Chapter 2

Glucosinolates, Myrosinase, and Isothiocyanates: Three Reasons for Eating Brassica Vegetables

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Numerous epidemiological studies provide consistent evidence that individuals who consume the highest quantiles of fruit and vegetables experience lower risks of developing several types of cancer (reviewed by *1-3*). Although cruciferous vegetables (and especially *Brassica* sp.) are believed to play a special role in this risk reduction (*4,5*), it is by no means clear how much of the protective effect can be attributed to the nutrient or the non-nutrient components of these foods, or to what extent indirect effects such as a parallel reduction in fat consumption and increases in fiber or vitamin and carotenoid intake may be responsible for protection. Epidemiological studies do, however, support the contention that dietary modification could reduce cancer risk by one third to one half in economically developed countries (*6,7*). Furthermore, it is believed that the majority of cancer cases (1.4 million new cases are expected in the U.S. alone in 1997) could be avoided by reducing various risk factors (tobacco, radiation, alcohol, exposure to certain drugs and chemicals) in combination with dietary modification (*8*). More recently, the prospect of reducing susceptibility to carcinogens by the administration of various chemical (including dietary) protectors has become widely recognized as both a feasible and a rational strategy in the battle against cancer (*9-12*). This approach has been termed chemoprotection, chemoprevention, or chemoprophylaxis.

One major strategy for achieving chemoprotection against cancer depends on the induction of Phase 2 detoxication enzymes in mammalian tissue by the administration of dietary or synthetic chemical agents (*13-15*). Although chronic exposure to various metabolic stresses cannot be always regarded as beneficial, exposure of animals and their cultured cells to low levels of certain of these stresses, especially electrophilic chemicals, causes an acceleration of reduced glutathione synthesis. In addition, electrophile exposure causes a coordinated induction of many of the Phase 2 enzymes that detoxify these compounds, thus reducing the susceptibility of cells to higher concentrations of the inducer as well as other related electrophiles (*16,17*). These Phase 2 enzymes (e.g. glutathione transferases, NAD(P)H:quinone oxidoreductase, glucuronosyltransferase, and epoxide hydrolase) inactivate ultimate carcinogens by destruction of their reactive centers or by conjugation with endogenous ligands thereby

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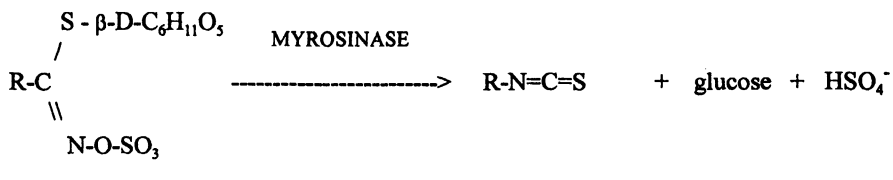
neutralizing their toxic properties and accelerating their elimination from the body. Evidence for a causal relationship between Phase 2 enzyme induction and protection has been mounting and can be considered as firmly established (*14,15*). Recently, considerable attention has been focused on edible plants which are rich in phytochemicals that induce Phase 2 enzymes (*18-20*). This approach is consistent with the epidemiological evidence which also strongly suggests the involvement of plants in cancer chemoprotection . Thus, cruciferous vegetables (e.g. broccoli, cauliflower, kale, turnips, collards, Brussels sprouts, cabbage, kohlrabi, rutabaga, Chinese cabbage, bok choy) contain water soluble secondary metabolites called glucosinolates, which are converted by endogenous enzymes (myrosinases) into highly reactive isothiocyanates as a defense response to predation or injury. The resultant isothiocyanates (R-N=C=S) or mustard oils are the principal Phase 2 enzyme inducers of cruciferous plants. The most potent naturally-occurring inducer isothiocyanate is sulforaphane (*19*). Sulforaphane was isolated in this laboratory from broccoli by monitoring the inducer activity of extracts of this plant. The inducer and anticarcinogenic properties of sulforaphane have also been demonstrated in animal tissues (*21,22*). The latent inducer activity of extracts of 3-day-old broccoli seedlings (sprouts) is attributable almost entirely to the presence of glucoraphanin (the glucosinolate precursor of sulforaphane). The concentration of inducer activity in such sprouts (200,000 - 800,000 units per gram fresh weight) is much higher than that of mature broccoli heads (5,000 - 80,000 units per gram fresh weight). When comparable doses of sulforaphane and of broccoli sprout extracts (based on inducer activity) were used to treat rats that also received single doses of the potent carcinogen DMBA (dimethylbenz[a]anthracene), a quantitatively similar reduction in mammary tumor development was observed (*20*).

The very stable glucosinolate precursors of isothiocyanates are typically present in some plants (e.g. crucifers) at much higher concentrations than their cognate isothiocyanates. Glucosinolate levels can amount to as much as 1% (w/w) in tissues of some cruciferous plant species. Glucosinolates comprise about 0.05 - 0.1% of the fresh weight of broccoli or about 50 - 100 mg of these compounds per 100 g portion. Glucosinolates are hydrolyzed to isothiocyanates by the coexisting enzyme myrosinase which is physically segregated within plant cells. It is commonly accepted that myrosinase activity is initiated when plants are damaged by insects or other predators, by food preparation, by chewing, or by other forms of damage, such as bruising or freeze-thawing during cultivation, harvest, shipping or handling, and in animal digestive systems. These types of tissue damage release myrosinase [thioglucohydrolase; EC 3.2.3.1] which cleaves the thioglucose linkage thereby releasing glucose. The resulting unstable intermediate undergoes [a probably nonenzymatic] rearrangement in which sulfate is released and isothiocyanates as well as



other products are formed. Nearly all of the inducer activity in extracts of crucifers arises from glucosinolates which are not biologically active as such, but must undergo hydrolysis to isothiocyanates in order to manifest Phase 2 enzyme inducer activity (*20*).

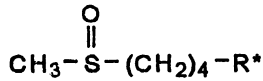
Glucosinolates were first isolated in the middle of the last century and much effort has been devoted to developing methods for their efficient isolation and identification. Existing methods for separation involved ion exchange, GC, and HPLC, mostly after chemical modification (enzymatic sulfate removal and/or silylation of sugar moieties), but chromatographic standards are difficult to obtain and in many methods biological activity of the molecule is destroyed. We have therefore developed methods for the separation and identification of individual glucosinolates in plant extracts by use of paired-ion chromatography in the presence of tetraoctyl- or tetradecyl-ammonium bromide in combination with mass and NMR spectroscopy for final confirmation of identity (*23*). The paired-ion chromatography methods are extensions of methods developed by others (*24,25*).

Paired-ion chromatography of glucosinolates in conjunction with myrosinase hydrolysis and the quantitation of the resultant isothiocyanates by cyclocondensation with 1,2-benzenedithiol provides a powerful and comprehensive procedure for rapidly characterizing and quantitating glucosinolates in plant extracts (*23*). The cyclocondensation reaction with vicinal dithiols was developed for the quantitation of isothiocyanates. It exploits the ability of isothiocyanates to form cyclic thiocarbonyl derivatives with vicinal dithiols. When 1,2-benzenedithiol is the analytical reagent, the resultant 1,3-benzodithiole-2-thione has highly favorable properties for spectroscopic quantitation (a_m = 23,000 $M^{-1}cm^{-1}$ at λ_{max} = 365 nm)(*26*). The cyclocondensation procedure has been recently improved and can now be used to measure as little as 10 picomoles of isothiocyanates in complex biological mixtures such as plant extracts (*27*). In all cases examined, inducer activity of broccoli extracts correlated with their potential isothiocyanate content (i.e., endogenous isothiocyanate plus isothiocyanate released by the action of an excess of added purified myrosinase).

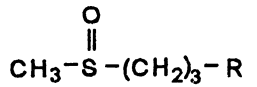
Phase 2 enzyme inducer potency varies according to a number of factors. We have obtained preliminary data showing that there are large cultivar effects as well as tremendous environmentally induced variation due to cultivation, handling and storage parameters. Once tissue is damaged (e.g. at harvest, or due to improper handling), the enzyme myrosinase initiates the conversion of relatively non-reactive glucosinolates to isothiocyanates and other breakdown products which are more highly reactive than glucosinolates. There is thus an immediate opportunity for loss of Phase 2 inducer potency since glucosinolate breakdown products such as isothiocyanates initiate further degradative reactions within the plant tissue. A sampling of fresh broccoli collected randomly from 22 Baltimore area supermarkets had qualitatively similar glucosinolate profiles, but had a greater than 8-fold range of inducer potencies between the highest and lowest sample (*20*). This tremendous variability in glucosinolate content [and hence Phase 2 inducer potency] thus translates to an inability of the consumer to rationally select broccoli for inducer potency using criteria such as appearance and odor for there are no such clues as to their ranking.

The common alkylthioalkyl glucosinolates (e.g. glucoraphanin, glucoiberin and glucoerucin; see Figure 1) form isothiocyanates (sulforaphane, iberin and erucin

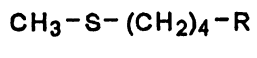
Glucoraphanin
(4-methylsulfinylbutyl)



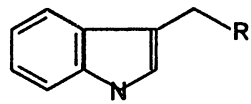
Glucoiberin
(3-methylsulfinylpropyl glucosinolate)



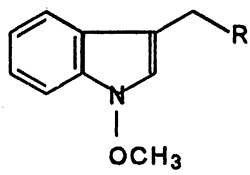
Glucoerucin
(4-methylthiobutyl glucosinolate)



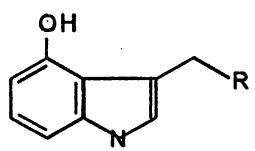
Glucobrassicin
(indole-3-ylmethyl glucosinolate)



Neoglucobrassicin
(1-methoxyindole-3-ylmethyl glucosinolate)



4-Hydroxyglucobrassicin
(4-hydroxyindole-3-ylmethyl glucosinolate)



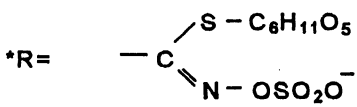


Figure 1. Glucosinolates commonly found in broccoli.

respectively) with very low cytotoxicity and extremely high Phase 2 enzyme inducing potency. In fact sulforaphane is the most potent naturally occurring Phase 2 enzyme inducer. These compounds are monofunctional inducers since they do not also induce Phase 1 enzymes which can activate xenobiotics thus potentially generating carcinogens (*19,28*).

Indole glucosinolates (e.g. glucobrassicin, neoglucobrassicin, 4-hydroxyglucobrassicin, 1-hydroxyglucobrassicin and 4-methoxyglucobrassicin) do not form stable isothiocyanates when hydrolysed by myrosinase. Unstable intermediates are formed in the enzymatic reaction and give rise to compounds such as indole-3-carbinol, indole-3-acetonitrile and 3,3'-diindolylmethane (*29-31*). These compounds are only weak inducers of Phase 2 detoxification enzymes (unpublished obervations). For example, the inducer potency of indole-3-acetonitrile is over 200 times lower than that of sulforaphane. However, these compounds also induce Phase 1 enzymes, i.e. they are bifunctional inducers (*28*), so that they can participate in the formation of carcinogens by modulating the metabolic activation of procarcinogens. Furthermore, in the acid conditions in the stomach, indole-3-carbinol can also spontaneously condense to cyclic structures which resemble TCDD (dioxin), and bind with high affinity to the Aryl hydrocarbon (Ah) receptor, and induce certain cytochromes P-450 that can activate procarcinogens. Metabolic derivatives of indole glucosinolates may therefore act simultaneously both to promote tumors and to prevent their initiation (*30,32-34*).

Myrosinase hydrolysis of another class of glucosinolates, the β-hydroxyalkenyl glucosinolates (e.g. progoitrin and epiprogoitrin), gives rise to intermediates that apparently cyclize spontaneously to goitrogenic oxazolidonethiones. Over-consumption of certain *Brassica* vegetables such as kale and cabbage has been known for many years to cause goiter in both experimental animals and humans and may be responsible for the development of endemic goiter in certain regions (*35*). Although some crucifers such as cabbage and especially the oilseed crops crambe and rapeseed contain large quantities of these goitrogenic glucosinolates, broccoli does not contain significant quantities of these compounds.

Efforts are being made to understand the complex effects of diet on cancer incidence. We have focused on minor dietary constituents that elevate the activity of cellular detoxication or Phase 2 enzymes based on evidence indicating that induction of these enzymes blocks the formation of tumors in experimental animals. Sensitive analytical techniques have been developed for the quantitation of glucosinolates and of isothiocyanates and these methods are being used to evaluate the anticancer potential of a range of cruciferous vegetables and the environmental and genetic components of glucosinolate production. Since glucosinolates are water-soluble, cooking and other forms of processing should be performed, if at all, in such a way (e.g. by steaming, microwaving, stir-frying or rapid boiling with minimal water) as to avoid excessive leaching of the compounds.

The utility of these plants in selectively elevating Phase 2 enzymes (and not Phase 1 enzymes) is a promising facet in the rational development of dietary strategies for reducing the risk of cancer.

Acknowledgments

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