Enzymatic, Chemical, and Thermal Breakdown of ^{3}H-Labeled Glucobrassicin, the Parent Indole Glucosinolate

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The enzymatic, chemical, and thermal breakdown pathways of glucobrassicin, the major indolylmethyl glucosinolate of cruciferous vegetables, have been studied using synthetic ^{3}H-labeled glucobrassicin (GBS). Radio-HPLC was used to analyze qualitatively and quantitatively the resulting products as well as their kinetics of formation. Enzymatic breakdown of GBS under myrosinase action gave rise to different indole compounds [indole-3-carbinol (I3C), indole-3-acetonitrile (IAN), and 3,3′-diindolylmethane (DIM)]. At neutral pH, GBS degradation was almost complete after 1 h, and the major breakdown product was I3C, which could be converted to DIM. The formation of this self-condensation product was observed as photosensitive. In acidic conditions, enzymatic degradation of GBS was a slower phenomenon, requiring 24 h to be nearly complete. IAN and I3C were the only two products occurring, and it was observed that the light had no effect either on the rate of formation or on the relative proportions of the breakdown products observed. GBS appeared as a very stable compound since no chemical degradation could be observed after 2 h in different aqueous media with pH in the 2−11 range. Moreover, after exposure to heat treatment, GBS was weakly degraded (10% in 1 h), giving rise to a new minor indole condensation product corresponding to a 3-(indolylmethyl)glucobrassicin (IM-GBS).

Keywords: *Glucosinolates; glucobrassicin; indole derivatives; anticarcinogenic substances; enzymatic breakdown; chemical breakdown; thermal breakdown*

INTRODUCTION

Glucosinolates constitute a wide class of natural compounds from which approximately a hundred have been identified to date. They possess a common chemical structure consisting in a β-D-1-thioglucopyranose moiety bearing on the anomeric site an O-sulfated thiohydroximate function, and they only differ by their side chain, which can be aliphatic, aromatic, or heterocyclic. Glucosinolates occurring in cultivated plants are responsible for the pungent flavor of the condiments horseradish and mustard and contribute to the characteristic flavors of turnip, Swedish rutabaga, cabbage, and related vegetables. In certain cruciferous plants, some of these substances have also been associated with endemic goiter.

Glucobrassicin (GBS; Figure 1) represents the most widespread indole glucosinolate and is present in cruciferous vegetables of the *Brassica* genus (Fenwick *et al.*, 1983; McDanell *et al.*, 1988). This indolylmethyl glucosinolate appears to be involved, via its breakdown products, in the inhibition of chemically induced carcinogenesis in animal (Stresser *et al.*, 1994; Takahashi *et al.*, 1995). One mechanism by which these chemicals may inhibit carcinogenesis is through the induction of enzymes, such as cytochrome P-450 dependent monooxygenases, glutathione *S*-transferases (GST), or epoxide hydrolases (EH) (Loub *et al.*, 1975; McDanell *et al.*, 1987), involved in the biotransformation of carcinogens to more polar and excretable forms.

Most of the biological effects as well as the flavors appreciated by man are not caused by the glucosinolates *per se* but by the degradation products of these compounds, and the active indole breakdown products issued from GBS may be the result of various degradation processes: enzymatic, chemical, or thermal.

In plant tissue, glucosinolates are always accompanied by a glucosinolate-hydrolyzing thioglucosidase, myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1). In the intact plant, enzyme and substrate occur in separate plant compartments, presumably as an adaptative measure to avoid autotoxicity, and during storage or processing of the vegetables, the enzyme action is the most common pathway leading to the indole breakdown products.

Gmelin (Gmelin and Virtanen, 1961; Gmelin, 1964) pioneered studies on the enzymatic breakdown of GBS. He proposed, on the basis of experimental data obtained using GBS extracted from kohlrabi leaves, a general scheme in which various indole derivatives [*i.e.* indole-3-carbinol (I3C), indole-3-acetonitrile (IAN), and 3,3′-diindolylmethane (DIM)] are generated, depending on the pH of the reaction. This scheme, which indicates the formation of the presumably unstable isothiocyanate, has often been referred to by various authors (Loub *et al.*, 1975; McDanell *et al.*, 1988; Hanley *et al.*, 1990).

In the 1980s, several studies were carried out to investigate the chemical and physiological properties of the compounds resulting from indole glucosinolate hydrolysis. The influence of pH on the formation of enzymatic breakdown products has been particularly investigated, showing that different amounts of all the indole products described above were formed (Searle *et al.*, 1982; Bradfield *et al.*, 1987). More recently, studies on enzymatic degradation products were conducted with synthesized GBS (Latxague *et al.*, 1991), and the breakdown products identified by gas chromatography/mass spectrometry (GC/MS) were similar to those found from the plant extracts.

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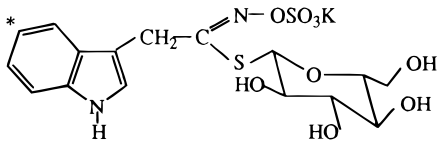
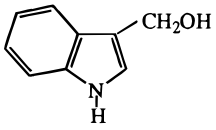


[5-^{3}H]-Glucobrassicin (GBS)



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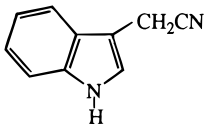
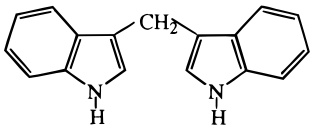


Indole-3-acetonitrile (IAN)



3,3'-Diindolylmethane (DIM)

Figure 1. Chemical structures of [5-^{3}H]GBS and indole breakdown compounds. (*) Labeling position.

Besides, myrosinase may be inactivated by temperatures in the 100−110 °C range, which are those encountered during cooking of vegetables. As a consequence, large amounts of intact glucosinolates, and particularly of indole glucosinolates when Brussels sprouts are concerned, may be ingested as such by humans and submitted to the low pH conditions of the stomach. Moreover, thermal degradation may first occur. In fact, there are very few available data on the chemical breakdown of GBS. An early work (Gmelin and Virtanen, 1961; Gmelin, 1964) proposed a scheme for different chemical breakdown pathways of GBS. The qualitative detection of the products formed by acid or alkaline conditions was achieved using thin layer and paper chromatography. More recently, the acidic hydrolysis of GBS has been investigated (Tiedink *et al.*, 1991), and it was found that only 17% of GBS was degraded. However, the final breakdown products were not examined.

With regard to the thermal degradation of indole glucosinolates, the main work has been conducted by Slominski and Campbell (1987, 1988, 1989a,b). These authors analyzed the degradation products resulting from the heat treatment used to inactivate myrosinase enzyme in commercial low-glucosinolate rapeseed meal samples. IAN was identified as the major indole derivative, and a scheme was proposed for the thermal degradation of GBS, which is quite similar to that proposed by Gmelin and Virtanen (1961) for enzymatic hydrolysis.

The aim of the present work was to re-examine the three headings (enzymatic, chemical, and thermal degradation) as well as the influence of light during the enzymatic degradation, using a chemically pure indole glucosinolate as starting material. Synthetic GBS and 5-^{3}H-labeled GBS allowed a complete followup (on both qualitative and quantitative bases in the different conditions) of the labeled indole products formed.

MATERIALS AND METHODS

Chemicals. Synthetic GBS with a chemical purity (determined by HPLC) better than 97% was prepared according to the method of Rollin (Viaud *et al.*, 1990, 1992). [5-^{3}H]-Glucobrassicin (specific activity = 14 Ci/mmol) was synthesized in our laboratory as previously described (Chevolleau *et al.*, 1993). Its radiochemical purity was at least 98% when analyzed by HPLC. I3C and IAN were purchased from Aldrich (Saint-Quentin-Fallavier, France). DIM was synthesized by the reflux method described by Leete and Marion (1953) and modified by Latxague *et al.* (1991). The product was characterized by GC/MS after silylation. The mass spectrum of DIM presented a molecular ion at *m*/*z* 390 and characteristic fragments at *m*/*z* 317 and 202 as previously described (Latxague *et al.*, 1991). Myrosinase (*β*-thioglucoside glucohydrolase, EC 3.2.3.1) was purchased from Sigma (Saint-Quentin-Fallavier, France), and its activity was *ca.* 200 units/g. Sodium dihydrogenophosphate buffer (0.05 M) (Merck, Darmstadt, Germany) was adjusted to pH 7 with sodium hydroxide. Trisodium citrate buffer (0.05 M) (Merck) was adjusted to pH 3 with citric acid. Disodium tetraborate buffer (0.05 M) (Prolabo, Paris, France) was adjusted to pH 9 and 11 with sodium hydroxide. The pH 2 solution was obtained with hydrochloric acid. Acetonitrile HPLC grade was purchased from Scharlau (Barcelone, Spain).

HPLC Analyses. Separation of glucobrassicin and indole breakdown products was performed on a Hewlett-Packard HPLC series HP1050 (Hewlett-Packard, Waldbronn, Germany) equipped with a Rheodyne 7125 injection valve, a 100 μL sample loop, an Ultrabase C_{18} column (250 mm, 4.6 mm, 5 μm) (SFCC, Eragny, France), and an Ultrabase C_{18} guard column (18 mm, 4.6 mm, 10 μm) (Shandon-LSI, Cergy-Pontoise, France). Indole derivatives were eluted in a gradient system. The HPLC mobile phases consisted in acetonitrile/phosphate buffer, 5 mM, pH 7.0 mixtures, 10/90 (v/v) and 70/30 (v/v) for solvents A and B, respectively. Elution was achieved with three successive linear increases of B in A, *i.e.* 0−34% in 10 min, then 34−46% in 15 min, and 46−100% in 20 min, and finally isocratic conditions at 100% B for 5 min. The flow rate was 1 mL/min, over controlled temperature of 30 °C. Ultraviolet (UV) detection was carried out with a Hewlett-Packard UV detector Series HP1050 (Hewlett-Packard) at 280 nm and radioactivity detection with a radiomatic Flo-one β A250 instrument (Radiomatic, La-Queue-lez-Yvelines, France) and Flo-scint II scintillation cocktail (Packard Instrument Co., Downers Grove, IL).

HPLC retention times of indole breakdown products were compared with those of indole standards. Retention times for GBS and its breakdown products were as follows: GBS, 5.90 min; IM-GBS, 11.90 min; I3C, 12.20 min; IAN, 22.30 min; and DIM, 42.00 min.

GC/MS. GC/MS was used for the structure confirmation of I3C, IAN, and DIM. HPLC fractions corresponding to these compounds were collected, desalted, and evaporated under a stream of nitrogen. The residue was silylated by addition of 100 μL of BSTFA/pyridine (1:1) and heating for 30 min at 60 °C. The mixture was evaporated to dryness and redissolved in hexane before injection.

Mass spectra were acquired on a Nermag R-10-10-T single-quadrupole mass spectrometer (Delsi Nermag Insitut, Argenteuil, France). Ionization was achieved by means of electron impact (70 eV). The mass spectrometer was fitted to a Delsi DI 200 gas chromatograph (Delsi Nermag Institut) equipped with a BPX5 (SGE, Villeneuve St Georges, France) capillary column (25 m × 0.22 mm × 0.25 μm). Injections of samples were made in the splitless mode using the following temperature program: 50 °C during 50 s, then raised from 50 to 230 °C at 25 °C/min and from 230 to 280 °C at 5 °C/min, and finally 10 min at 280 °C. Helium was used as the vector gas at a flow rate of 1 mL/min.

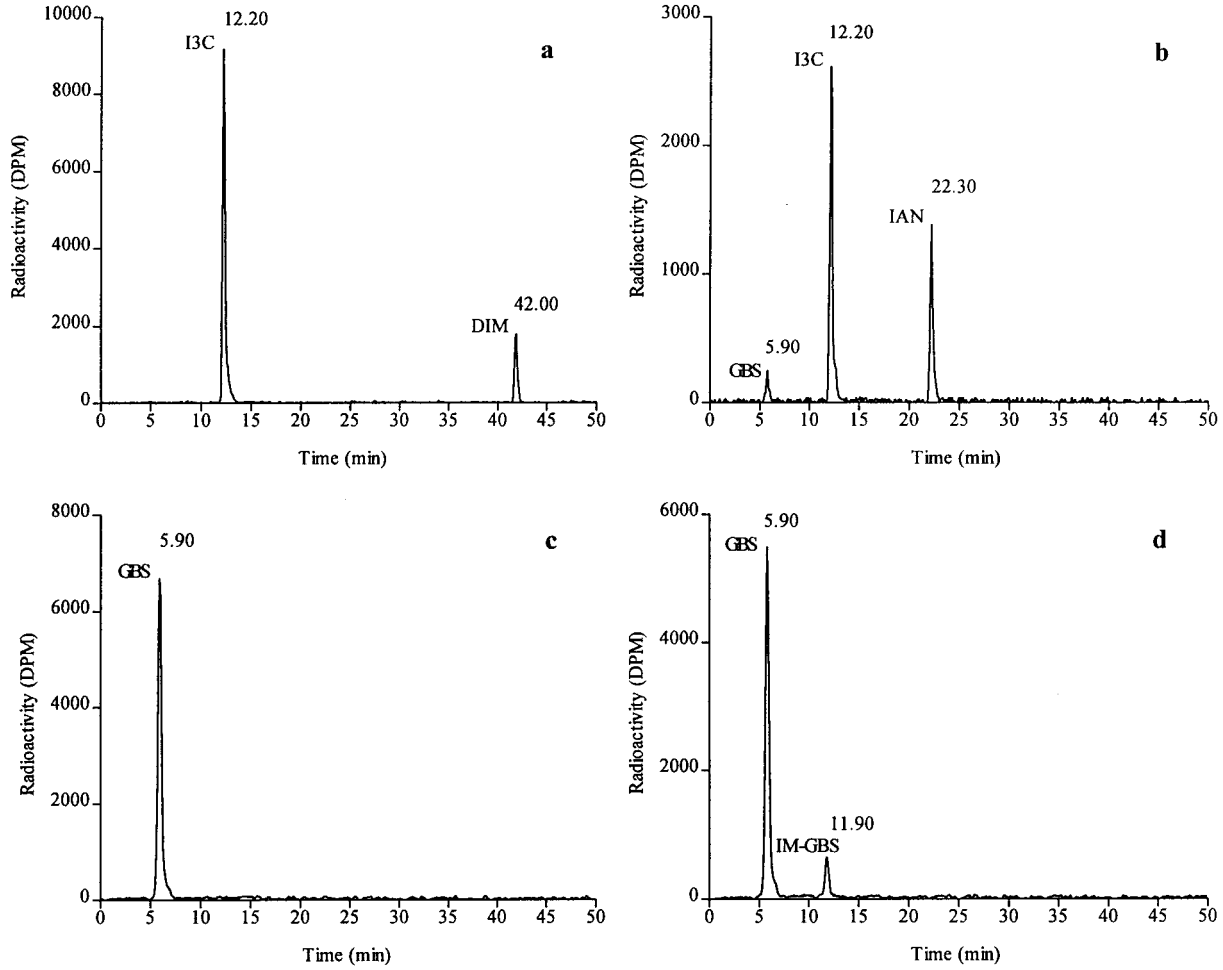


Figure 2. HPLC chromatograms of radioactive indole breakdown products from GBS degradation: (a) enzymatic breakdown at pH 7 during 24 h in daylight; (b) enzymatic breakdown at pH 3 during 24 h in daylight; (c) chemical breakdown at pH 2 during 24 h; (d) thermal breakdown at 100 °C during 1 h. Retention times were as follows: GBS, 5.90 min; IM-GBS, 11.90 min; I3C, 12.20 min; IAN, 22.30 min; and DIM, 42.00 min.

Liquid Chromatography/Mass Spectrometry. LC/MS was used for the analysis of GBS and IM-GBS, with a Finnigan LCQ quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA) coupled to a P2000 Thermo Separation LC pump (Thermo Separation Products, Les Ulis, France). Mass spectra were obtained by means of electrospray ionization. Separations were achieved on an Ultrabase C_{18} column (250 mm, 2 mm, 5 μm) (SFCC). Mobile phases consisted in acetonitrile/0.5% acetic acid mixtures, 10:90 (v/v) for A and 70:30 (v/v) for B, respectively. A linear gradient from 0 to 46% of B in 15 min followed by isocratic conditions at 46% of B during 10 min were used for the elution, at a flow rate of 0.2 mL/min. Under these conditions, GBS and IM-GBS eluted at 11.4 and 20.7 min, respectively.

Enzymatic Breakdown. GBS (15 μg) and [5-^{3}H]GBS (2500 Bq) were prepared either in 1 mL of phosphate buffer (0.05 M, pH 7) containing 0.16 mg of myrosinase or in 1 mL of citrate buffer (0.05 M, pH 3) containing 0.16 mg of myrosinase. These samples were introduced into separated closed vials and allowed to react at room temperature, in daylight or darkness, during 1, 3, and 24 h.

Chemical Breakdown. GBS (15 μg) and [5-^{3}H]GBS (2500 Bq) were prepared in 1 mL of buffer solutions (pH 2, 3, 7, 9, and 11) in separated closed vials and were kept at room temperature during 2 h (a 24 h experiment has been carried out at pH 2).

Thermal Breakdown. GBS (15 μg) and [5-^{3}H]GBS (2500 Bq) were prepared in 1 mL of water, introduced into four closed vials, and kept at 100 °C during 30 min and 1, 3, and 24 h.

Radioactivity Recovery and Degradation Product Analysis. At the end of the degradation time, the vials were opened and the volumes were checked and readjusted to 1 mL when necessary. Radioactivity remaining in the vials was compared to the initial 2500 Bq by measuring a 50 μL aliquot. Radioactivity measurements were achieved on a Tri-Carb 2200CA liquid scintillation analyzer (Packard Instrument Co.) using Ultima gold as scintillation cocktail (Packard Instrument Co., Meriden, CT).

For each of the breakdown assays described above and that were conducted in quadruplicate, 200 μL aliquots were sampled, evaporated to dryness with a nitrogen stream, and then reconstituted in 100 μL of mobile phase for radio-HPLC analysis.

RESULTS AND DISCUSSION

HPLC chromatograms of the radioactive indole breakdown products formed during enzymatic, chemical, and thermal degradation of GBS are presented in Figure 2. The same scheme is observed (data not shown) if the UV trace is considered. Moreover, it has been verified that the total radioactivity injected was recovered in the different peaks eluting during the analysis.

Enzymatic Breakdown. Parts a and b of Figure 2 show the HPLC chromatograms obtained after 24 h of enzymatic breakdown in daylight, at pH 3 and 7, respectively. The complete results of rates of indole breakdown products formed at these pH values and in daylight as well as in darkness are shown in Figure 3.

Myrosinase action at pH 7 and at room temperature leads to the complete breakdown of glucobrassicin after 1 h whatever the lighting conditions. It gives rise to

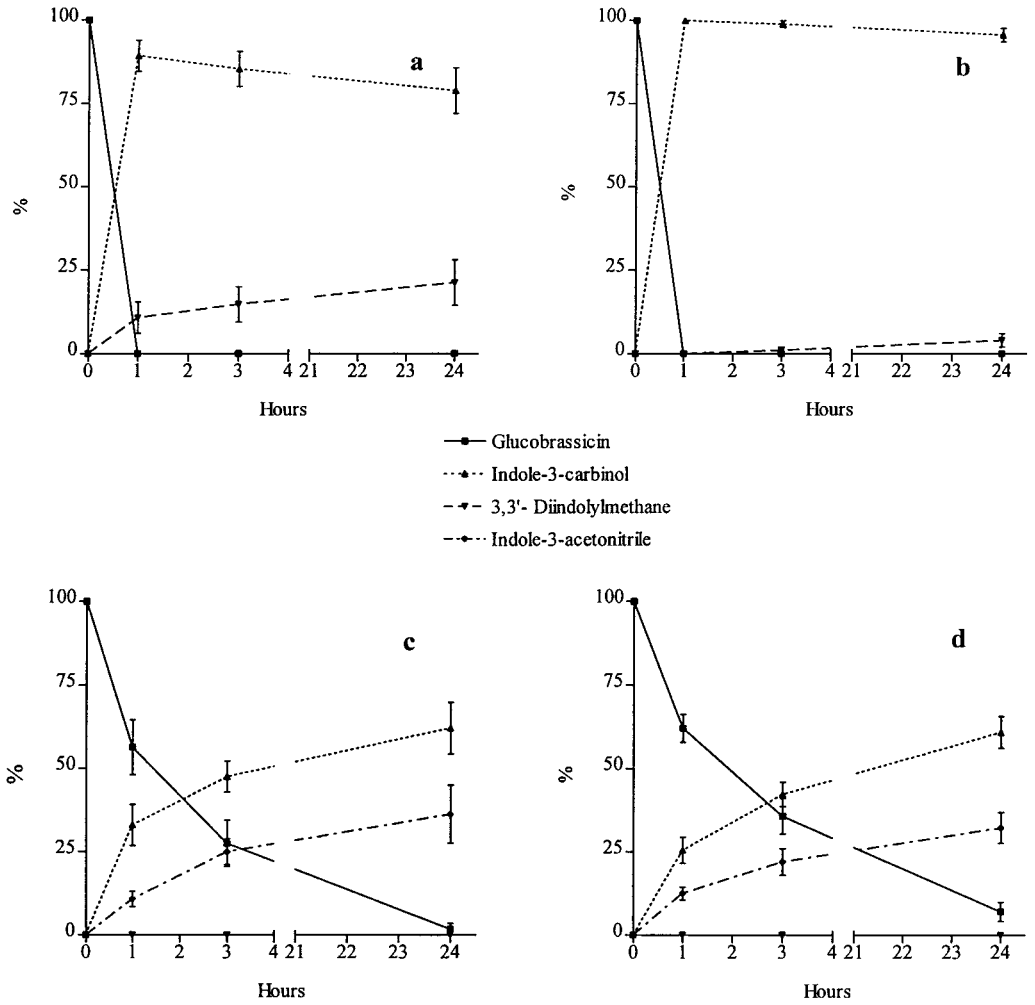


Figure 3. Rates of indole breakdown products formed by enzymatic breakdown of GBS at pH 7 (a and b) and pH 3 (c and d) in daylight and darkness, respectively. Values represent the mean ± SD ($N = 4$).

the formation of only two products, I3C and DIM as previously described in the literature (Loub *et al.*, 1975; McDanell *et al.*, 1988). No trace of IAN was observed at this pH, a result that is also in agreement with previous studies performed in similar conditions (Gmelin, 1964; Latxague *et al.*, 1991).

Within the action of the daylight, the relative amount of I3C (89% of the total radioactivity after 1 h of incubation) slowly decreased to represent 79% after 24 h, whereas the opposite was seen for DIM, which increased from 11 to 21% at the end of the 24 h period. These two compounds accounted for the total radioactivity after 1 h of incubation, meaning that they were rapidly generated and that a small part of the I3C formed could give rise to DIM through a self-condensation reaction according to previous works (BeMiller *et al.*, 1972; Grose *et al.*, 1992). However, in an other paper Latxague *et al.* (1991) identified DIM as the unique breakdown product of GBS at pH 7. Nevertheless, no data were produced concerning a correspondence in terms of molarity between GBS and DIM, and they very likely failed to observe I3C. Indeed, the analysis of the breakdown products was only conducted on an hexane extract, and I3C is not extractable with such a nonpolar solvent.

In darkness, I3C was the unique compound formed that accounted for the total radioactivity after 1 h. A very weak proportion of DIM (4%) was observed only after a 24 h incubation period. This indicates that the enzymatic activity of myrosinase is not influenced by the lighting conditions, in contrast to the self-condensation of I3C, which is almost not observed in darkness. With regard to this point, some results have previously shown that indole derivatives are more reactive in the presence of light and that condensation must be photochemically initiated (Bergman, 1970; Amat-Guerri *et al.*, 1983).

When the action of myrosinase was checked under acidic conditions, the formation of I3C and IAN was observed as previously described by some authors (Loub *et al.*, 1975; Bradfield *et al.*, 1987), but in contrast with their results, formation of DIM was never detected in our work. This was somewhat unexpected since I3C is known to be unstable in acidic conditions and to undergo self-condensation, thus leading to oligomerization products as previously reported by de Kruif *et al.* (1991) and Grose and Bjeldanes (1992).

In daylight and at room temperature, incubation with myrosinase at pH 3 resulted in only a partial degradation of glucobrassicin. After 1 h, the proportion of unchanged GBS was 56% and the breakdown was almost complete only after 24 h (Figure 3c). In similar conditions, Bradfield *et al.* (1987), using GBS and myrosinase extracted from plant material, reported GBS degradations of *ca.* 10% at pH 3 and 50% at pH 7. These quite low values, when compared to ours, can be attributed either to the use of lower enzyme/GBS ratio or to a different enzymatic activity of the myrosinase used.

The relative proportions of I3C and IAN expressed as percent of initial ^3H increased, respectively, from 33 to 62% and from 11 to 36%, after 1 and 24 h of incubation (Figure 3c). The same results were obtained when incubations were conducted in darkness (Figure 3d), meaning that light had no influence on the enzymatic degradation pathway of GBS in acidic conditions.

I3C was found to be formed together with IAN, whereas Latxague *et al.* (1991) mentioned IAN as the only enzymatic breakdown product of GBS at pH 3, in a similar study achieved on synthetic GBS. Our results rather support a degradation scheme in which the two main degradation pathways coexist at this pH, as previously proposed elsewhere (Gmelin, 1964; Loub *et al.*, 1975).

Chemical Breakdown. The chemical breakdown of GBS was studied using aqueous buffered solutions with pH in the 2−11 range. In Figure 2c the radiochromatogram obtained after a 24 h of incubation at pH 2 is shown; it clearly indicates that no degradation occurred. Moreover, whatever the pH and after 2 h, no degradation products of GBS were noticed. This result is in opposition to the scheme proposed by Gmelin (Gmelin and Virtanen, 1961; Gmelin, 1964) and indicates two pathways of chemical degradation. The first leads to I3C and DIM as in the enzymatic process at pH 7. The second way initially yields IAN, which gives rise to subsequent hydrolysis byproducts such as indole-3-carboxaldehyde, indole-3-carboxylic acid and indole or indole-3-acetic acid and skatole, depending on the pH conditions. The qualitative detection of these breakdown products was achieved by thin layer and paper chromatography, bringing evidence for only some of them. Moreover, this study was conducted with GBS extracted from plant material, and its purity was not reported. Nevertheless, the author mentioned that the conditions under which the plant material was processed greatly influenced the nature of the degradation products preferentially formed. Thus, it may be concluded that some interfering processes may have occurred in this study.

In another work (Tiedink *et al.*, 1991), the acid hydrolysis of several glucosinolates has also been studied at pH 2 by incubation at 40 °C for 2 h. The intact glucosinolates were analyzed by HPLC, and the results showed that 17% of GBS was broken down during this period. However, the nature of the resulting products was not examined in this work. Moreover, in this study, a number of other glucosinolates were found to be stable in the same conditions.

Our work, which constitutes the first study achieved with synthetic radiolabeled material, indicates that GBS is not sensitive to chemical breakdown.

Thermal Breakdown. The HPLC profile of the thermal breakdown of GBS after 1 h of incubation at 100 °C is reported (Figure 2d). After 30 min and 1 h incubations at 100 °C, respectively 93 and 90% of GBS remained unchanged. After 3 and 24 h, 50 and 100% of the GBS was damaged, respectively. Besides, after 24 h, 30% of the total radioactivity disappeared from the solution, very likely due to the formation of volatile products that were lost when the vials were opened. During these incubations, several compounds were formed in small proportion that did not correspond to any of the indole standard compounds expected to be formed and that were not identified.

GBS appeared to be very stable to heat treatment, at least for heating periods that did not exceed 1 h. This result is in contrast with those obtained in previous works conducted with raw vegetable material. Slominski and Campbell (1989b) have reported substantial decomposition of indole glucosinolates during heat treatment of cabbage, broccoli, cauliflower, and Brussels sprouts. The main degradation products consisted of indole-3-carbinols (on the basis of thiocyanate ion release) and indole-3-acetonitriles, after heat treatment at 100 °C during 50 min. From samples of raw collard and kale, Wall *et al.* (1988) also reported a major increase in the IAN content after 3−30 min of cooking.

These discrepancies may originate from the nature of the starting material submitted to the heat treatments in the different studies. Indeed, in the plant, GBS is associated with other compounds, and it is very likely that the overall GBS degradation observed from raw vegetable heating is the result of several simultaneous processes. Nevertheless, in our work we really analyze thermal breakdown of GBS, whereas in the two above-mentioned studies, the efficiency of the heat treatment used to inactivate myrosinase is questionable. Indeed, Bradfield and Bjeldanes (1987) have observed that cooking practices (100 °C/10 min) do not inactivate the thioglucosidase to a significant extent.

Yet, it is noteworthy to consider the formation of a compound accounting for 10% of the total radioactivity after 1 h. This compound had a retention time of 11.90 min (see Figure 2d), which corresponds neither to I3C nor to IAN, which were mentioned to be formed by heating *Brassica* samples at 100 °C during 40 min (Slominski *et al.*, 1988, 1989a,b).

An aliquot of the incubation mixture was analyzed using LC/MS. The ESI-MS spectrum obtained exhibited a quasimolecular ion at *m/z* 578 and fragment ions at *m/z* 498, 259, and 130, which were consistent with a reaction product of I3C with GBS that could be called 3-(indolylmethyl)glucobrassicin (IM-GBS). Several I3C condensation products have already been described, consisting in I3C 1- or 2- substituted by an indolylmethyl group (Amat-Guerri *et al.*, 1984; Stresser *et al.*, 1995). Nevertheless, the occurrence of such a condensation process has never been reported in the case of an intact indole glucosinolate. Work is now in progress to achieve the complete structural identification of this compound, which is able to be formed in cruciferous vegetables during the myrosinase inactivating thermal treatments.

CONCLUSION

In this work, the use of synthetic GBS (unlabeled and ^3H-labeled) allowed quantitative followup and characterization of the breakdown compounds occurring when this model of indole glucosinolate was submitted to three degradation processes. The different degradation pathways are summarized in Figure 4.

When the enzymatic breakdown under myrosinase action was concerned, the classical schemes reported in the literature and leading to I3C and IAN were confirmed. However, the essential role of daylight in the formation of condensation products from I3C has been established.

Moreover, new data were obtained concerning the chemical and thermal degradations. Almost no GBS was damaged after treatment at acidic, neutral, or

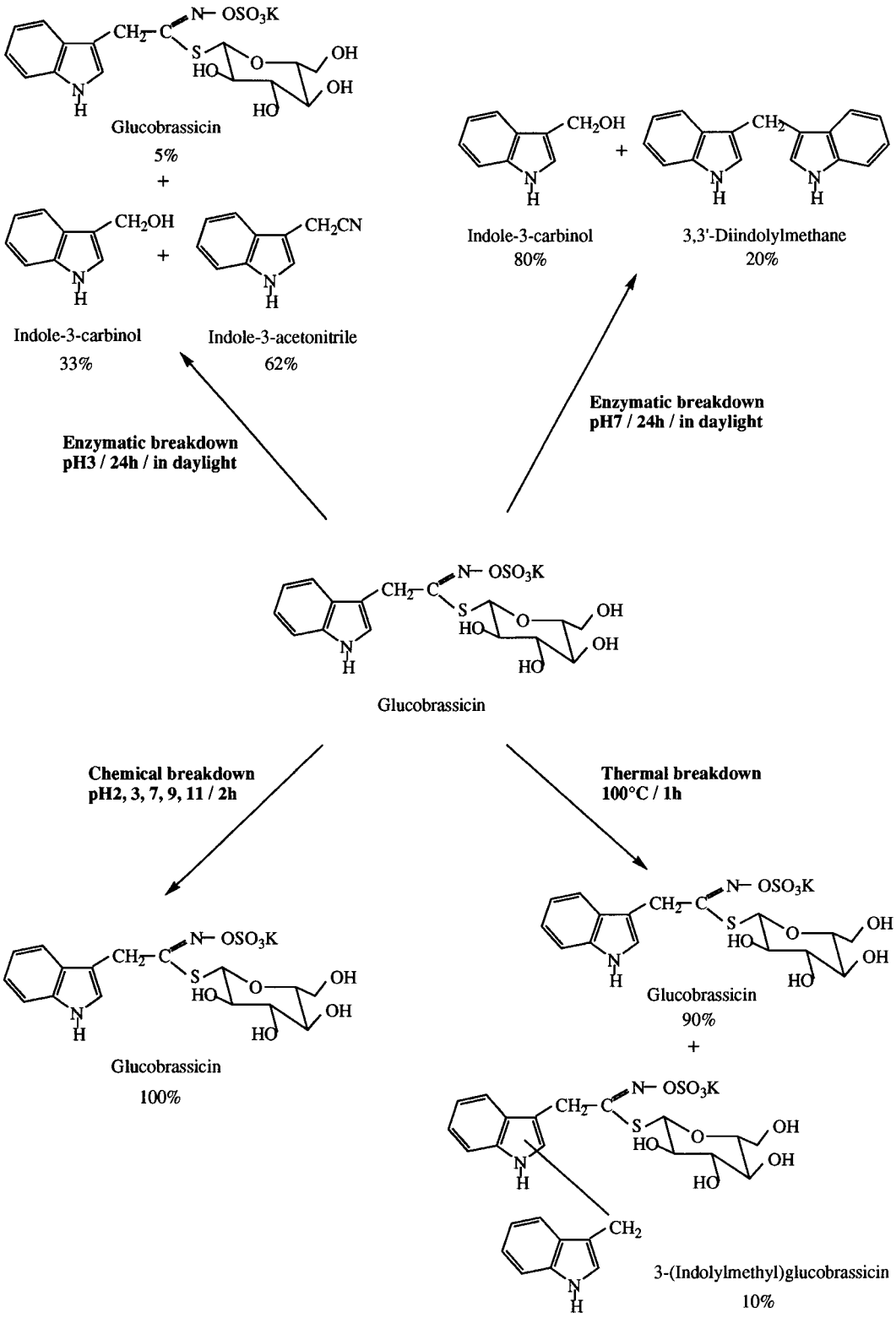


Figure 4. General scheme proposed for enzymatic, chemical, and thermal breakdowns of GBS.

alkaline pH values, and the main part of GBS remained unchanged when exposed at 100 °C. The unique heat-modified product observed was a new chemical entity corresponding to a condensation adduct.

This work gives a new highlight for the possible role of the indole glucosinolates of cruciferous vegetables in man; it gives a good knowledge of what could happen to GBS in the acidic conditions of the stomach and then when submitted to the thioglucosidase activity of some of the bacteria strains of the digestive tract.

ABBREVIATIONS USED

GBS, glucobrassicin; I3C, indole-3-carbinol (also called 3-indolylmethanol); IAN, indole-3-acetonitrile (also called 3-indolylacetonitrile); DIM, 3,3′-diindolylmethane [also called bis(3-indolyl)methane]; IM-GBS, 3-(indolylmethyl)glucobrassicin; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; HPLC, high-performance liquid chromatography; GC/MS, gas chromatography/mass spectrometry; LC/MS, liquid chromatography/mass spec-

trometry; ESI-MS, electrospray ionization mass spectrometry.

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