, the ITC isolated from broccoli but found also in cabbage (Fahey and Talalay 1999). However, to our best knowledge, the modulation of activity of enzymes responsible for the repair of ROS-induced DNA damage by natural food component has been reported before only for kiwifruit and the enhancement was ascribed to an increased stability of OGG1 protein (Collins *et al.* 2003).

The ROS scavenging ability of FCJ and SJ was also investigated in cellular system. The HT29 cells exposed to H_2O_2 concomitantly with these juices showed much lower degree of DNA degradation compared with cells treated with H_2O_2 on its own. The assessment of growth inhibition of combined treatment suggested that HT29 cells seemed to recover more efficiently from oxidative stress because of accelerated rate of proliferation. Only in cell cultures treated with cabbage juices, the decline of cell number measured right after treatment was no longer seen after 48 h postincubation in fresh medium. The treatment of HT29 cells with cabbage juices brought about more surprises. Firstly, both FCJ and SJ turned out to cause substantial DNA fragmentation in cells as demonstrated by comet assay. However, the extent of DNA damage was easily tolerated by the cells and no inhibition of their growth was seen after 48 h of postincubation in fresh medium. Secondly, preincubation with the cabbage juices, in a broad range of nontoxic concentrations from 1 to 10% v/v did not "immune" the cells against ROS attack as could be expected, taking into account the presence of antioxidants as well as the induction of enzymes involved in the protection against oxidative stress. On the contrary, the exposure of HT29 cells for 24 h to FCJ and SJ prior to H_2O_2 treatment increased the genotoxic effect of the latter as well as overall cytotoxicity of such combined treatment.

The said observations make it obvious that cabbage phytocomplex acts via different route(s) than those anticipated in the case of direct scavenging of ROS or products of their reaction with biomolecules. It is difficult to decide what caused the enhancement of H_2O_2 cytotoxicity shown in the case of cells pretreated with cabbage juices for 24 h. It is even more difficult to explain the appearance of comets in the case of cell cultures exposed to FCJ and SJ for 30 min, though this observation is in accord with earlier reports describing such genotoxic effects as the induction of chromosomal aberrations and sister chromatid exchanges by cabbage juices in mammalian cells following 60-min treatment (Kassie *et al.* 1996). The lack of correlation between genotoxic effect and growth inhibition after 48-h postincubation in fresh medium, typical procedure in cell growth inhibition assays, is extremely puzzling and we cannot explain these results at the moment. It is tempting to speculate that the ability of ITCs to react with sulfhydryl and amino groups might alter genome function in many ways. It may cause already postulated transient depletion of GSH altering cellular redox status and thereby modulate expression of ARE dependent as well as redox-sensitive genes (Loo 2003; Eberhardt and Jeffery 2006). However, it is also possible that ITCs may become involved in chemical modifications of chromatin components and thereby influence chromatin structure with difficult to predict consequences. The depletion of GSH could be responsible for the sensitization to H_2O_2 exposure observed by us following 24-h pretreatment of HT29 cells with cabbage juices. The modification of chromatin could explain the genotoxicity of cabbage phytochemicals and clearly require closer examination as regards risk/benefit ratio for at least cells lining alimentary tract, both normal as well as those entering the pathway of carcinogenic transformation.

To conclude, the presented investigations have shown that the biological activities demonstrated so far mainly for pure bioactive compounds isolated from cruciferous vegetables are seen for the food item in its natural form or after typical culinary processing at concentrations that can be expected in alimentary tract after cabbage consumption.

NOMENCLATURE

ABTS, 2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diamonium salt; ARE, antioxidant response element; DAPI, 4′,6-diamino-2-phenylindol; DPPH, 1,1-diphenyl-2-picrylhydrazyl; FCJ, fresh cabbage juice; GLS, glucosinolates; GST, glutathione-S-transferases; GSH, glutathione; ITC, isothiocyanate; LMP agarose, low melting point agarose; PBS, phosphate buffered saline; OPT, o-phthalaldehyd; ROS, reactive oxygen species; SJ,

sauerkraut juice, TEAC, Trolox equivalent anti-oxidant capacity; TLC, thin layer chromatography.

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