Available online at www.sciencedirect.com
SCIENCE DIRECT®
Phytochemistry 65 (2004) 1273–1281
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Heating decreases epithiospecifier protein activity and increases sulforaphane formation in broccoli

Nathan V. Matusheski [a], John A. Juvik [b], Elizabeth H. Jeffery [a,*]

[a] Department of Food Science and Human Nutrition, University of Illinois, 499 Bevier Hall, 905 South Goodwin Avenue, Urbana, IL 61801, USA
[b] Department of Natural Resources and Environmental Sciences, University of Illinois, 1102 South Goodwin Avenue, Urbana, IL 61801, USA

Received 12 December 2003; received in revised form 19 March 2004
Available online 19 May 2004

Abstract

Sulforaphane, an isothiocyanate from broccoli, is one of the most potent food-derived anticarcinogens. This compound is not present in the intact vegetable, rather it is formed from its glucosinolate precursor, glucoraphanin, by the action of myrosinase, a thioglucosidase enzyme, when broccoli tissue is crushed or chewed. However, a number of studies have demonstrated that sulforaphane yield from glucoraphanin is low, and that a non-bioactive nitrile analog, sulforaphane nitrile, is the primary hydrolysis product when plant tissue is crushed at room temperature. Recent evidence suggests that in *Arabidopsis*, nitrile formation from glucosinolates is controlled by a heat-sensitive protein, epithiospecifier protein (ESP), a non-catalytic cofactor of myrosinase. Our objectives were to examine the effects of heating broccoli florets and sprouts on sulforaphane and sulforaphane nitrile formation, to determine if broccoli contains ESP activity, then to correlate heat-dependent changes in ESP activity, sulforaphane content and bioactivity, as measured by induction of the phase II detoxification enzyme quinone reductase (QR) in cell culture. Heating fresh broccoli florets or broccoli sprouts to 60 °C prior to homogenization simultaneously increased sulforaphane formation and decreased sulforaphane nitrile formation. A significant loss of ESP activity paralleled the decrease in sulforaphane nitrile formation. Heating to 70 °C and above decreased the formation of both products in broccoli florets, but not in broccoli sprouts. The induction of QR in cultured mouse hepatoma Hepa lclc7 cells paralleled increases in sulforaphane formation.

Keywords: Broccoli; *Brassica oleracea*; Cruciferae; Cancer; Anticarcinogen; Sulforaphane; Sulforaphane nitrile; Epithiospecifier protein; Quinone reductase

1. Introduction

Cruciferous plants such as broccoli (*Brassica oleracea* var. *italica*) contain glucosinolates, secondary metabolites that are part of an elegant herbivory defense mechanism (reviewed in Bones and Rossiter, 1996). When the tissue of these plants is damaged, glucosinolates are released from the vacuoles of myrosin cells and are hydrolyzed by cytosolic myrosinase, a thioglucoside glucohydrolase enzyme (EC 3.2.3.1). The spontaneous products of this reaction at neutral pH have been shown to be isothiocyanates (Gil and MacLeod, 1980b), and these compounds are the principal products of glucosinolate hydrolysis in a number of crucifers (Cole, 1976). However, other cruciferous plants, such as rape (*Brassica napus*) form predominantly nitriles from these glucosinolates (Gil and MacLeod, 1980c; Gil and MacLeod, 1980a; Lambrix et al., 2001), indicating that different products may be formed from the same glucosinolate, depending upon the plant species.

Sulforaphane (**1**) [4-(methylsulfinyl)butyl isothiocyanate], an isothiocyanate from broccoli, was initially identified as a potential anticarcinogen by its capacity to induce quinone reductase (QR), a phase II detoxification enzyme, in Hepa lclc7 cell culture (Zhang et al., 1992). The phase II detoxification enzyme system is an inducible

* Corresponding author. Present address: Division of Nutritional Sciences, National Soyabean Research Center, University of Illinois at Urbana Champaign, 1101 W Peabody Dr, Urbana, IL 61801, USA. Tel.: +1-217-333-3820; fax: +1-217-333-8046.
E-mail address: ejeffery@uiuc.edu (E.H. Jeffery).

0031-9422/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2004.04.013

set of xenobiotic defense enzymes that can intercept bioactivated carcinogens, protecting against DNA damage in the mammal (Talalay et al., 1995). Since its identification, experimental evidence has accumulated regarding the efficacy of sulforaphane as a cancer preventative agent in a number of animal models of carcinogenesis. Sulforaphane has been shown to inhibit chemically-induced cancer in rats and mice (Zhang et al., 1994; Fahey et al., 2002). Although there are no clinical studies showing prevention of cancer with sulforaphane, a recent clinical study evaluated absorption of broccoli extracts perfused into the small intestine and found that approximately 75% of the sulforaphane was absorbed (Petri et al., 2003). In that study levels of two detoxification enzymes, glutathione-*S*-transferase and UDP-glucuronosyl transferase, were induced more than 2-fold in exfoliated enterocytes released into the perfusate, indicating the potential for anticarcinogenic action of sulforaphane in these human subjects. Sulforaphane has also been observed to inhibit growth and stimulate apoptosis in several cancer cell lines in culture (Gamet-Payrastre et al., 2000; Chiao et al., 2002), indicating potential antiproliferative activity.

Although investigations of the effects of storage and cooking on the glucosinolate content of broccoli have been performed (Goodrich et al., 1989; Vallejo et al., 2003), surprisingly little research has examined the effects of storage or cooking on formation of sulforaphane in dietary broccoli. In current literature examining the chemoprotective effects of sulforaphane, the assumption is made that sulforaphane is the sole product of the hydrolysis of its precursor glucosinolate, glucoraphanin (**2**) (Fahey et al., 1997; Shapiro et al., 1998). However, a nitrile analog to sulforaphane, sulforaphane nitrile (**3**) [5-(methylsulfinyl)pentane nitrile] may actually be the predominant hydrolysis product of glucoraphanin (Matusheski et al., 2001; Mithen et al., 2003). Sulforaphane nitrile has recently been shown not to possess the anticarcinogenic properties of sulforaphane (Matusheski and Jeffery, 2001; Basten et al., 2002). Thus the potential health benefit of broccoli as a result of sulforaphane formation is compromised by the alternative formation of an inactive nitrile when broccoli is crushed.

Other cruciferous plants, including crambe seed (*Crambe abyssinica*), garden cress (*Lepidium sativum*) and other members of the *Brassica* species such as rapeseed (*Brassica napus*) and white cabbage (*Brassica oleracea*) have all been shown to form nitriles (Daxenbichler et al., 1977). A protein, called epithiospecifier protein (ESP), has been identified in some crucifers that appears responsible for the formation of epithionitriles (Tookey, 1973; Petroski and Tookey, 1982). This protein does not catalyze glucosinolate hydrolysis by itself, but instead directs the products of glucosinolate hydrolysis toward epithionitriles, rather than isothiocyanates. Epithiospecifier protein requires iron for its activity, and heat treatment has been shown to decrease the formation of epithionitriles in seeds of turnip rape (*Brassica campestris*; Kirk and Macdonald, 1974). A recent study in the experimental plant *Arabidopsis thaliana* suggests that ESP may regulate nitrile formation in addition to epithionitrile formation (Lambrix et al., 2001). Here, we test the hypothesis that ESP is present in broccoli, and supports the formation of inactive sulforaphane nitrile at the expense of formation of the potent anticarcinogen sulforaphane (Fig. 1). Our objectives were to examine the formation of sulforaphane and sulforaphane nitrile from glucoraphanin in fresh broccoli from several commercial cultivars, and to examine the effects of heating on sulforaphane and sulforaphane nitrile formation, ESP activity, and potential bioactivity of extracts prepared from fresh broccoli florets and broccoli sprouts.

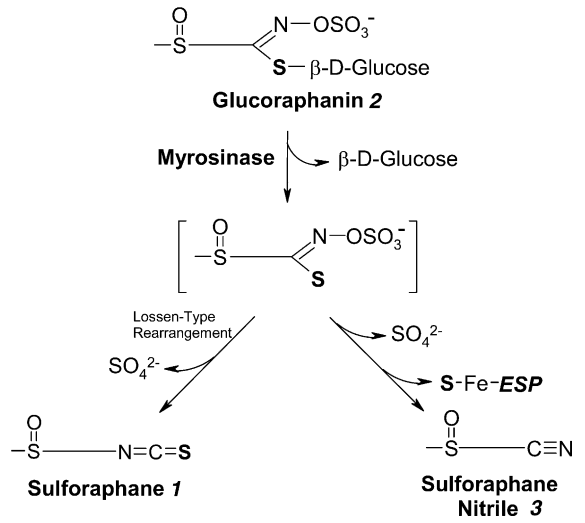

Fig. 1. Proposed mechanism for the formation of sulforaphane (**1**) and sulforaphane nitrile (**3**) in broccoli florets and sprouts.

2. Results and discussion

2.1. Glucoraphanin content of commercial broccoli cultivars

Glucoraphanin content determined on lyophilized powders of 7 commercial broccoli cultivars varied significantly, ranging from 4.4 ± 0.4 to 16.4 ± 0.9 μmol/g dry weight. Several of the cultivars examined were also represented in a previous study, where 50 broccoli accessions and a number of other cruciferous vegetable crops were examined for glucosinolate content (Kushad et al., 1999). Our results were in agreement with the earlier study, cv. Brigadier containing the highest glucoraphanin content and cv. Baccus containing the lowest. Purchased broccoli (unknown cultivar) fell within this range. Minor differences in broccoli gluco-

sinolate content may be attributable to environmental differences, but genetic differences among cultivars have been shown to play a key role in determining aliphatic glucosinolate content (Brown et al., 2002).

The large variation in glucoraphanin content observed among different cultivars currently makes it difficult to determine how much glucoraphanin may be present in dietary broccoli because of a lack of cultivar identification at the retail level. However, this large variability also indicates that an effective breeding program has the potential to produce a broccoli cultivar stably expressing increased glucoraphanin content. In a recent study, the wild species *Brassica drepanensis* and *Brassica villosa* were crossed with a double haploid broccoli breeding line (cv. Green Duke) and the resulting crosses produced a 10 fold higher concentration of glucoraphanin compared to Green Duke and Marathon commercial cultivars (Faulkner et al., 1998). A more recent study from the same group describes the development of advanced backcrossed lines of Green Duke cultivar broccoli and *B. villosa*, containing specific genomic segments from *B. villosa* capable of enhancing glucoraphanin content (Mithen et al., 2003). While increased glucoraphanin content gives greater potential for sulforaphane formation, this does not specifically address the problem of nitrile formation in fresh broccoli.

2.2. Formation of sulforaphane and sulforaphane nitrile in commercial broccoli cultivars

When florets of fresh broccoli were homogenized with water, sulforaphane nitrile was the predominant product of glucoraphanin hydrolysis in each cultivar on a fresh weight basis. Sulforaphane and sulforaphane nitrile formation varied significantly among the cultivars, ranging from 0.08 ± 0.00 to 0.62 ± 0.08 μmol/g fresh weight for sulforaphane and from 0.35 ± 0.04 to 1.50 ± 0.06 μmol/g fresh weight for sulforaphane nitrile. Interestingly, sulforaphane nitrile, not sulforaphane, was the principal product of glucoraphanin hydrolysis measured in each cultivar tested (Fig. 2).

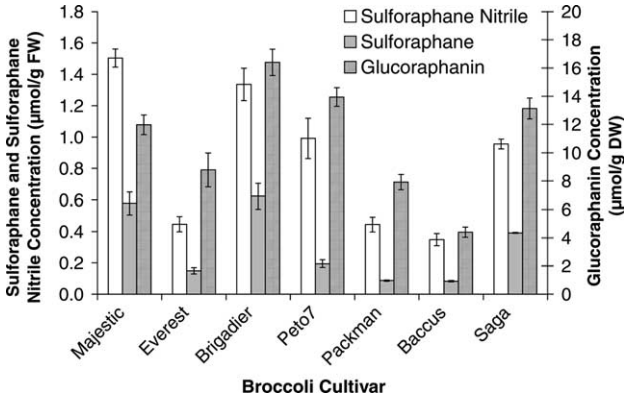

Fig. 2. Glucoraphanin (**2**) concentration (μmol/g dry weight) and subsequent formation of sulforaphane (**1**) and sulforaphane nitrile (**3**) (μmol/g fresh weight) in commercial broccoli cultivars after crushing.

These results are in agreement with previous data we have obtained when freeze-dried broccoli powder was rehydrated with distilled deionized water, in which case the nitrile was the predominant product of glucoraphanin hydrolysis (Matusheski et al., 2001). Several other studies have also reported the formation of sulforaphane nitrile from glucoraphanin in broccoli. Kore et al. (1993) demonstrated that extraction of broccoli seed yielded approximately equal amounts of sulforaphane and sulforaphane nitrile. In a study examining the effects of post-harvest storage and processing, sulforaphane nitrile made up greater than 50% of the glucoraphanin hydrolysis products under certain conditions (Howard et al., 1997). Mithen and coworkers (2003) recently reported that approximately 80% of the glucoraphanin from the broccoli cultivar Marathon hydrolyzed to sulforaphane nitrile and only 20% was converted to sulforaphane.

A common assumption in the current literature regarding the health benefits of sulforaphane from broccoli is that glucoraphanin is entirely converted to sulforaphane when broccoli is crushed. As a result of this assumption, an analysis method has been utilized for broccoli and broccoli sprouts involving the extraction of glucosinolates and subsequent treatment with purified myrosinase or crude extracts of daikon (Fahey et al., 1997; Shapiro et al., 1998; Farnham et al., 2000; Pereira et al., 2002). It has been documented that some cruciferous plants form nitriles as their principal hydrolysis products while others produce primarily isothiocyanate products (Gil and MacLeod, 1980a; Hasapis and MacLeod, 1982b; Lambrix et al., 2001). According to the results presented here and the results of others (Kore et al., 1993; Kyung et al., 1995; Mithen et al., 2003), broccoli and other members of the *Brassica oleracea* species are nitrile-forming plants. Daikon, horseradish and white mustard, however, have been shown to form only isothiocyanates from their glucosinolates (Cole, 1976; Cole, 1980; Petroski and Tookey, 1982). Thus, the source of myrosinase activity should be carefully considered when studying the products derived from glucosinolate hydrolysis, since the amount of isothiocyanate formed when plant tissue is disrupted may or may not be a measure of the glucosinolate concentration, depending upon the plant species under investigation. It should also be noted that these concerns do not address the situation when broccoli is cooked then eaten, where the plant myrosinase is destroyed by the cooking and glucosinolates are hydrolyzed in the human gastrointestinal tract by gut microflora (Shapiro et al., 2001).

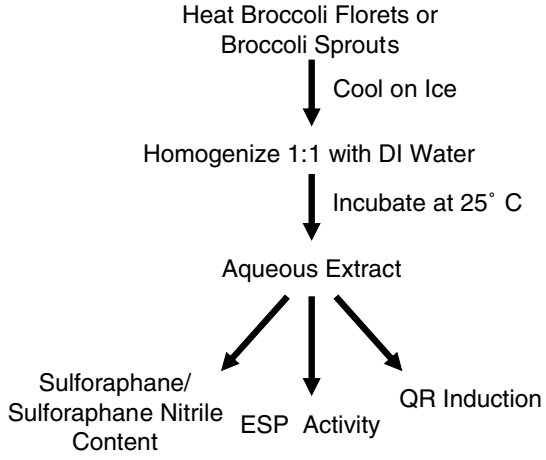

Fig. 3. Scheme for sample preparation and analysis of heat treated broccoli florets and sprouts. As detailed in the text, vegetable tissue was heat treated, cooled to room temperature and homogenized. Aqueous extracts were analyzed for the formation of sulforaphane (**1**) and sulforaphane nitrile (**3**), ESP activity, and bioactivity (induction of QR in Hepa lclc7 cells).

2.3. Effect of heat treatment on sulforaphane formation, ESP activity and capacity to induce QR in broccoli florets and broccoli sprouts

Broccoli florets and sprouts were heat treated and analyzed according to the scheme presented in Fig. 3. Glucoraphanin content of purchased broccoli was 6.2 ± 0.2 μmol/g dry weight. In the unheated sample, the majority of the glucoraphanin hydrolysis products were sulforaphane nitrile, similar to the results obtained in commercial broccoli cultivars. All 5 and 10 min preheat treatments significantly decreased the formation of sulforaphane nitrile compared to the unheated control (Fig. 4(a)). Sulforaphane formation was significantly increased in the sample preheated to 60 °C with 5 and 10 min treatments, but heating to 70 or 100 °C resulted in a significant decrease in total hydrolysis product formation compared to the other treatments. Epithiospecifier protein activity was significantly decreased at all preheating temperatures of 50 °C or higher compared to the unheated sample (Fig. 4(b)). This result is consistent with the observed decrease in nitrile formation. Induction of QR by broccoli floret extracts reflected the observed changes in sulforaphane formation (Fig. 4(b)). Heating treatments of 5 or 10 min at 60 °C gave significantly greater QR induction compared to the unheated sample. Preheating at 70 °C for 5 min or 100 °C for 5 or 10 min significantly decreased induction of QR compared to unheated samples, reflecting the decrease observed in total hydrolysis product formation. In a separate experiment, we monitored homogenates for changes in acidity during hydrolysis. The initial pH of the homogenate standing at room temperature (22 °C) was 6.3, and the pH of the homogenate from tissue that

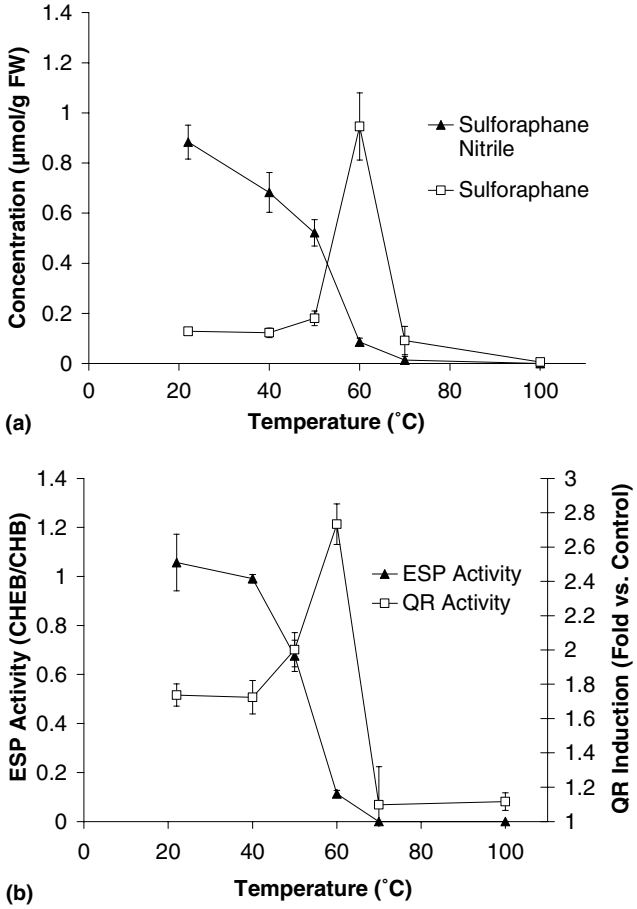

Fig. 4. Effect of pre-heating temperature (5 min treatment) on (a) sulforaphane (**1**) and sulforaphane nitrile (**3**) formation in broccoli florets, and (b) ESP activity (defined as the ratio of CHEB/CHB) and bioactivity (induction of QR in Hepa lclc7 cells) of broccoli floret extracts. Average QR specific activity of untreated cells was 82.0 ± 12.7, and 1 μM β-napthaflavone induced QR by 4.6 ± 2.6 fold compared to control.

had been heated to 100 °C ("cooked") and then chilled to room temperature prior to homogenization and hydrolysis, was 6.2. The pH of both cooked and fresh homogenates stayed within 0.2 pH units of the initial sample over the course of the 8-h hydrolysis. However, extending the hydrolysis period to 22 h resulted in a pH decrease to 5.4 in the homogenate of fresh broccoli, and to 4.0 in the homogenate of cooked broccoli (data not shown).

Glucoraphanin content of broccoli sprouts (cv. Majestic) was 10.3 ± 0.8 μmol/g dry weight. Similar to broccoli florets, the principal product of glucoraphanin hydrolysis in broccoli sprouts was sulforaphane nitrile (Fig. 5(a)). Preheating to 50 °C for 20 min or 60 °C for 10 or 20 min significantly decreased nitrile formation compared to the unheated control. Sulforaphane was significantly increased by preheating sprouts to 60 °C or above for 10 min or to 50 °C or above for 20 min. Preheating sprouts to higher temperatures resulted in a

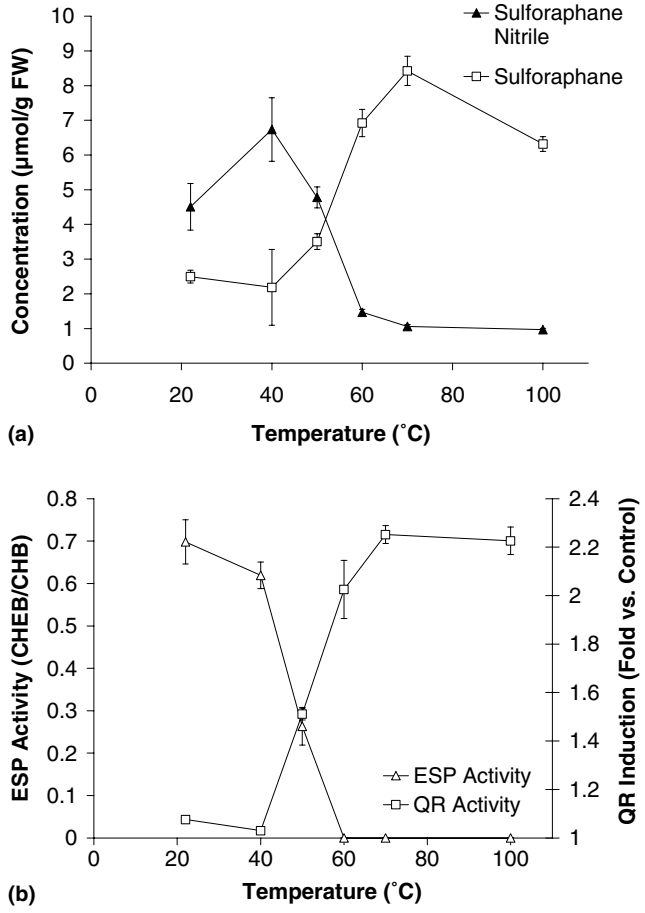

Fig. 5. Effect of pre-heating temperature (10 min treatment) on (a) sulforaphane (**1**) and sulforaphane nitrile (**3**) formation in broccoli sprouts, and (b) ESP activity (defined as the ratio of CHEB/CHB) and bioactivity (induction of QR in Hepa lclc7 cells) of broccoli sprout extracts. Average QR specific activity of untreated cells was 78.4 ± 4.7, and 1 μM β-napthaflavone induced QR by 4.3 ± 2.5 fold compared to control.

significantly decreased total hydrolysis product formation in the maximum time-temperature treatment, 100 °C for 20 min (data not shown). Similar to broccoli florets, ESP activity was significantly decreased in sprouts heated to 50 °C and higher for 10 min and in sprouts heated to temperatures of 40 °C for 20 min compared to the unheated sample (Fig. 5(b)). This result is again consistent with the observed decrease in nitrile formation. Induction of QR by broccoli sprout extracts also reflected the observed changes in sulforaphane formation. All preheating treatments of greater than 50 °C gave significantly greater QR induction compared to the unheated sample (Fig. 5(b)). Preheating at 100 °C for 20 min significantly decreased induction of QR compared to treatments of 70 °C for 20 min or 100 °C for 10 min, reflecting the decrease observed in total hydrolysis product formation (data not shown).

These results suggest that a reduction in ESP activity as a result of heat processing may allow for greater formation of sulforaphane from glucoraphanin because of a decrease in the formation of the alternative product, sulforaphane nitrile. Epithiospecifier protein was first identified as a non-catalytic myrosinase accessory protein in Crambe (*Crambe abyssinica*) seed that was capable of directing the hydrolysis of an alkenylglucosinolate to form an epithionitrile (Tookey, 1973). Since its identification, ESP has been identified in a number of nitrile-forming cruciferous plants (Cole, 1978; Kaoulla et al., 1980; Petroski and Tookey, 1982; MacLeod and Rossiter, 1985). The formation of epithionitriles has been shown to be heat sensitive in the seed of turnip rape (*Brassica campestris*), where a mild heat treatment was shown to decrease the formation of epithionitriles and increase the formation of isothiocyanates from benzylglucosinolate without retarding myrosinase activity (Kirk and Macdonald, 1974).

More recent work with ESP has identified the intriguing possibility that ESP may be capable of controlling the formation of 'simple nitriles' in addition to epithionitriles during myrosinase-catalyzed hydrolysis of glucosinolates. A study by Lambrix and coworkers (Lambrix et al., 2001) surveyed 122 ecotypes of *Arabidopsis thaliana* for the formation of glucosinolate hydrolysis products when the leaf tissue was macerated with water. Some ecotypes were found to produce mostly nitriles, and others mostly isothiocyanates, from their glucosinolates. A quantitative trait locus was identified that mapped closely to a gene with homology to the ESP gene previously identified in *Brassica napus.* The gene product was cloned and expressed in *E. coli* and found to possess the activity of ESP. Since glucoraphanin was a principal glucosinolate in a number of ecotypes, it and several other glucosinolates were assayed with myrosinase and *E. coli* extracts expressing the gene product. Interestingly, the ESP-containing extracts were capable of directing the hydrolysis of glucoraphanin toward the formation of sulforaphane nitrile. Here we report for the first time that broccoli also possesses ESP activity, and demonstrate that ESP activity tracks closely with sulforaphane nitrile formation in broccoli florets and sprouts.

Interestingly, we found that myrosinase-catalyzed formation of sulforaphane was much more heat sensitive in broccoli florets compared to broccoli sprouts. Myrosinase from broccoli vegetable tissue has previously been reported to be heat-labile, with loss of approximately 90% of activity after only 3 min of heat treatment at 60 °C (Ludikhuyze et al., 1999). However, although not reported for broccoli seeds, myrosinase activity from the seeds of several other crucifers has been reported to be relatively more heat stable (Van Etten et al., 1966; Hasapis and MacLeod, 1982a). It is possible that the temperature resistance of myrosinase activity in the sprouts may be a result of its content of a specific seed myrosinase family, as has been reported in white

mustard seedlings (Eriksson et al., 2001). The heat sensitivity of myrosinase that we observed in broccoli florets suggests that with normal commercial or domestic cooking procedures, myrosinase activity is completely lost. The work of Shapiro and coworkers provides an initial view towards understanding how much glucoraphanin may be converted to bioactive sulforaphane in the human intestine as a result of consuming the intact glucosinolates in cooked broccoli sprouts (Shapiro et al., 2001). These investigators also examined the contribution of plant myrosinase indirectly, by evaluating the effects of chewing on the ultimate appearance of sulforaphane metabolites in the urine. However, more research is clearly needed to fully characterize the hydrolysis of glucoraphanin by gut micoflora.

We have shown that mild heat treatment of broccoli and broccoli sprouts increases formation of the anticarcinogen sulforaphane, and subsequently improves the health benefit of broccoli or broccoli sprouts as evidenced by an increased capacity to induce QR in Hepa lclc7 cell culture. The results we describe suggest that sulforaphane nitrile formation in broccoli may be under the control of ESP, however further research is required to verify this proposal. If so, then optimal conversion of glucoraphanin to sulforaphane in fresh broccoli may be achieved by identifying processing methods, such as temperature or pressure treatment, that target labile ESP but still allow myrosinase to act. Specific broccoli cultivars or crosses may be identified that express decreased levels of ESP, allowing optimal amounts of sulforaphane formation under natural conditions, and providing increased health benefit.

3. Experimental procedures

3.1. Materials

Broccoli seeds for the production of sprouts and mature broccoli were gifts from Asgrow Seed Co. (cv. Majestic, Everest, Packman and Baccus), from Peto Seed Co. (Seminis Seeds; cv. Brigadier, Peto 7) and from Sakata Seed Co. (cv. Saga). Organic solvents (HPLC grade) were purchased from Fisher Scientific (Fairlawn, NJ). Purified epi-progoitrin and benzyl glucosinolate were purchased from Dr. Jens Sørensen at the Bioraf Denmark Foundation (Copenhagen, Denmark). Isothiocyanates, nitriles and epithionitriles for GC calibration were purified from broccoli or crambe seed using previously described extraction and purification methods, and verified by mass spectrometry (Matusheski et al., 2001; Niedoborski et al., 2001). All other chemical reagents, including phenyl and benzyl isothiocyanate, were purchased from Sigma (St. Louis, MO). Hepa lclc7 cells were from American Type Culture Collection (ATCC).

3.2. Preparation of broccoli sprouts

Dry broccoli seeds (15 g, cv. Majestic) were soaked in 2% sodium hypochlorite for 10 min, then washed 5 times with distilled deionized water. The washed seeds were soaked in water for 90 min to initiate germination, and spread into 5×6 inch 'Sproutmaster' sprouting trays (Sproutpeople, Viroqua, WI). Sprouts were grown for 5 days at ambient temp (22 °C) using a 16 h light, 8 h dark cycle (40W incandescent bulb). An automatic spraying system provided a 5 s mist every 15 min. Sprouts were loosened on day 3 to prevent root mass formation. On day 5 sprouts were harvested and de-hulled by immersing in excess cold water, then gently shaken dry. The sprouts were stored at 4 °C until analysis.

3.3. Formation of sulforaphane and sulforaphane nitrile in fresh commercial broccoli cultivars

Seven broccoli cultivars were grown on-site for analysis of sulforaphane and sulforaphane nitrile formation. Cultivars were grown to commercial harvest maturity at the University of Illinois Vegetable Research Farm (Champaign, IL) according to standard cultural practices developed for cruciferous vegetables in Illinois (Foster and Maynard, 1998) during the Fall season of 2001. At the time of harvest, broccoli heads were cut and kept on ice until florets were sampled by cutting 5 cm from the top of the crown, then homogenized with 1 volume of distilled deionized water in triplicate. Homogenates were incubated at room temp for 8 h in capped tubes to assure complete glucosinolate hydrolysis, then squeezed through cheesecloth into microcentrifuge tubes and centrifuged at 4 °C under 16,000*g* for 20 min. Supernatants were collected and stored at −20 °C prior to analysis.

3.4. Effects of mild heat treatment on broccoli and broccoli sprouts

Fresh broccoli heads were purchased from a local supermarket (Meijer Inc., Champaign, IL) and stored at 4 °C until analysis. Florets were cut 5 cm from the top of the crown and heat treated directly in distilled deionized water for either 5 or 10 min, at temperatures ranging from ambient (22 °C) to 100 °C in triplicate. The samples were removed from the water, cooled immediately on ice, and chopped and homogenized with 1 volume of distilled deionized water using a Tekmar Tissumizer. Broccoli sprouts were heat treated in sealed tubes containing 10 volumes of distilled deionized water for either 10 or 20 min at temperatures ranging from ambient (22 °C) to 100 °C, in triplicate. Tubes were removed from

the water bath, cooled immediately on ice, and the contents homogenized as above.

Homogenates of broccoli florets or sprouts intended for ESP activity determination were kept on ice, then squeezed through cheesecloth into microcentrifuge tubes and centrifuged at 4 °C under 16,000*g* for 20 min. Supernatants were collected and stored at −20 °C prior to analysis. Homogenates intended for quantification of sulforaphane and sulforaphane nitrile were allowed to incubate at room temperature for 8 h prior to extraction and centrifugation as described above. Eight hours was chosen as an adequate time period for the completion of glucosinolate hydrolysis since, in a preliminary experiment, no additional glucose was released from the homogenate after this time (data not shown). Aqueous extracts incubated at room temperature were divided. One sample was used to quantify sulforaphane and sulforaphane nitrile formation by GC. The second sample was sterile-filtered through a 0.22 μm nitrocellulose membrane and analyzed for the capacity to induce QR.

3.5. Analysis of glucoraphanin content and sulforaphane and sulforaphane nitrile formation

For glucosinolate analysis, unheated intact samples of broccoli and broccoli sprouts were snap-frozen in liquid nitrogen and stored at −80 °C. Samples were lyophilized and ground to a fine powder using a Tekmar analytical grinder. Desulfoglucosinolates were analyzed in triplicate by HPLC as previously described (Kushad et al., 1999).

For analysis of sulforaphane and sulforaphane nitrile formation, 20 μl of 0.5 mg/ml benzyl isothiocyanate (Sigma, St. Louis, MO) in acetonitrile (internal standard) was added to 500 μl of aqueous extract. After vortexing, the solution was transferred to a Teflon microcentrifuge tube and extracted with 1 ml of methylene chloride. The tubes were pulsed in a microcentrifuge to fully separate layers, and the organic phase was collected. For GC analysis, 1 μl of methylene chloride extract was injected into a splitless HP 5890 GC system using a 7363A autosampler. The flowpath consisted of a deactivated cyclo-double gooseneck liner (Restek Inc., Bellefonte, PA), a 3 m J&W DB-5 guard column and a 30 m J&W DB-5 capillary column (0.25 mm id, 0.25 μm film) with flame ionization detection. The injector was held at 200 °C and detector was 280 °C. Initial temperature was 40 °C for 2 min, and ramped from 40–260 °C at 10 °C per min. Final temperature of 260 °C was held for 10 min. Helium was used as carrier gas, with a head pressure of 25 psi. The GC was calibrated using standard curves of 1–100 mg/ml BITC and purified sulforaphane and sulforaphane nitrile in methylene chloride (Matusheski et al., 2001).

3.6. Measurement of ESP activity

Activity of ESP was measured by examining the products formed from the purified glucosinolate epi-progoitrin when hydrolyzed by a purified myrosinase enzyme in the presence of the extract under study (Tookey, 1973; Bernardi et al., 2000). Epithiospecifier protein activity was defined as the ratio of epithionitrile [(2*S*)-1-cyano-2-hydroxy-3,4-epithiobutane] (CHEB) to simple nitrile [(*S*)1-cyano-2-hydroxy-3-butene] (CHB) formed in the presence of excess myrosinase and iron. For each sample, 350 μl of 50 mM acetate buffer containing 1 mM ferrous sulfate and 1 mM dithiothreitol (DTT; pH 5.5) was combined with 50 μl of myrosinase solution (0.5 U/ml) and 50 μl of ESP-containing extract. To initiate the reaction, 50 μl of 5 mg/ml epi-progoitrin solution was added, and the samples were allowed to incubate for 1 h in a 25 °C shaking water bath. After incubation, 20 μl of 0.5 mg/ml phenyl isothiocyanate (Sigma, St. Louis, MO) in acetonitrile (internal standard) was added to each sample. After vortexing, the solution was transferred to a Teflon microcentrifuge tube and extracted with 1 ml of methylene chloride. The tubes were pulsed in a microcentrifuge to fully separate layers, and the organic phase was collected. The methylene chloride extracts were analyzed by GC as described above, only with modifications to the column oven temperature program. An initial temperature of 60 °C was held for 4 min, then ramped to 95 °C at 10 °C per min. Temperature was then immediately ramped from 95 to 110 °C at 2 °C per min then from 110 °C to a final temperature of 200 °C at 10 °C per min. Final temperature was held for 10 min. The GC was calibrated using standard curves of 1–100 mg/ml phenyl isothiocyanate, CHB [(*S*)1-cyano-2-hydroxy-3-butene], CHEB [(2*S*)-1-cyano-2-hydroxy-3,4-epithiobutane], and goitrin [(5*R*)-5-vinyl-1,3-oxazolidine-2-thione] in methylene chloride, purified from seeds of *C. abyssinica* (Matusheski et al., 2001; Niedoborski et al., 2001). In preliminary experiments, assays performed in the absence of myrosinase resulted in no detectable hydrolysis products; assays performed in the absence of iron produced no nitriles (goitrin, a cyclic isothiocyanate derivative was formed exclusively) and assays performed without plant extract resulted in formation of the simple nitrile CHB only, with no detectable formation of the epithionitrile CHEB. The assay was linear when serial sample dilutions were made (data not shown).

3.7. Induction of QR in hepa lclc7 cells

Hepa lclc7 cells were plated into 96 well plates (Costar # 3595, Corning, Inc., Corning, NY) at 10,000 cells/well using α-minimum essential medium (without ribonucleosides or deoxyribonucleosides) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and

100 μg/ml streptomycin (final concentrations). Cells were grown for 24 h, then the medium was replaced with medium containing sterile filtered aqueous extracts of broccoli (3.13 mg fresh weight/ml) or broccoli sprouts (0.57 mg fresh weight/ml) from each experiment. One plate was assayed for each experimental replicate. β-Naphtho-flavone (l μM) was also used as a positive control for each experiment. After 24 h, cells were lysed with digitonin and QR was measured using a common 96 well plate assay procedure (Prochaska and Santamaria, 1988). Protein was measured in duplicate plates by a modification of the method of Bradford (Bradford, 1976) using a Bio-Rad kit (Bio-Rad Laboratories, Hercules, CA). Results are expressed as fold induction vs control to account for inter-plate variability.

3.8. Statistical analysis

Statistical analysis for each experiment was performed with ANOVA and Fisher's protected LSD ($\alpha = 0.05$) using Statistical Analysis Software (SAS; Cary, NC).

Acknowledgements

This research was supported by grants from the USDA (National Research Initiative 99-35503-7010). The authors thank William R. Bagby Jr. of Flatland Hydroponics, Thomasboro, IL for providing equipment and guidance for broccoli seed sprouting. Also thanks to Asgrow Seed Co., Peto Seed Co. and Sakata Seed Co. for donating seed stock utilized for broccoli production in this work.

References

Basten, G.P., Bao, Y., Williamson, G., 2002. Sulforaphane and its glutathione conjugate but not sulforaphane nitrile induce UDP-glucuronosyl transferase (UGT1A1) and glutathione transferase (GSTA1) in cultured cells. Carcinogenesis 23, 1399–1404.

Bernardi, R., Negri, A., Ronchi, S., Palmieri, S., 2000. Isolation of the epithiospecifier protein from oil-rape (*Brassica napus* ssp. *oleifera*) seed and its characterization. FEES Lett. 467, 296–298.

Bones, A.M., Rossiter, J.T., 1996. The myrosinase–glucosinolate system, its organisation and biochemistry. Physiol. Plant. 97, 194–208.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Brown, A.F., Yousef, G.G., Jeffery, E.H., Klein, B.P., Kushad, M.M., Wallig, M.A., Juvik, J.A., 2002. Glucosinolate profiles in broccoli (*Brassica oleracea* L.): stability over environments and implications for cancer chemoprotection. J. Am. Soc. Hort. Sci. 127, 807–813.

Chiao, J.W., Chung, F.L., Kancherla, R., Ahmed, T., Mittelman, A., Conaway, C.C., 2002. Sulforaphane and its metabolite mediate growth arrest and apoptosis in human prostate cancer cells. Int. J. Oncol. 20, 631–636.

Cole, R.A., 1976. Isothiocyanates, nitriles and thiocyanates as products of autolysis of glucosinolates in *Cruciferae*. Phytochemistry 15, 759–762.

Cole, R.A., 1978. Epithiospecifier protein in turnip and changes in products of autolysis during ontogeny. Phytochemistry 17, 1563–1565.

Cole, R.A., 1980. Volatile components produced during ontogeny of some cultivated crucifers. J. Sci. Food Agric. 31, 549–557.

Daxenbichler, M.E., VanEtten, C.H., Spencer, G.F., 1977. Glucosinolates and derived products in cruciferous vegetables. Identification of organic nitriles from cabbage. J. Agric. Food Chem., 25121–25124.

Eriksson, S., Ek, B., Xue, J., Rask, L., Meijer, J., 2001. Identification and characterization of soluble and insoluble myrosinase isoenzymes in different organs *of Sinapis alba*. Physiol. Plant. 111, 353–364.

Fahey, J.W., Haristoy, X., Dolan, P.M., Kensler, T.W., Scholtus, I., Stephenson, K.K., Talalay, P., Lozniewski, A., 2002. Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of helicobacter pylori and prevents benzo[*a*]pyrene-induced stomach tumors. Proc. Natl. Acad. Sci. USA 99, 7610–7615.

Fahey, J.W., Zhang, Y., Talalay, P., 1997. Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. Proc. Natl. Acad. Sci. USA 94, 10367–10372.

Farnham, M.W., Stephenson, K.K., Fahey, J.W., 2000. Capacity of broccoli to induce a mammalian chemoprotective enzyme varies among inbred lines. J. Am. Soc. Hort. Sci. 125, 482–488.

Faulkner, K., Mithen, R., Williamson, G., 1998. Selective increase of the potential anticarcinogen 4-methylsulphinylbutyl glucosinolate in broccoli. Carcinogenesis 19, 605–609.

Foster, R., Maynard, E., 1998. Midwest vegetable production guide for commercial growers, University of Illinois Coop. Exten. Ser. C-1356, pp. 40–44.

Gamet-Payrastre, L., Li, P., Lumeau, S., Cassar, G., Dupont, M.A., Chevolleau, S., Gasc, N., Tulliez, J., Terce, F., 2000. Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. Cancer Res. 60, 1426–1433.

Gil, V., MacLeod, A.J., 1980a. Degradation of glucosinolates of *Nasturtium officinale* seeds. Phytochemistry 19, 1657–1660.

Gil, V., MacLeod, A.J., 1980b. The effects of pH hydrogen-ion concentration on glucosinolate degradation by a thioglucoside glucohydrolase preparation. Phytochemistry 19, 2547–2551.

Gil, V., MacLeod, A.J., 1980c. Some glucosinolates *of Farsetia aegyptia* and *Farsetia ramosissima*. Phytochemistry 19, 227–231.

Goodrich, R.M., Anderson, J.L., Stoewsand, G.S., 1989. Glucosinolate changes in blanched broccoli and brussels sprouts. J. Food Process. Preserv. 13, 275–280.

Hasapis, X., MacLeod, A.J., 1982a. Benzylglucosinolate degradation in heat-treated *Lepidium sativum* seeds and detection of a thiocyanate forming factor. Phytochemistry 21, 1009–1013.

Hasapis, X., MacLeod, A.J., 1982b. Effects of pH and ascorbate on benzylglucosinolate degradation in seed extracts *of Lepidium sativum*. Phytochemistry 21, 291–296.

Howard, L.A., Jeffery, E.H., Wallig, M.A., Klein, B.P., 1997. Retention of phytochemicals in fresh and processed broccoli. J. Food Sci. 62, 1098–1100, 1104.

Kaoulla, N., MacLeod, A.J., Gil, V., 1980. Investigation of *Brassica oleracea* and *Nasturtium officinale* seeds for the presence of epithiospecifier protein. Phytochemistry 19, 1053–1056.

Kirk, J.T.O., Macdonald, C.G., 1974. 1-Cyano-3,4-epithiobutane: a major product of glucosinolate hydrolysis in seeds from certain varieties *of Brassica campestris*. Phytochemistry 13, 2611–2615.

Kore, A.M., Spencer, G.F., Wallig, M.A., 1993. Purification of the omega-(methylsulfinyl)alkyl glucosinolate hydrolysis products: 1-isothiocyanato-3-(methylsulfinyl)propane, 1-isothiocyanato-4-

(methylsulfinyl)butane, 4-(methylsulfinyl)butanenitrile, and 5-(methylsulfinyl)pentanenitrile from broccoli and *Lesquerella fendleri*. J. Agric. Food Chem. 41, 89–95.

Kushad, M.M., Brown, A.F., Kurilich, A.C., Juvik, J.A., Klein, B.P., Wallig, M.A., Jeffery, E.H., 1999. Variation of glucosinolates in vegetable crops of *Brassica oleracea*. J. Agric. Food Chem. 47, 1541–1548.

Kyung, K.H., Fleming, H.P., Young, C.T., Haney, C.A., 1995. 1-Cyano-2,3-epithiopropane as the primary sinigrin hydrolysis product of fresh cabbage. J. Food Sci. 60, 157–159.

Lambrix, V., Reichelt, M., Mitchell-Olds, T., Kliebenstein, D.J., Gershenzon, J., 2001. The Arabidopsis epithiospecifier protein promotes the hydrolysis of glucosinolates to nitriles and influences *Trichoplusia ni* herbivory. Plant Cell 13, 2793–2807.

Ludikhuyze, L., Ooms, V., Weemaes, C., Hendrickx, M., 1999. Kinetic study of the irreversible thermal and pressure inactivation of myrosinase from broccoli (*Brassica oleracea* L cv *italica*). J. Agric. Food Chem. 47, 1794–1800.

MacLeod, A.J., Rossiter, J.T., 1985. The occurrence and activity of epithiospecifier protein in some cruciferae seeds. Phytochemistry 24, 1895–1898.

Matusheski, N.V., Jeffery, E.H., 2001. Comparison of the bioactivity of two glucoraphanin hydrolysis products found in broccoli, sulforaphane and sulforaphane nitrile. J. Agric. Food Chem. 49, 5743–5749.

Matusheski, N.V., Wallig, M.A., Juvik, J.A., Klein, B.P., Kushad, M.M., Jeffery, E.H., 2001. Preparative HPLC method for the purification of sulforaphane and sulforaphane nitrile from *Brassica oleracea*. J. Agric. Food Chem. 49, 1867–1872.

Mithen, R., Faulkner, K., Magrath, R., Rose, P., Williamson, G., Marquez, J., 2003. Development of isothiocyanate-enriched broccoli, and its enhanced ability to induce phase 2 detoxification enzymes in mammalian cells. Theor. Appl. Genet. 106, 727–734.

Niedoborski, T.E., Klein, B.P., Wallig, M.A., 2001. Rapid isolation and purification of 1-cyano-2-hydroxy-3-butene (crambene) from *Crambe abyssinica* seed meal using immiscible solvent extraction and high-performance liquid chromatography. J. Agric. Food Chem. 49, 3594–3599.

Pereira, F.M., Rosa, E., Fahey, J.W., Stephenson, K.K., Carvalho, R., Aires, A., 2002. Influence of temperature and ontogeny on the levels of glucosinolates in broccoli (*Brassica oleracea* var. *italica*) sprouts and their effect on the induction of mammalian phase 2 enzymes. J. Agric. Food Chem. 50, 6239–6244.

Petri, N., Tannergren, C., Holst, B., Mellon, F.A., Bao, Y., Plumb, G.W., Bacon, J., O'Leary, K.A., Kroon, P.A., Knutson, L., Forsell, P., Eriksson, T., Lennernas, H., Williamson, G., 2003. Absorption/metabolism of sulforaphane and quercetin, and regulation of phase II enzymes, in human jejunum in vivo. Drug Metab. Dispos. 31, 805–813.

Petroski, R.J., Tookey, H.L., 1982. Interactions of thioglucoside glucohydrolase and epithiospecifier protein of cruciferous plants to form 1-cyanoepithioalkanes. Phytochemistry 21, 1903–1905.

Prochaska, H.J., Santamaria, A.B., 1988. Direct measurement of NAD(P)H:quinone reductase from cells cultured in microtiter wells: a screening assay for anticarcinogenic enzyme inducers. Anal. Biochem. 169, 328–336.

Shapiro, T.A., Fahey, J.W., Wade, K.L., Stephenson, K.K., Talalay, P., 2001. Chemoprotective glucosinolates and isothiocyanates of broccoli sprouts: metabolism and excretion in humans. Cancer Epidemiol. Biomarkers Prev. 10, 501–508.

Shapiro, T.A., Fahey, J.W., Wade, K.L., Stephenson, K.K., Talalay, P., 1998. Human metabolism and excretion of cancer chemoprotective glucosinolates and isothiocyanates of cruciferous vegetables. Cancer Epidemiol. Biomarkers Prev. 7, 1091–1100.

Talalay, P., Fahey, J.W., Holtzclaw, W.D., Prestera, T., Zhang, Y., 1995. Chemoprotection against cancer by phase 2 enzyme induction. Toxicol. Lett. 82-83, 173–179.

Tookey, H.L., 1973. Crambe thioglucoside glucohydrolase (EC 3.2.3.1): separation of a protein required for epithiobutane formation. Can. J. Biochem. 51, 1654–1660.

Vallejo, F., Tomas-Barberan, F., Garcia-Viguera, C., 2003. Health-promoting compounds in broccoli as influenced by refrigerated transport and retail sale period. J. Agric. Food Chem. 51, 3029–3034.

Van Etten, C.H., Daxenbichler, J.E., Peters, J.E., Tookey, H.L., 1966. Variation in enzymatic degradation products from the major thioglucosides in *Crambe abyssinica* and *Brassica napus* seed meals. J. Agric. Food Chem. 14, 426–430.

Zhang, Y., Kensler, T.W., Cho, C.G., Posner, G.H., Talalay, P., 1994. Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates. Proc. Natl. Acad. Sci. USA 91, 3147–3150.

Zhang, Y., Talalay, P., Cho, C.G., Posner, G.H., 1992. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. Proc. Natl. Acad. Sci. USA 89, 2399–2403.