Conversion of Glucosinolates to Isothiocyanates in Humans after Ingestion of Cooked Watercress[1]

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Abstract

Isothiocyanates (ITCs), major constituents of cruciferous vegetables, can inhibit tumorigenesis in rodents by modulating the metabolism of carcinogens. ITCs that occur as glucosinolates are released by myrosinase-mediated hydrolysis when raw vegetables are chopped or chewed. However, because cruciferous vegetables are commonly consumed by humans after being cooked, it is important to examine whether dietary glucosinolates are converted to ITCs after cooked cruciferous vegetables in which myrosinase is deactivated have been consumed. This information is useful for evaluating the potential role of ITCs in cruciferous vegetables in the protection against human cancers. A urinary marker, based on a cyclocondensation product formed by the reaction of ITCs and their conjugates with 1,2-benzenedithiol, was used to quantify the uptake of dietary ITCs in humans. At breakfast and lunch, nine volunteers consumed a total of 350 g of cooked watercress in which the myrosinase activity was completely deactivated. On the basis of the analysis of ITCs in the cooked watercress upon adding exogenous myrosinase, the amount of glucosinolates ingested by each subject was estimated to be 475 μmol. The 24-h urine samples showed that the total urinary excretion of ITC conjugates in the subjects ranged from 5.6 to 34.8 μmol, corresponding to 1.2–7.3% of the total amount ingested. On the basis of our previous results that ~50% of dietary ITCs were excreted in the urine as conjugates, these values represent the minimal *in vivo* conversion of glucosinolates to ITCs. For purposes of comparison, we carried out a second experiment in which 150 g of uncooked watercress were consumed. The percentage of urinary ITC conjugates excreted in this study ranged from 17.2 to 77.7% of the total ingested ITCs. These results indicate that glucosinolates are converted to ITCs in humans after ingestion of cooked watercress, in which the myrosinase has been completely inactivated. The extent of conversion, however, is considerably less than that after ingesting uncooked vegetables. Furthermore, upon incubation of the cooked watercress juice with fresh human feces under anaerobic conditions, ~18% of glucosinolates was hydrolyzed to ITCs in 2 h. These results suggest that the microflora in the intestinal tract are a likely source for the hydrolysis of glucosinolates to ITCs in humans.

Introduction

ITCs,[3] which occur as major constituents in cruciferous vegetables, have been shown to inhibit tumorigenesis in rodents treated with carcinogens (1–4). Studies *in vitro* and *in vivo* showed that the inhibitory activity of ITCs against carcinogenesis can be attributed to their ability to deactivate phase I enzymes and/or to activate phase II enzymes (3, 5). Recent studies in humans also demonstrated that the consumption of cruciferous vegetables resulted in an increase in glutathione transferase activity and a decrease in the metabolic activation of carcinogens in smokers (6–8). These results support a potential role of ITCs in cruciferous vegetables in the prevention of human cancers.

ITCs are products of degradation of glucosinolate precursors in cruciferous vegetables, a reaction usually catalyzed by myrosinase, an enzyme that is activated upon crushing the vegetables by chopping or chewing (Fig. 1*a*; Refs. 9 and 10). We and others have demonstrated that significant amounts of ITCs are released after ingesting raw cruciferous vegetables, as indicated by the presence of ITC mercapturic acid in the urine (11–13). Little is known, however, about the fate of glucosinolates in humans upon consumption of cooked vegetables in which the myrosinase activity is absent. Because crucifers are commonly consumed by humans after being cooked, it is important to know whether glucosinolates in cooked vegetables are converted to ITCs. This information can be useful in the investigation of the potential roles of dietary ITCs in human cancers. Here, we studied the metabolic conversion of glucosinolates to ITCs in humans after ingesting cooked and uncooked watercress and compared the extent of conversion. Because ITCs are primarily metabolized via the mercapturic acid pathway, the *N*-acetylcysteine conjugate of ITC in the urine serves as a marker of ITCs released from glucosinolates. Using a previously developed assay based on the reaction of ITCs or their thiol conjugates with 1,2-benzenedithiol forming a cyclic dithiol thione product (Fig. 1*b*; Refs. 14 and 15), we quantified the urinary metabolites of ITCs after eating watercress with and without myrosinase activity. In addition, we also examined whether intestinal microflora are a possible source for the degradation of glucosinolates in humans by incubating cooked watercress juice with human fecal samples.

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[3] The abbreviations used are: ITC, isothiocyanate; HPLC, high-performance liquid chromatography.

Table 1 Cumulative amounts of ITC conjugates in 24-h human urine before (baseline) and after ingestion of cooked watercress meal

Subject no.	Sex	Urine excreted before consumption (liters)	ITCs in baseline urine (μmol)[a]	Urine excreted after consumption (liters)	ITCs in urine after consumption (μmol)[a]	% of administered dose excreted as ITC conjugates[b]
1	M	1.2	5.4 ± 0.2	2.0	34.8 ± 0.8[c]	7.3
2	F	0.6	0.6 ± 0.2	1.5	33.0 ± 2.5	6.9
3	F	1.5	0.5 ± 0.1	3.2	28.1 ± 0.2	5.9
4	F	1.3	1.1 ± 0.1	0.8	17.8 ± 0.7	3.7
5	M	2.8	1.0 ± 0.1	3.0	17.4 ± 0.1	3.6
6	F	0.3	0.3 ± 0.1	0.7	15.2 ± 0.6	3.2
7	F	1.4	1.6 ± 0.4	1.4	15.4 ± 0.3	3.2
8	M	2.1	2.2 ± 0.1	1.4	5.6 ± 0.1	1.2
9	M	1.8	0.7 ± 0.1	3.0	5.7 ± 0.2	1.2

[a] Mean ± SD (triplicate data).
[b] On the basis of 475 μmol of ITCs in cooked watercress juice treated with myrosinase.
[c] Values obtained after subtracting the baseline concentration.

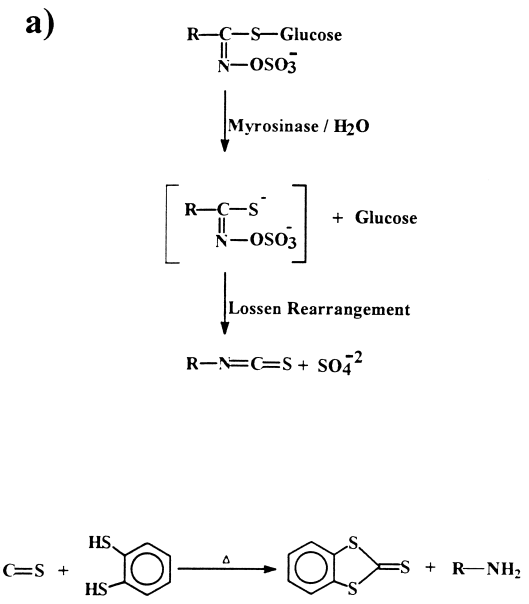


Fig. 1. *a*, myrosinase-catalyzed conversion of glucosinolates to ITCs via Lossen rearrangement. *b*, reaction of ITC with 1,2-benzenedithiol forming the cyclocondensation product, 1,3-benzodithiole-2-thione, and amine.

Materials and Methods

Chemicals. 1,2-Benzenedithiol was purchased from Lancaster Synthesis, Inc. (Windham, NH). 1,3-Benzodithiole-2-thione was prepared and characterized according to a published method (14, 15). Myrosinase was prepared from mustard seeds according to a published procedure (16). Nutrient broth medium was purchased from Difco Laboratories (Detroit, MI). Sodium thioglycolate was purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals and solvents were reagent grade and were obtained from commercial sources.

Study Protocol. Nine healthy volunteers (designated 1–9; five females and four males; Table 1) ages 23–45 years were recruited for the study. All subjects signed a consent form before participating in the 4-day study. The experimental protocol is outlined in Table 2.

Volunteers were asked to avoid foods containing ITCs such as mustard and cruciferous vegetables, including broccoli, cabbage, horseradish, turnips, brussels sprouts, collard greens, and tender greens, for the entire experimental period. A 24-h urine sample was collected on day 2 as baseline prior to the consumption of watercress on day 3. Fresh watercress (*Nasturtium officinale*) purchased from a local supermarket was cooked in boiling water for 3 min (85.7 g/liter), and a total of 350 g was ingested by each volunteer, 200 g at breakfast and 150 g at lunch. After breakfast, a 27-h urine sample was collected from each subject. Another 18-h urine sample was collected after lunch on day 4 as the follow-up. After a 7-day period, a second experiment was conducted. The same protocol was used with the exception that uncooked watercress was consumed. Because we have previously conducted a similar study using uncooked watercress (12), in this experiment, only four subjects (subjects 1, 2, 7, and 8; see Table 3) participated who consumed 100 g for breakfast and 50 g for lunch on day 3. In both experiments, a 10-ml aliquot of each pooled urine sample was stored at −20°C until ITC analysis.

Table 2 Experimental Protocol

Days 1 and 2	Day 3	Day 4
Baseline	Watercress consumption	Follow-up

Analysis of ITC in Urine. The amount of ITCs in the urine samples was analyzed by a previously described method (15). Briefly, the urine sample was thawed and vortexed. A 1-ml aliquot was placed in a 2-ml centrifuge tube and centrifuged for 20 min at 1000 × *g*. An aliquot (100 μl) of the supernatant was mixed with 600 μl of 1,2-benzenedithiol in 2-propanol (10 mM, degassed) and 500 μl of phosphate buffer (0.1 M, pH 8.5, degassed) in a 2-ml Chromacol autosampler vial with a screw cap (Chromacol, Inc., Trumbull, CT). The mixture was then vortexed for 1 min and incubated at 65°C for 2 h in a water bath shaker. The product 1,3-benezodithiol-2-thione was analyzed by reverse-phase HPLC using a Waters μBondapak C_{18} (150 × 3.9 mm) with a C_{18} Waters μBondapak guard column and a detection wavelength of 365 nm. The mobile phase consisted of a mixture of methanol and H_2O (7:3 v/v) with a flow rate of 1.75 ml/min. A standard curve was constructed using a series of 1,3-benzodithiole-2-thione solutions with concentrations ranging from 0 to 20 μM in 2-propanol:water (1:1 mixture). Each urine sample was aliquoted three times and analyzed in triplicate. A negative control sample contained only deionized water.

Analysis of ITC in Watercress. The cooked watercress (113 g) was blended for 2 min in deionized water (100 ml) using a Mini-Prep blender (Cuisinart) until it became a fine paste. The paste was then poured through six layers of cheesecloth, and the liquid was squeezed into a 500-ml beaker. Another portion of

Table 3 Cumulative amounts of ITC conjugates in 24-h human urine before (baseline) and after ingestion of uncooked watercress meal[a]

Subject no.	Sex	Urine excreted before consumption (liters)	ITCs in baseline urine (μmol)[a]	Urine excreted after consumption (liters)	ITCs in urine after consumption (μmol)[a]	% of administered dose excreted as ITC conjugates[b]
2	F	1.4	0.7 ± 0.0	2.0	755.2 ± 0.4[c]	77.7
1	M	2.0	1.6 ± 0.0	3.2	447.3 ± 0.5	46.3
7	F	1.4	0.7 ± 0.1	2.8	388.0 ± 2.4	39.6
8	M	2.5	1.7 ± 0.1	1.9	167.2 ± 0.1	17.2

[a] Mean ± SD (triplicate data).
[b] On the basis of 972 μmol of ITCs in uncooked watercress extract.
[c] Values obtained after subtracting the baseline concentration.

deionized water (125 ml) was added, and the liquid was again squeezed into the beaker. This process was repeated a third time, and the combined liquid (350 ml) was transferred to a 500-ml volumetric flask and deionized water was added to 500 ml. A 10-ml aliquot of this solution was then filtered through Whatman filter paper no. 1 using a Buchner funnel to obtain a clear solution. One ml of myrosinase (2 mg/ml) in 0.1 M potassium phosphate buffer (pH 6.6) was then added to the filtrate; the mixture was then incubated at 37°C for 2 h. For the analysis of ITCs released in the incubation mixture, 100 μl of the myrosinase-treated watercress juice were subjected to the cyclocondensation reaction as described above. The glucosinolates and free ITCs in the broth after cooking watercress were also determined by the same method.

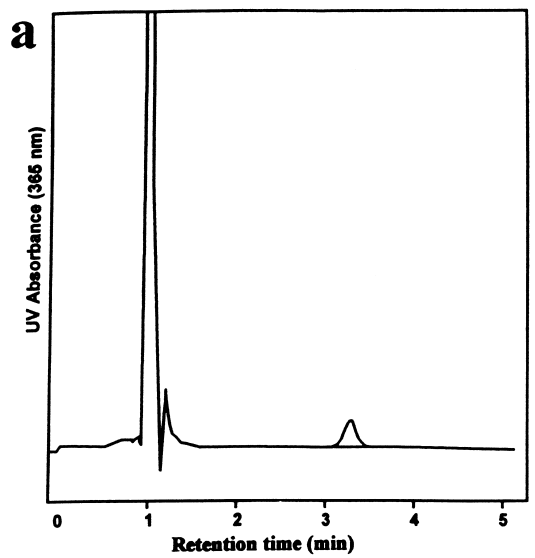
For the determination of ITC content of uncooked watercress, 10 g was finely chopped in Mini-Prep blender, followed by the addition of methanol (50 ml). In this experiment, methanol was used for efficient extraction of ITCs. The mixture was then blended into a fine paste. The resulting paste was filtered through six layers of cheesecloth, and the residue was again washed with 50 ml of methanol. The procedure was repeated two more times, and the final volume was adjusted to 200 ml in a volumetric flask. A 10-ml aliquot of this solution was filtered through a Whatman filter paper no. 1 using a Buchner funnel; a 100-μl aliquot of the filtrate was subjected to the cyclocondensation reaction as described above.

Release of ITCs from Cooked Watercress Juice upon Incubation with Human Feces. A fresh human fecal sample was stored in a disposable anaerobic system (Gas pak; Becton Dickinson Co., Cockeysville, MD) with a hydrogen-carbon dioxide generator container. The stool specimen (20 g) was placed in a 50-ml centrifuge tube containing 15 ml of 0.01% sodium thioglycolate in 50 mM sodium phosphate (pH 7.2). The above mixture was vortexed under nitrogen in a flow hood and centrifuged at 2000 × *g* for 5 min. The supernatant was placed in another tube, and the sediment was vortexed with an additional 30 ml of buffer. The supernatants were combined and sonicated at 0°C for 30 s and centrifuged again at 2000 × *g* for 30 min. A 3-ml aliquot of the supernatant was mixed with 15 ml of sterilized nutrient broth media (0.65%, pH 7.2) and 2 ml of cooked watercress juice. The mixture was placed in a serum bottle and sealed with a rubber stopper and an aluminum cap. The serum bottle was incubated at 37°C with agitation for 30 min and 1, 2, 3, 4, and 6 h. As negative control, the fecal homogenate was boiled at 100°C for 10 min. In addition, 2 ml of watercress juice were incubated under identical conditions with 1 ml of myrosinase (2 mg/ml) and 17 ml of nutrient broth for 2 h as the positive control. The incubation was terminated by mixing 500 μl of the incubation mixture with 800 μl of 10 mM 1,2-benzenedithiol in 2-propanol. After addition of 500 μl of phosphate buffer pH 8.5, the mixture was incubated at 65°C for 2 h. The cyclocondensation reaction product was analyzed by HPLC, as described above.

Results and Discussion

We have previously shown that the cyclocondensation product 1,3-benzodithiol-2-thione provides a useful and specific urinary biomarker for quantifying uptake of ITCs after ingesting uncooked watercress and mustard (15). Because the ITC conjugates are in equilibrium with free ITCs and can react with thiols through an exchange reaction, the mercapturic acid conjugates excreted in the urine readily undergo the cyclocondensation reaction with 1,2-benzenedithiol (15, 17). Using this assay, we demonstrated that thiol conjugates of ITCs were excreted in the urine after consumption of cooked watercress. These results provide clear evidence that glucosinolates in cooked vegetables are converted to free ITCs in humans.

The cooked watercress used in this study was completely devoid of myrosinase activity, as indicated by the absence of ITCs in the juice of the homogenized watercress. Upon addition of exogenous myrosinase to the cooked watercress juice followed by incubation at 37°C for 2 h, 1.4 μmol of ITCs per 1 g of vegetable were released. It is estimated, based on this value, that a total of 475 μmol of ITCs were consumed by each subject after eating 350 g of cooked watercress. The amount of myrosinase used and the incubation time were determined by monitoring the formation of ITCs from known concentrations of sinigrin ranging from 0.7 μM to 0.4 mM. Under these conditions, the conversion of sinigrin to allyl ITC was quantitative at all concentrations examined. Therefore, the amount of 475 μmol is likely to represent the total glucosinolate content in the cooked watercress because increasing the incubation time or amount of myrosinase did not result in further hydrolysis. Gluconasturtiin, the precursor of phenethyl ITC, constitutes >30% of the total glucosinolate content in watercress (12). The glucobrassicins, minor components in watercress that are converted to indoles, do not hydrolyze to ITC by myrosinase (10). In this study, we noted a 6-fold difference in the total levels of ITC metabolites excreted in the 24-h urine collected from each of the nine subjects, ranging from 5.6 to 34.8 μmol after subtracting the background levels (Fig. 2 and Table 1). The background levels of cyclocondensation product found in baseline urine samples are intriguing. Similar background levels were found in the urine samples collected during the follow-up period (day 4). It is not clear whether they come from residual ITCs or other related compounds. On the basis of the total glucosinolates (475 μmol) consumed, the rate of conversion to ITC ranged from 1.2 to 7.3%. If one assumes that ~50% administered ITCs are excreted in the 24-h urine as reported previously (12, 13), this represents a total conversion of 2.4–14.6%.



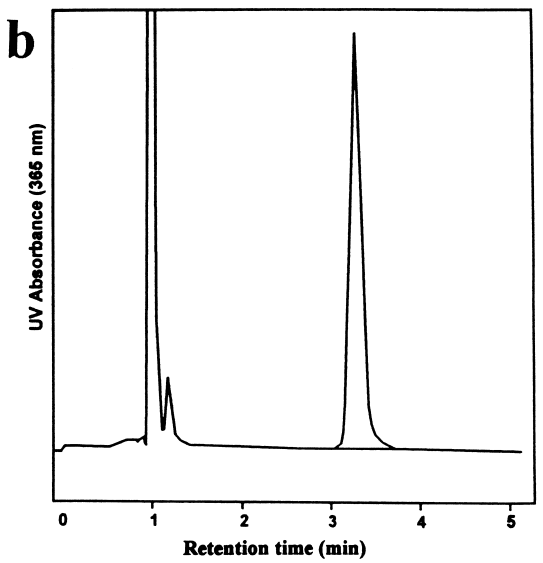


Fig. 2. Reverse-phase HPLC chromatograms obtained from analysis of a 24-h human urine sample after consumption of cooked watercress (*a*) and raw watercress (*b*). Peak at 3.2 min is identified as 1,3-benzodithiole-2-thione by its retention time and UV spectrum as compared to the synthetic standard.

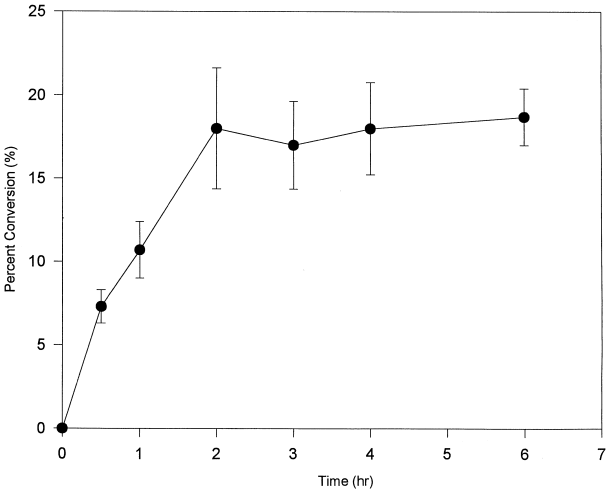


Fig. 3. The percentage of glucosinolates converted to ITCs upon incubation with human fecal homogenates.

To compare the extent of conversion in humans after ingesting cooked and uncooked watercress, we carried out a second experiment in which four subjects who participated in the first experiment with cooked watercress were asked to eat a total of 150 g of uncooked watercress for breakfast and lunch. The total ITCs in the 150 g of uncooked watercress were 972 μmol or 6.5 μmol/g, as compared to 1.4 μmol/g in the cooked watercress after myrosinase treatment. A substantial amount of glucosinolates was found in the broth (2.8 μmol/g) of the cooked watercress after myrosinase treatment. These results are consistent with the notion that cooking may cause a significant loss of glucosinolates in cruciferous vegetables (18). In the experiment with the uncooked watercress, the amount of ITC metabolites in the 24 h urine was found to range from 167 to 755 μmol, equivalent to a conversion rate from 17.2 to 77.7% (Table 3). These rates of conversion are comparable to those reported previously for phenethyl and allyl ITCs, which ranged from 30 to 67% (12, 13). A 4-fold difference in the metabolism of dietary ITCs among the subjects in this experiment was observed. These results indicate that the extent of conversion in humans after consumption of the uncooked watercress was at least 10-fold greater than that of the cooked watercress. Interestingly, in both experiments, subjects 1 and 2 had a higher level of urinary metabolites than those found in subjects 7 and 8, suggesting there may be individual variabilities in the metabolism of ITCs via the mercapturic acid pathways.

Oginsky *et al.* (19) previously showed that goitrin was detected in urine and blood of humans after oral administration of pure progoitrin, the glucosinolate precursor of goitrin. This group reported that a variety of bacterial species, including those present in human feces, appeared to have myrosinase-like activity (20). Goodman *et al.* (21) described thioglucosidase activity in the tissues of mammals, including humans. Our study is the first to show that the intact glucosinolates in cooked cruciferous vegetables can be converted to ITCs in humans. To verify the potential role of intestinal microflora in the conversion, we examined whether human feces possess such activity under anaerobic conditions. Upon incubating cooked watercress juice with human fecal samples, we observed a linear increase of ITC up to 17.7% for the first 2 h of incubation. No further increase of ITCs was seen after 2 h, possibly due to the ITC-mediated inhibition of enzyme activities (Fig. 3). ITCs were not detected in the incubations with the heat-deactivated fecal homogenate. These results demonstrated that a myrosinase-like activity is present in human feces, supporting the notion that microflora in the gut may contribute to the degradation of glucosinolates to ITCs in humans. We cannot, however, exclude the possibility that nonspecific thioglycosidase activity is present in tissues (21).

Studies have shown that glucosinolates inhibited tumorigenesis in carcinogen-treated rodents (22, 23). The mechanism(s) by which glucosinolates inhibited tumorigenesis is not

known; however, it is possible that ITCs released *in vivo* could, in part, be responsible for the inhibition. We showed that in A/J mice 21% of the total gluconasturtiin given in the diet was excreted as the *N*-acetylcysteine conjugate of phenethyl ITC (12), indicating *in vivo* hydrolysis of gluconasturtiin to its ITC. This study shows that humans can degrade glucosinolates to ITCs upon ingesting cooked crucifers, although the extent is considerably less than that after ingesting uncooked vegetables. These results could explain our recent observations that a significant amount of ITC metabolites is present in the urine from samples accrued from cohort studies in Singapore and Shanghai; both populations are known to consume daily cooked cruciferous vegetables (15, 17). These findings may have significant implications in epidemiological investigations toward a better understanding of the role of cruciferous vegetables in the protection against human cancers.

Acknowledgments

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