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Epithiospecifier Protein from Broccoli (*Brassica oleracea* L. ssp. *italica*) Inhibits Formation of the Anticancer Agent Sulforaphane

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In some cruciferous plants, epithiospecifier protein (ESP) directs myrosinase (EC 3.2.3.1)-catalyzed hydrolysis of alkenyl glucosinolates toward epithionitrile formation. Here, for the first time, we show that ESP activity is negatively correlated with the extent of formation of the health-promoting phytochemical sulforaphane in broccoli (*Brassica oleracea* L. ssp. *italica*). A 43 kDa protein with ESP activity and sequence homology to the ESP of *Arabidopsis thaliana* was cloned from the broccoli cv. Packman and expressed in *Escherichia coli*. In a model system, the recombinant protein not only directed myrosinase-dependent metabolism of the alkenyl glucosinolate *epi*-progoitrin [(2*S*)-2-hydroxy-3-butenyl glucosinolate] toward formation of an epithionitrile but also directed myrosinase-dependent hydrolysis of the glucosinolate glucoraphanin [4-(methylsulfinyl)butyl glucosinolate] to form sulforaphane nitrile, in place of the isothiocyanate sulforaphane. The importance of this finding is that, whereas sulforaphane has been shown to have anticarcinogenic properties, sulforaphane nitrile has not. Genetic manipulation designed to attenuate or eliminate expression of ESP in broccoli could increase the fractional conversion of glucoraphanin to sulforaphane, enhancing potential health benefits.

KEYWORDS: Epithiospecifier protein; *Brassica oleracea*; broccoli; glucosinolates; sulforaphane; sulforaphane nitrile

INTRODUCTION

Cruciferous plants contain glucosinolates, secondary metabolites that are hydrolyzed by an endogenous thioglucosidase enzyme, myrosinase (EC 3.2.3.1), to produce isothiocyanates, nitriles, and, to a lesser extent, thiocyanates and epithionitriles (**Figure 1**; reviewed in ref *1*). The effects of dietary isothiocyanates on human health have been studied in detail and are considered to be responsible for the distinct anticarcinogenic action of cruciferous vegetables, including cabbage, broccoli, cauliflower, and Brussels sprouts (reviewed in ref *2*). Glucoraphanin [4-(methylsulfinyl)butyl glucosinolate], the primary glucosinolate in broccoli (*3*), is hydrolyzed to sulforaphane [4-(methylsulfinyl)butyl isothiocyanate]. Sulforaphane has been identified as a particularly potent anticarcinogen in humans and animals, able to induce a battery of xenobiotic metabolizing enzymes that detoxify carcinogens, protecting DNA (reviewed in ref *4*). Sulforaphane also has antitumor activity, inhibiting

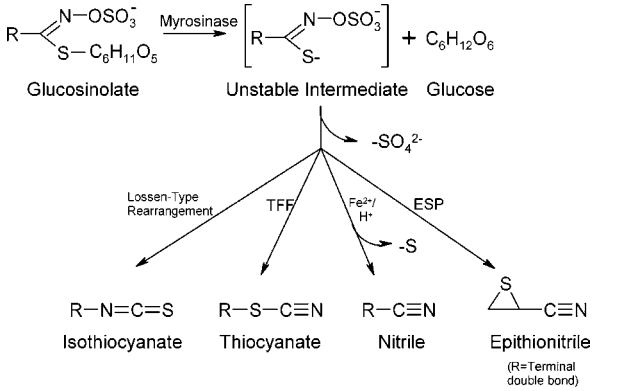

Figure 1. Generic model of glucosinolate hydrolysis. TFF is the thiocyanate-forming factor and ESP epithiospecifier protein. Adapted from ref *27*.

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cell growth and stimulating apoptosis in cultured cancer cells (*5*) and decreasing the rate of tumor growth in animals with chemically induced cancers (*6*, *7*). Despite the growing body of evidence supporting the positive health benefits of crucifers, little modern research has been devoted to the formation of the bioactive components of interest from their precursor glucosinolates in the plant tissue of broccoli. Because of the powerful health benefits associated with sulforaphane, a detailed understanding of the factors controlling its formation in the fresh vegetable is critical for enabling breeders and food manufacturers to provide broccoli products with optimal health benefit.

When purified glucosinolates and myrosinase are incubated together at neutral pH, isothiocyanates are the sole products of this reaction (*8*). For example, when a glucosinolate extract from broccoli is incubated with excess purified myrosinase, glucoraphanin undergoes stoichiometric conversion to the isothiocyanate sulforaphane, leading to the assumption that the isothiocyanate is the sole product of glucoraphanin hydrolysis in broccoli florets and sprouts (*9*−*12*). However, when endogenous glucoraphanin is hydrolyzed by crushing the broccoli plant tissue, as when chewed in the mouth, sulforaphane nitrile [5-(methylsulfinyl)pentane nitrile] is the preponderant product formed rather than sulforaphane (*13*−*15*). In contrast to sulforaphane, the nitrile has been found to be ineffective as an inducer of the detoxification enzymes glutathione *S*-transferase and quinone reductase in the rat (*16*), and quinone reductase, UDP-glucuronosyl transferase, and glutathione *S*-transferase in cultured mammalian cells (*16*, *17*). These data suggest that in fresh broccoli, there exists a biochemical pathway that directs glucosinolate hydrolysis toward nitrile formation, compromising both sulforaphane production and its associated health benefits.

A number of plants, including rapeseed (*Brassica napus*), garden cress (*Lepidium sativum*), and several *Brassica* species, have been shown to form principally nitriles during glucosinolate hydrolysis (*18*−*20*). Other cruciferous plants, including radish (*Raphanus sativus*), white mustard (*Sinapis alba*), and horseradish (*Armoracia rusticana*), have been shown to produce exclusively isothiocyanates (*21*, *22*), yet the reason for these differences among crucifers has not been determined previously.

A myrosinase cofactor, the epithiospecifier protein (ESP), is known to influence the products formed from myrosinase-catalyzed hydrolysis by directing the formation of an epithionitrile [(2*S*)-1-cyano-2-hydroxy-3,4-epithiobutane] from the alkenyl glucosinolate *epi*-progoitrin [(2*S*)-2-hydroxy-3-butenyl glucosinolate], in seeds of *Crambe abyssinica* (*23*), *B. napus*, *Brassica campestris*, and *L. sativum* (*24*). The biochemical mechanism of ESP action was demonstrated to be an iron-dependent intramolecular rearrangement of the sulfur atom derived from the thioglucoside bond of the glucosinolate (*25*). We have previously found that mild heat treatment of broccoli inhibits formation of an epithionitrile from *epi*-progoitrin, suggesting the presence of ESP in broccoli. In addition, we showed that sulforaphane nitrile formation was similarly heat sensitive, allowing us to propose that ESP in broccoli may support simple nitrile formation from alkyl glucosinolates as well as epithionitrile formation from alkenyl glucosinolates. In the heat-treated broccoli, sulforaphane was formed in place of sulforaphane nitrile, resulting in increased bioactivity as measured by induction of quinone reductase in cultured cells (*26*).

A study using the experimental plant *Arabidopsis thaliana* found that a primary gene locus affecting nitrile versus isothiocyanate formation mapped to the same region as a gene encoding a homologue of ESP from *B. napus* (*27*). Plants not expressing the protein formed no nitriles, and the isolated protein supported nitrile formation from glucoraphanin and several other glucosinolates in vitro. In a recent study, also in *Arabidopsis*, transgenic plants overexpressing ESP hydrolyzed glucoraphanin to the simple nitrile in substantially greater amounts (>60% of products) than did the wild type (<10% of products) (*28*). These findings further support the hypothesis that ESP or an ESP-like protein in broccoli may be responsible for sulforaphane nitrile formation, binding the sulfur of the proximal aglucon intermediate formed by myrosinase-dependent hydrolysis and thus preventing the sulfur from participating in the Lossen-type rearrangement required to form an isothiocyanate, resulting in nitrile formation in its stead (**Figure 2**).

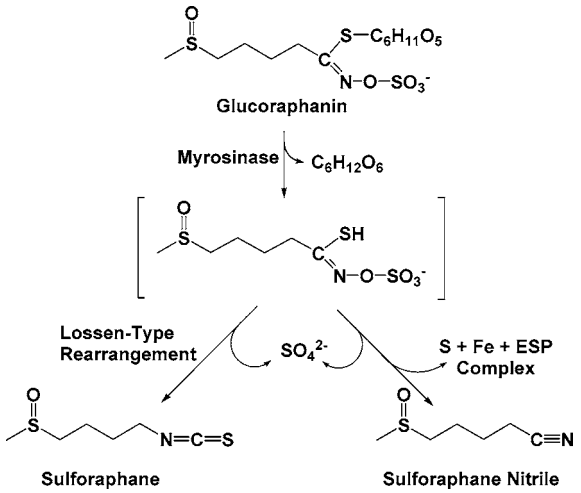

Figure 2. Proposed mechanism for the formation of sulforaphane and sulforaphane nitrile in broccoli florets and sprouts.

If the expression of ESP in broccoli is the primary factor controlling the formation of sulforaphane nitrile in the vegetable, a cultivar with low levels of this protein might convert a higher percentage of glucoraphanin to sulforaphane during hydrolysis, providing a broccoli with improved potency as an anticarcinogenic food.

Here we tested the hypotheses that (1) ESP activity measured in the tissues of 20 commercial broccoli cultivars is negatively correlated with the extent of sulforaphane formation during endogenous glucosinolate hydrolysis and (2) recombinant ESP from broccoli directs the formation of sulforaphane nitrile from glucoraphanin in a model system.

MATERIALS AND METHODS

Materials. Broccoli seeds of the cultivars Marathon, Brigadier, Peto 6, Peto 7, and Peto 13 were from Seminis Seeds (Oxnard, CA). Cultivar Pirate was from Sakata Seeds (Morgan Hill, CA). Cultivars Majestic, Packman, Baccus, and Legacy were from Asgrow Seeds (Monsanto, St. Louis, MO). The inbred genotypes Eu 8-1, Ev 6-1, VI 158, MA 191, and SU 003 were provided by M. Farnham of the USDA Vegetable Research Center (Charleston, NC). Cultivars BNC, High Sierra, Gem, Atlantic, Shogun, and Zeus were from the USDA Plant Genetic Resource Unit (Cornell University, Ithaca, NY). Broccoli plants were grown to commercial harvest maturity under standard conditions at the University of Illinois South Farms (Champaign, IL), placed on ice, and brought to the laboratory for sample preparation and extraction. Horseradish, cabbage, and daikon were purchased locally (Meijer Inc., Champaign, IL). Organic solvents (HPLC-grade) were purchased from Fisher Scientific (Fair Lawn, NJ). Purified *epi*-progoitrin, benzyl glucosinolate, and glucoraphanin were purchased from J. Sørensen at the Bioraf Denmark Foundation (Copenhagen, Denmark). Isothiocyanates, nitriles, and epithionitriles for GC calibration were purified from broccoli (sulforaphane and sulforaphane nitrile) or crambe (crambene and epithiocrambene) seed using previously described extraction and

purification methods (*14*, *29*) and verified by mass spectrometry. All other chemical reagents, including phenyl and benzyl isothiocyanate and components for Western blot analysis, were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Analysis of Glucoraphanin Content and Sulforaphane and Sulforaphane Nitrile Formation. For glucosinolate analysis, intact samples of broccoli florets, cut 5 cm from the top of the crown, 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 by HPLC as previously described (*3*).

For analysis of glucoraphanin hydrolysis products (sulforaphane and sulforaphane nitrile), 150 mg of lyophilized broccoli powder was mixed with 1.5 mL of distilled deionized water in a 2 mL microcentrifuge tube, and the mixture was allowed to incubate for 8 h at room temperature. We have previously shown that lyophilization of broccoli does not alter hydrolysis product formation (*30*). The slurry was drained through cheesecloth and centrifuged (20 min at 15000*g*). The supernatant (500 μL) was transferred to a glass test tube, and benzyl isothiocyanate (20 μL of 0.5 mg/mL acetonitrile) was added as an internal standard. After being vortexed, the solution was transferred to a Teflon microcentrifuge tube containing 1 mL of methylene chloride and pulsed in a microcentrifuge. The organic phase was collected and injected onto the gas chromatograph, using the conditions described previously (*14*).

Reverse Transcription PCR and cDNA Cloning. Messenger RNA was extracted from 25 mg of freeze-dried broccoli powder (cv. Packman) using the RNeasy kit (Qiagen Ltd., Crawley, U.K.). The integrity of the extracted mRNA was verified using an Agilent 2100 Bioanalyzer on an RNA 6000 chip. An RNA ligase-mediated rapid amplification of the 5′ cDNA end (5′ RLM-RACE) was performed using a commercial kit (GeneRacer, Invitrogen Ltd., Paisley, U.K.) to identify a target sequence for amplification of the full-length cDNA. A cDNA library was generated by ligating a GeneRacer mRNA oligo to dephosphorylated and decapped mRNA and reverse transcribing with reverse polymerase (Superscript II) using a GeneRacer oligo-dT primer. The 5′ end was amplified by PCR using the supplied GeneRacer forward primer, and a reverse degenerate primer (IACICCICC[G/A]AAIAC-[G/A]TAIAC) was created, on the basis of the published ESP partial amino acid sequence from *B. napus* (*31*). The reaction mix consisted of 12.4 μL of autoclaved water and 4 μL of 5× PCR buffer containing 10 mM $MgCl_2$, 0.4 μL of 10 mM dNTP mix, 1 μL of DNA sample, and 1 μL each of the forward and reverse primer (100 μM). The reaction mixture was preincubated at 94 °C for 2 min and then hot-started at 72 °C by adding 0.2 μL of Red-Hot Taq polymerase (ABGene, Epsom, U.K.). The PCR was carried out for 35 cycles of 30 s at 94 °C, 30 s at 52 °C, and 60 s at 72 °C, with a final extension step of 10 min at 72 °C. A nested PCR was performed under identical conditions on the product of this reaction using the supplied GeneRacer nested forward primer and a nested reverse degenerate primer (CCAIGT[A/G]TGIGT-[C/T]TT[A/G]AA[A/G]TC[A/G]AA), also designed from the *B. napus* partial amino acid sequence. The resulting product was visualized on a 1% agarose gel, isolated and purified with a Qiagen gel purification column, and cloned into DH5α-T1^R *Escherichia coli* cells (Invitrogen Ltd.). Sequencing was performed at the University of Nottingham (Sutton-Bonington, U.K.) using an ABI 373A automated sequencer (Perkin-Elmer Applied Biosystems, Warrington, U.K.).

The full-length cDNA was cloned using a Gateway Cloning kit (Invitrogen Ltd.). Using the sequencing results of the 5′ RLM-RACE procedure, a gene-specific forward primer (ATGGCTCCGAGTGTG-CAAGGC) was designed, linked at the 5′ end to a Gateway-specific *att*B1 oligo. An oligo-dT sequence, linked to a Gateway-specific *att*B2 oligo, was used as the reverse primer. The full-length sequence was PCR-amplified under the conditions described above, except using a high-fidelity polymerase (Platinum Pfx, Invitrogen Ltd.) with an annealing temperature of 55 °C and an extension temperature of 68 °C. The resulting product was visualized on a 1% agarose gel, purified, and cloned into the pDONR 201 entry vector using the provided BP-clonase enzyme mix. The resulting entry vector was recombined with the pDEST 17 destination vector using the LR-clonase enzyme mix and transformed into BL21-AI *E. coli* cells (Invitrogen Ltd.) for expression and purification. To facilitate purification, a six-histidine tag was attached to the N-terminus of *Bo*ESP upon successful recombination of the entry vector to the pDEST 17 destination vector.

Protein Expression and Purification. To express the recombinant protein, a single positive colony was inoculated into 25 mL of LB medium containing 0.1 mg/mL ampicillin and grown overnight at 37 °C. This culture was used to inoculate an additional 500 mL of LB medium and incubated for 1 h, before induction of the six-His *Bo*ESP fusion protein with 0.2% arabinose for 4 h.

For isolation of the native recombinant protein, cultures (530 mL) were centrifuged at 10000*g* for 20 min at 4 °C, and then pelleted cells were lysed in 4 mL of 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 10 mM imidazole, and 0.5% Triton X-100. Lysates were frozen at −80 °C, thawed, homogenized briefly with a Tekmar tissuemizer, and then recentrifuged at 10000*g* for 20 min. One milliliter of a Ni−NTA agarose gel solution (Qiagen Ltd.) was added to the supernatant, and the lysate/gel mixture was agitated on a rotary shaker for 1 h at 4 °C and then drained through a 5 mL poly-prep column (Bio-Rad Laboratories, Hercules, CA). The column was washed twice with 4 mL of wash buffer, containing 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, and 20 mM imidazole. For elution of the recombinant protein, 600 μL of elution buffer [50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, and 250 mM imidazole] was applied to purge the wash buffer from the column (fraction 1). An additional 1 mL of elution buffer was applied to then fully elute the protein (fraction 2). After dialysis overnight into 25 mM Tris buffer (pH 6.8), the protein concentration was measured in each fraction by a modification of the method of Bradford (*32*) using a Bio-Rad kit (Bio-Rad Laboratories). Fractions 1 and 2 were separated on a 10% SDS−PAGE gel and visualized by staining with Coomassie brilliant blue (**Figure 4**).

Antibody Production and Immunoblotting. A polyclonal antibody was prepared against the recombinant protein in a New Zealand White rabbit (University of Illinois Immunological Resource Center, Urbana, IL). For western blot analysis, 10 g fresh vegetable samples were homogenized with 10 mL of extraction buffer, consisting of 2% (w/v) polyvinylpolypyrrolidone, 10 mM glutathione, and 33 μL/mL plant-specific protease inhibitor cocktail (Sigma) in 25 mM Tris buffer (pH 6.8). After centrifugation at 4 °C, 20 μg of protein was separated on a 10% Tris-HEPES-SDS−polyacrylamide gel (150 V) and transferred onto a 0.45 μm nitrocellulose membrane for 1 h at 100 V. The membrane was blocked with 5% nonfat dry milk and 0.1% Tween 20 in Tris-buffered saline for 90 min on an orbital shaker. Rabbit serum was used directly as the primary antibody at a titer of 1:500 in blocking buffer and incubated for 1 h at room temperature. Membranes were washed and then incubated in a 1:5000 alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody/5% bovine serum albumin mixture. After being washed, membranes were developed using a color substrate mix (5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium, Sigma). Densitometric analysis of bands and molecular mass determination were performed using UnScanIt image digitizing software (Silk Scientific, Orem, UT).

Characterization of ESP in Vitro. The activity of ESP was measured by incubating the glucosinolate *epi*-progoitrin with myrosinase in the presence or absence of recombinant ESP as previously described, with some modifications (*22*). Epithiospecifier protein activity is defined here as the molar percentage of epithionitrile to total *epi*-progoitrin hydrolysis products formed during the incubation. Briefly, 350 μL of 50 mM acetate buffer (pH 5.5) containing 1 mM ferrous sulfate and 1 mM dithiothreitol was combined with 50 μL of myrosinase (0.5 unit/mL), purified from *S. alba* (Sigma) and 50 μL of the ESP-containing extract. *epi*-Progoitrin (50 μL of a 5 mg/mL mixture) was added to initiate the reaction and incubated for 1 h in a shaking water bath at 25 °C. Following incubation, 20 μL of 0.5 mg/mL phenyl isothiocyanate in acetonitrile (internal standard) was added to each sample, which was transferred to a Teflon microcentrifuge tube and extracted into 1 mL of methylene chloride. Extracts (1 μL) were analyzed using a splitless HP 5890 GC system (Hewlett-Packard, Wilmington, DE) with a 7363A autosampler, using helium as a carrier gas (25 psi head pressure) and flame ionization detection. A deactivated cyclo-double gooseneck liner was used (Restek Inc., Bellefonte, PA), connected to a 3 m J&W DB-5 capillary guard column with a 30 m J&W DB-5 capillary column (0.25

mm inside diameter, 0.25 μm film) (J&W, Folsom, CA). The injector temperature was 200 °C, and the detector temperature was 280 °C. The oven temperature program consisted of 60 °C for 4 min, ramped to 95 °C at 10 °C/min, and immediately ramped from 95 to 110 °C at 2 °C/min and then from 110 °C to a final temperature of 200 °C at 10 °C/min, holding at 200 °C for 10 min. Standard curves were constructed for phenyl isothiocyanate, crambene, [(2*S*)-1-cyano-2-hydroxy-3-butene], epithionitrile [(2*S*)-1-cyano-2-hydroxy-3,4-epithiobutane], and goitrin [(5*R*)-5-vinyl-1,3-oxazolidine-2-thione], using methylene chloride as the solvent. Curves were linear over the concentration range that was used (1−100 μg/mL, data not shown).

The extent to which recombinant ESP drives glucoraphanin hydrolysis toward sulforaphane nitrile formation was examined with a similar procedure, except that a solution of 10 mg/mL purified glucoraphanin was used as the substrate and a 5-fold higher concentration of myrosinase was required for complete hydrolysis within 1 h. Benzyl isothiocyanate (20 μL of a 0.5 mg/mL solution in acetonitrile) was added to each sample, and the samples were extracted into methylene chloride as described above. Detection by GC was as described above, except that the initial oven temperature was 40 °C for 2 min and then was ramped from 40 to 260 °C at 10 °C/min. The final temperature of 260 °C was held for 10 min.

Statistical Analysis. The sequence alignment between broccoli and *Arabidopsis* ESP protein sequences was performed using EMBOSS-Align (EMBL-European Bioinformatics Institute, Cambridge, U.K.). Statistical analysis of each experiment was performed with ANOVA and Fisher's protected LSD ($\alpha = 0.05$) using Statistical Analysis Software (SAS; Cary, NC). A linear correlation between ESP activity and fractional sulforaphane formation was analyzed using "proc reg" ($\alpha = 0.05$).

RESULTS

Glucoraphanin Content, Fractional Sulforaphane Formation, and Epithiospecifier Activity in Commercial Broccoli Cultivars. Glucoraphanin content varied significantly across genotypes, as did the extent of sulforaphane nitrile formation (**Table 1**). The activity of ESP also varied significantly ($p = 0.027$). A significant, negative correlation ($p = 0.012$, $R^2 = 0.305$) was observed between ESP activity and the extent of fractional sulforaphane formation.

Cloning of ESP from *B. oleracea* L. ssp. *italica*. Using the published partial amino acid sequence for *B. napus* ESP (*31*), a degenerate 5′ sequence was designed. Using this sequence, the 5′ RLM-RACE procedure yielded a PCR fragment from the broccoli cv. Packman of approximately 190 bp, with a 5′ sequence similar to that of *A. thaliana* and *B. napus* ESP. This sequence was used to design a gene-specific forward primer in conjunction with an oligo-dT reverse primer to amplify the full-length cDNA with end-terminal oligonucleotides for Gateway cloning. The full-length amplification yielded a PCR fragment of approximately 1 kb, which was cloned into the Gateway donor vector pDNO201. The full-length nucleotide sequence (GenBank accession number DQ059298) was obtained by primer walking (**Figure 3**, expressed as the amino acid sequence). A BLAST (NCBI) search using the translated sequence identified significant alignment with the ESP from *A. thaliana*. A 77% level of identity and an 83.4% level of similarity were observed using EMBOSS-Align (EMBL-EBI; **Figure 3**). The BLAST search also identified four Kelch domains and two Kelch-repeat β-propeller domains, not previously identified in any ESP.

To facilitate purification, the cloned *Bo*ESP was expressed as a six-His fusion protein in *E. coli* BL-21AI cells induced with arabinose (0.2%) for 4 h. After Ni−NTA agarose affinity chromatography and overnight dialysis, one major protein band of approximately 43 kDa was observed (**Figure 4**). Each 500 mL of culture produced 2−3 mg of native ESP protein.

Table 1. Distribution of Glucoraphanin Content, Fractional Sulforaphane Formation,[a] and ESP Activity[b] in Floret Tissue of 20 Commercial Broccoli Cultivars

genotype	glucoraphanin[c] (μmol/g of dry weight)	fractional sulforaphane[d] (mol % of hydrolysis products)	ESP activity[c,d] (mol % ETN in assay)
BNC[e]	10.4 ± 1.3	30.5 ± 3.51	42.9 ± 1.82
Brigadier[e]	8.3 ± 0.8	46.4 ± 10.9	29.0 ± 4.34
Eu 8-1[e]	7.9 ± 1.9	52.4 ± 14.2	30.0 ± 9.75
Ev 6-1[e]	8.3 ± 1.7	43.5 ± 7.68	28.9 ± 11.9
High Sierra[e]	3.7 ± 1.1	46.3 ± 2.91	46.0 ± 1.33
Majestic[e]	7.4 ± 0.7	39.2 ± 0.81	27.4 ± 4.90
Marathon[e]	6.5 ± 0.7	54.3 ± 13.1	30.0 ± 4.75
Peto 6[e]	4.3 ± 1.2	51.0 ± 6.84	28.6 ± 8.50
Peto 7[e]	10.0 ± 0.9	39.2 ± 3.90	44.4 ± 3.64
Pirate[e]	4.8 ± 0.1	41.3 ± 1.92	23.0 ± 1.46
VI158[e]	6.0 ± 1.6	54.5 ± 9.27	40.0 ± 7.00
Gem[f]	4.3 ± 1.1	48.8 ± 0.54	30.4 ± 3.78
Atlantic[f]	3.2 ± 1.8	49.2 ± 10.1	47.2 ± 2.89
Baccus[f]	3.4 ± 0.8	45.1 ± 6.21	33.3 ± 8.48
Legacy[f]	4.4 ± 0.2	68.7 ± 2.11	17.1 ± 0.98
MA191[f]	5.6 ± 0.7	58.2 ± 9.20	20.1 ± 3.00
Shogun[f]	9.6 ± 0.5	59.2 ± 15.8	18.0 ± 1.12
SU003[f]	2.9 ± 0.3	51.7 ± 0.89	18.7 ± 1.94
Zeus[f]	4.1 ± 1.3	52.9 ± 1.11	30.4 ± 5.80
Peto 13[g]	5.1	78.7	21.18

[a] Freeze-dried broccoli floret powders were reconstituted by homogenizing in water (1:9, w/v) and allowed to stand for 8 h before GC analysis for sulforaphane and nitrile. [b] Expressed as mole % epithionitrile (ETN) of total products formed from *epi*-progoitrin. [c] Genotypes varied significantly in their content of glucoraphanin ($p < 0.001$) and their ESP activity ($p = 0.027$). [d] A significant negative correlation was observed between ESP activity and fractional sulforaphane formation, where $p = 0.012$, $y = -0.6199x + 69.35$, and $R^2 = 0.305$. [e] Expressed as the mean ± the standard error of field replicates, where $n = 3$. [f] Expressed as the mean ± the standard error of field replicates, where $n = 2$. [g] Expressed as the mean ± the standard error of field replicates, where $n = 1$.

ESP Content in Crucifers, Including Two Commercial Broccoli Cultivars. Using the recombinant ESP protein, antibodies were generated in New Zealand White rabbits and used to evaluate variation in ESP levels in plant tissue from broccoli, daikon, cabbage, and horseradish. Protein extracts were separated by polyacrylamide gel electrophoresis and subjected to western blot analysis. For samples from broccoli and cabbage, two bands at approximately 43 and 37 kDa reacted with the ESP antibody (**Figure 5**). Neither daikon nor horseradish possessed any protein that reacted with the antibody. Protein samples from the head tissue of two broccoli cultivars, BNC and Pirate, each with three field-grown replications, also contained two protein bands that reacted with the ESP antibody (**Figure 6A**). Densitometric analysis revealed that the cultivars differed significantly in their expression of the 43 kDa band ($p = 0.004$) but not in their expression of the 37 kDa band ($p = 0.086$; **Figure 6B**).

Activity of Recombinant Broccoli Espithiospecifier Protein. Past work on ESP from *C. abyssinica* has utilized as a measure of ESP activity the formation of epithionitrile versus simple nitrile during hydrolysis of *epi*-progroitrin (*23*). We evaluated the activity of recombinant broccoli ESP using purified myrosinase and the alkenyl glucosinolate *epi*-progoitrin as a substrate. Consistent with the report that ESP requires iron for activity, no nitriles were formed at iron concentrations of ≤4 μM (**Figure 7**). The cyclized isothiocyanate goitrin was the sole product, when ESP was present. When iron was added in the absence of ESP, the nitrile crambene was formed, at the expense of goitrin (**Figure 7A**). When both ESP and iron were present, both nitrile and epithionitrile were formed at the expense of goitrin (**Figure 7B**).

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  1 MAPSVQGEWIKVEQKGGQTPGPRSSHGIAVVGDKLYSFGGELTPNISIDK     50
    |||::||:|||||||||..||||||||||.|||||||||||||||..|||
  1 MAPTLQGQWIKVEQKGGSGPGPRSSHGIAAVGDKLYSFGGELTPNKHIDK     50

 51 DLYVFDFNTHTWSISPSKGVAPDVKALGTRMVSVGTKLYLFGGRDENKKF     100
    |||||||||.||||:..||.||.|..||.|||:||||:|:|||||||:.|
 51 DLYVFDFNTQTWSIAQPKGDAPTVSCLGVRMVAVGTKIYIFGGRDENRNF     100

101 DDFYSYDTVTNEWTKLTILDQEGGPEARTYHSMASDENHVYVFGGVSKGG     150
    ::|.||||||:|||.||.||:.|||||||:||||||||||||||||||||
101 ENFRSYDTVTSEWTFLTKLDEVGGPEARTFHSMASDENHVYVFGGVSKGG     150

151 TNKTPFRFRTIEAYNIADGKWSQLPDPGEQFPRFERRGGAGFVVVQGKIW     200
    |..||.|||||||||||||:|||||:   .||:|||||||.|||||||
151 TMNTPTRFRTIEAYNIADGKWAQLPDPGD---NFEKRGGAGFAVVQGKIW     197

201 VVYGFATSPDPNGKNDYESDQVQFYDPATQKWTEVETKGDKPSARSVFGH     250
    ||||||||..|.||:||||:.|||||||::||||||||.|.|||||||.|
198 VVYGFATSIVPGGKDDYESNAVQFYDPASKKWTEVETTGAKPSARSVFAH     247

251 AVVGKYILIFGGETWPDPKAHLGPGTLSDEGFALDTETLVWERFG-GGAE     299
    |||||||:||.||.|||...|.|||||||:||:||||||||||:.| .||.
248 AVVGKYIIIFAGEVWPDLNGHYGPGTLSNEGYALDTETLVWEKLGEEGAP     297

300 PGQLGWPGYTTATVYGKKGLLMHGGKRPTNNRTDELYFYAVNSA    343
    ....||..||.|||.||.|||||||.|||.|||:||||||||||
298 AIPRGWTAYTPATVDGKNGLLMHGGKLPTNERTDDLYFYAVNSA    341
```

Figure 3. Amino acid sequence of ESP cloned from Packman cultivar broccoli (top row), translated from the expressed cDNA sequence. The region highlighted in gray corresponds to the initial sequence obtained from the 5′ RLM-RACE procedure. Alignment of amino acids with the reported sequence for ESP from *A. thaliana* (*27*; bottom row) returns levels of identity and similarity of 77.0 and 83.4%, respectively.

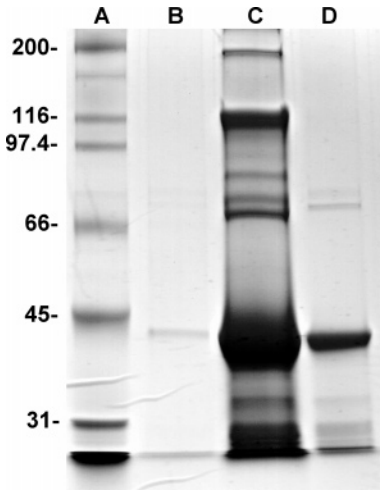

Figure 4. Coomassie-stained polyacrylamide gel showing purified recombinant ESP following elution from a Ni–NTA column. From left to right, 15 μL per well: (**A**) Bio-Rad molecular mass standard, (**B**) fraction 1 at 0.05 mg of protein/mL, (**C**) fraction 2 at 3.6 mg of protein/mL, and (**D**) fraction 2 diluted 10-fold.

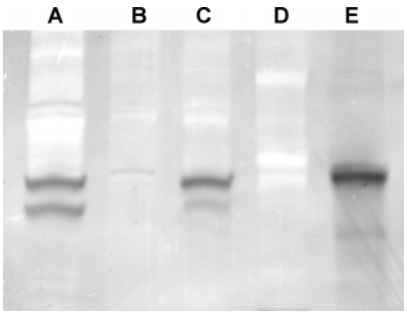

Figure 5. Broccoli proteins recognized by an antibody to recombinant ESP. Fresh, 50% (w/v) extracts (15 μL/well) of (**A**) broccoli, (**B**) daikon, (**C**) white cabbage, and (**D**) horseradish or (**E**) 75 ng of recombinant broccoli ESP was incubated with antibody to ESP (see Materials and Methods).

Dependence of Sulforaphane Nitrile Formation from Glucoraphanin on ESP. When purified glucoraphanin was incubated in buffer with myrosinase purified from white mustard seed (Sigma), the isothiocyanate sulforaphane was the sole product, as previously reported (*9*−*12*). In the presence of 0.1 mM iron and no ESP, some nitrile formation occurred at the expense of sulforaphane, accounting for ∼20% of the product (**Figure 8A**). When increasing concentrations of ESP were added to the incubation containing a fixed amount of iron (0.1 mM), the level of nitrile formation increased in a concentration-dependent manner with ESP, reaching >90% of the product formed (**Figure 8B**). This was in strict contrast to addition of ESP in the absence of added iron, where nitrile formation was below the level of detection.

DISCUSSION

Past work from both our laboratory and others has demonstrated that sulforaphane nitrile is the major hydrolysis product of glucoraphanin when fresh broccoli is crushed and that relatively little of the alternative product, anticarcinogenic sulforaphane, is formed (*13*−*15*). Our research has therefore focused on understanding why sulforaphane is not the major hydrolysis product and how conditions might be manipulated to enhance fractional sulforaphane formation. Recently, we showed that mild heating simultaneously decreased ESP activity and increased the level of sulforaphane formation in broccoli florets (*26*), leading us to hypothesize that a heat sensitive factor might be directing hydrolysis toward nitrile formation. Epithionitrile formation has been shown to be heat sensitive in other species of crucifers (*33*). Recently, it was suggested that the

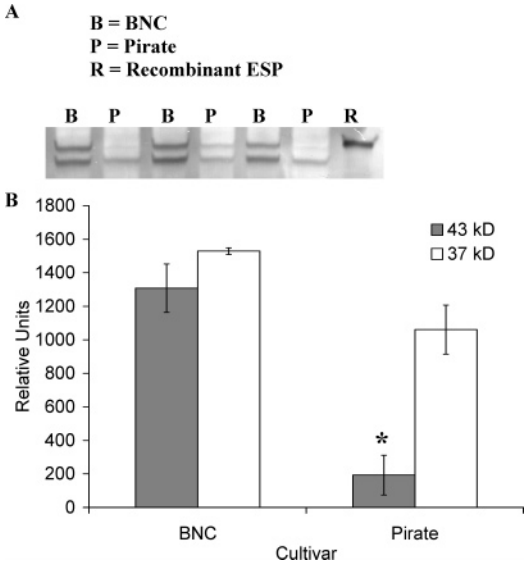

Figure 6. Comparison of ESP proteins in two broccoli cultivars. (**A**) Quantitative western blot analysis of three field replicates of freeze-dried broccoli cv. BNC and cv. Pirate (20 μg of protein/lane). (**B**) Densitometric analysis. The asterisk denotes significant difference; with a Student's *t* test, $\alpha = 0.05$.

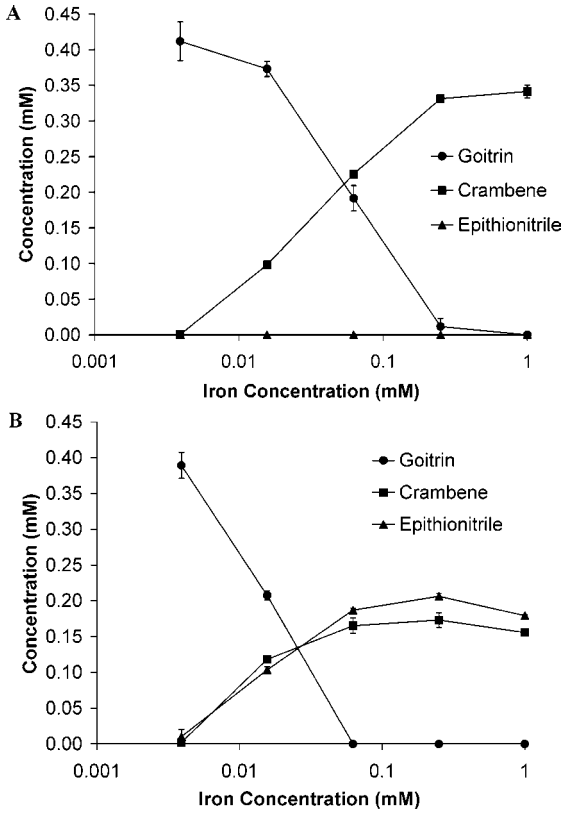

Figure 7. Effect of iron concentration on broccoli ESP activity. *epi*-Progoitrin (1.12 mM) was incubated with myrosinase (0.05 unit/mL) in the presence of increasing concentrations of $FeSO_4$ (**A**) without or (**B**) with 300 μg/mL recombinant ESP.

activity of ESP may go beyond its documented role of catalyzing the formation of an epithionitrile from an alkenyl glucosinolate, also serving the function of a more general "nitrile specifying protein" for glucosinolates such as glucoraphanin that lack a terminal double bond (*27*), although this proposal was recently challenged (*28*). Furthermore, neither the mechanism nor even the presence of ESP has previously been examined in a

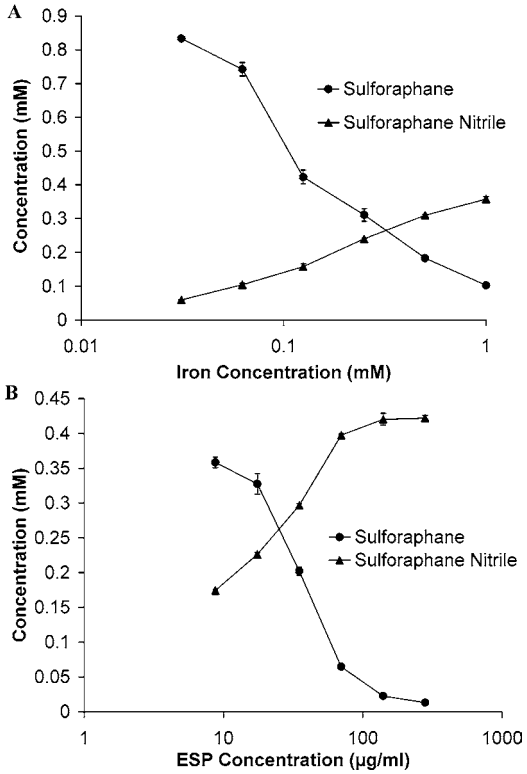

Figure 8. Effect of recombinant ESP on formation of sulforaphane nitrile from glucoraphanin. Purified glucoraphanin (2.02 mM) was incubated with purified myrosinase (0.25 unit/mL) and (**A**) iron (0.1–1.0 mM Fe^{2+}) or (**B**) submaximal iron (0.1 mM Fe^{2+}) and ESP (8.75–280 μg/mL).

commercial vegetable crop such as broccoli, where an improved understanding of glucosinolate hydrolysis could have a profound impact on human health.

Our initial aim was to determine if an association exists between ESP activity and the fractional formation of sulforaphane from glucoraphanin in broccoli. To accomplish this, we correlated these variables among 20 genetically distinct broccoli genotypes. Because different genotypes of broccoli vary significantly in their content of glucoraphanin (*3*), total sulforaphane and sulforaphane nitrile vary accordingly across genotypes. When the level of sulforaphane formation was expressed as fractional conversion, meaning the percent of total glucoraphanin hydrolysis products (sulforaphane + sulforaphane nitrile) formed, a negative correlation with ESP activity was observed (**Table 1**), consistent with ESP playing a role in the formation of the alternative product, sulforaphane nitrile, at the expense of sulforaphane. These data also indicate a diversity in ESP activity, suggesting a potential for decreasing ESP levels in broccoli through traditional hybridization technology.

To confirm a role for ESP in nitrile formation from glucoraphanin in broccoli, we chose to determine if broccoli expresses ESP and, if so, to characterize its activity. To our knowledge, this is the first report that broccoli expresses an ESP and that it is homologous to the ESP found in *A. thaliana* and *B. napus*. In this study, cloning was chosen as an alternative to conventional protein purification because broccoli florets contain only trace amounts of the protein. Thus, a very large amount of starting material would be required to purify sufficient protein for characterization studies. Previously, purification of ESP from the defatted meal of crambe (*C. abyssinica*) and rapeseed (*B. napus*) has been reported (*23*, *31*, *34*). Whereas broccoli seed could have been used for this study, our intent was to characterize the ESP present in floret tissue, the edible

portion of broccoli, because of the potential for ESP to oppose the formation of the anticarcinogen sulforaphane in the human diet. Using an antibody generated to broccoli ESP, we identified ESP in seed, stalk, and leaf in addition to florets (data not shown). However, it is not known if additional ESP isoenzymes are present in seed, possibly leading to activities different than those present in broccoli florets.

The amino acid sequence of rapeseed ESP has been shown to be 82% identical to that of ESP from *Arabidopsis* (*31*). Our finding that the translated sequence of the full-length ESP cDNA from broccoli is greater than 75% identical to that of *A. thaliana* (**Figure 3**) further supports the conserved nature of ESP across species. This homology suggests that these proteins may also share functional similarity. A BLAST search revealed conserved Kelch motifs and Kelch-repeat β-propeller domains within the broccoli ESP sequence, not previously reported for any ESP. The β-propeller domain is known to play a role in the binding of divalent cations and in protein−protein interactions (reviewed in ref *35*). It is possible that the β-propeller motif serves a functional role by binding to ferrous ion at the active site of ESP and/or facilitating a protein−protein interaction between ESP and the myrosinase enzyme.

Using a polyclonal antibody developed against the recombinant protein, we analyzed ESP expression in several cruciferous vegetables and found two reactive bands (43 and 37 kDa) in broccoli and cabbage but no proteins reacting with this antibody in daikon or horseradish (**Figure 5**). This is consistent with the report that neither daikon nor horseradish form nitriles as hydrolysis products of glucosinolates (*21*, *22*). We also examined two broccoli cultivars for their content of ESP and found that the cultivar BNC, which had greater ESP activity, possessed a significantly denser 43 kDa band compared to the cultivar Pirate. The density of the 37 kDa band was similar, however (**Figure 6A,B**). These data may indicate (1) that there are at least two distinct loci encoding homologous ESP proteins in broccoli germplasm, (2) that the smaller protein is an artifact of the extraction process, or (3) that the ESP gene product can undergo post-transcriptional modification. The BNC genotype is a traditional land race cultivar grown in Italy that has undergone more than 100 generations of inbreeding and is therefore unlikely to be polymorphic at the ESP locus (*36*). Recently, ESP purified from *B. napus* (rapeseed) was reported to have a molecular mass of 39 kDa (*31*, *34*), with a second, slightly smaller protein of 35 kDa found by one of these groups (*34*), although the relative activity of these two proteins was not investigated. The finding of two proteins in both rapeseed and broccoli is intriguing and suggests that this was not an artifact of our isolation method. The relative activity of the two ESP proteins and whether this duplicity stems from post-translational modification of a single gene product remain to be elucidated.

The activity of the recombinant protein was evaluated in the presence of commercially available purified myrosinase from *S. alba* and *epi*-progoitrin as the substrate. In the absence of recombinant ESP and iron, myrosinase supported the formation of the cyclic isothiocyanate goitrin from *epi*-progoitrin. Epithionitrile formation did not occur in the absence of the recombinant ESP protein, even when supraphysiologic concentrations of iron were present (**Figure 7A**). Nor did epithionitrile form in the presence of ESP without added iron. In the presence of both recombinant ESP and iron, there was an approximately equimolar appearance of the epithionitrile and nitrile (**Figure 7B**). These data clearly demonstrate that the recombinant ESP was able and necessary to support iron-dependent epithionitrile formation from progoitrin.

To determine whether the recombinant ESP was also capable of directing the formation of sulforaphane nitrile from glucoraphanin, we used glucoraphanin as a substrate for hydrolysis by myrosinase in the presence of iron and recombinant broccoli ESP. As expected, the extent of nitrile formation increased with increasing iron concentrations, even in the absence of ESP (**Figure 8A**). In the absence of iron, even the maximum amount of ESP did not support nitrile formation. In a similar published in vitro study using a crude bacterial preparation of *A. thaliana*, ESP supported sulforaphane nitrile formation during glucoraphanin hydrolysis in the absence of added iron (*27*). It is possible that iron was present in the crude bacterial extract used as a source of recombinant ESP in the latter study, either coincidentally or because the less stringent purification methods did not strip iron from the active center of ESP. Alternatively, it is possible that ESPs from broccoli and *A. thaliana* vary in their iron requirement. This latter possibility appears unlikely, since in a recent study investigating ESP from *A. thaliana* at least 0.1 mM iron was required for the formation of epithionitriles (*28*). However, their ESP preparation did not enhance the iron-dependent formation of the nitrile from glucoraphanin in vitro. The reason for this discrepancy, while not clarified at present, is unlikely to be differences in ESP homology, since these authors did see substantial ESP-enhanced sulforaphane nitrile formation in transgenic plants overexpressing ESP, compared to wild-type plants that did not express ESP (*28*).

The work described here supports the evolutionary conservation of ESP among crucifer species, as well as its role in deflecting glucoraphanin hydrolysis away from sulforaphane formation. Here we demonstrate that ESP is expressed in broccoli and that variation in the ESP content among broccoli cultivars is related inversely to the sulforaphane yield upon glucoraphanin hydrolysis. We also show that broccoli ESP is capable of directing the formation of sulforaphane nitrile, at the expense of the anticarcinogen sulforaphane. As we gain more information about the beneficial effects of health-promoting phytochemicals such as sulforaphane from broccoli, it becomes increasingly important that we understand the fundamental physiological and biochemical factors that control their formation and degradation in the plant so that cultivar production and processing parameters may be optimized for their preservation. One of us has recently produced a hybrid broccoli that has very high glucoraphanin levels (*15*). Research to optimize sulforaphane yield, through processing (*26*) or breeding technology, particularly when coupled with high-glucoraphanin broccoli cultivars, has the potential to provide the public with broccoli that provides greater health benefits.

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