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Enzymatic Degradation of some natural and synthetic glucosinolates

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Summary

The authors report some new results of their most recent study aimed at characterizing the specificity of the active site of myrosinase (Sinapis alba) using natural and synthetic glucosinolates (GLs). Focus is one of the most important aspects arising from a comparison of the enzymatic hydrolysis trends of glucotropaeolin (benzyl GL) and glucoviolin (phenyl GL), natural and synthetic GLs, respectively. This study, conducted with myrosinase solubilized in reverse micelles in AOT-isooctane, shows how the small difference of one CH2-group in the side chain of both GLs can affect the enzymatic catalysis.

Although in the last decades the chemical and biological knowledge of glucosinolates (GLs) has grown enormously, some important aspects of the chemistry and biochemistry of the myrosinase-GLs system appears to be still not completely clear. Among these, the relationship between chemical structures and biological activity, but, in particular, the detailed knowledge of the hydrolysis mechanism are the most important and necessary to understand better the role of the enyme in plants and potential in biotechnology.

With the aim of bringing a contribution in this field, we have studied the mechanism of myrosinase action in the hydrolytic catalysis of a series of natural and synthetic GLs. The natural Gls were extracted and purified from different cruciferous species and materials (1), whereas synthetic ones were modified, compared to those occurring naturally, both in the aglucone and, mainly in the glucosidic part.

The synthetic GLs belonged essentially to the classes of brassicins and violins, i.e. Phenyl-GLs. In these GLs, the glucoside moiety was replaced by different types of sugars, viz. -D-Glucose, -D-Galactose, -D-Arabinose, -D-Xylose, -L-Rhamnose, -D-Mannose, -Cellobiose and, finally, -Maltose (2). All these GLs were tested with an excess of pure myrosinase (3) to determine, without any doubt, which were hydrolyzed.

The enzyme activity was determined with a direct spectrophotometric assay at 227 nm, monitoring the decrease of absorbance due to conversion of the GLs structure to the corresponding isothiocyanate (4).

Only in the case of -Glucoviolin (GVL), the enzyme activity was determined at 279 nm, observing the increase of absorbance due to the phenyl isothiocyanate (PITC) and/or nitrile formation. For a better comprehension of the enzymatic degradation of GVL, the hydrolysis trend was also followed both by HPLC and polarographically by glucose oxidase.

The results of a previous preliminary approach (5) lead us to propose the following hypotheses :

1) Myrosinase would be considered a -(thio)-glucoside glucohydrolase

In fact, among the 24 synthetic GLs tested, we established that myrosinase only catalyzes the hydrolysis of those with the sugar moiety containing a monosaccharide with a pyrano structure, which cannot be stereochemically modified in any smallest detail, without preventing the enzymatic catalysis. All the other GLs are not hydrolyzed, including those which contain the anomer -Glucose.

2) Myrosinase, when acting as a β-thioglucosidase, needs a ligand with a negative charge

The negative charge is of great importance both for substrate binding and for ascorbate activation. In fact, desulfo-GLs and other synthetic phenyl thioglucosides are not practically hydrolyzed by myrosinase and, furthermore, do not appear to inhibit the hydrolysis of other natural GLs. Clearly, these neutral compounds cannot compete for the active site, because the anion has been lost with the sulfate. Thus the anionic charge appears to be crucial for binding substrates in their thioglucosidic form.

3) Ascorbate induces a conformational change in the entire active center of myrosinase

The active center of myrosinase, as for many other glycosidases, has been proposed to be constitutd by an aglycon site an a glycon site. The ascorbate induces a favourable conformational change in the entire active center of myrosinase, and not only the aglycon-binding site, as previously postulated in 1968 by Tsuruo and Hata (6). In fact, their model cannot explain, for instance, why the enzymatic hydrolysis of galactobrassicin is not activated by ascorbate (5).

4) The role of the glycon site of myrosinase seems to be more important than that of the aglycon site

While the role of the glycon site appears to be very important for substrate selectivity, the aglycon site seems to affect the kinetic parameters of hydrolysis. In fact, myrosinase can act on about 100 different natural GLs with specific Vmax and Km values. The structure of these GLs differ only in their side chains, whereas they all contain a beta-D-gluco moiety. For instance, we have demonstrated that the aromatic GLs are better substrates than the aliphatic ones (5).

5) Myrosinase is able to hydrolyze some synthetic unnatural glucosinolates such as glucoviolin, in which the hydrolysis seems to produce a sufficiently stable intermediate, such as the phenyl thiohydroximate-O-sulfate

This last hypothesis was also proposed on the basis of our preliminary results (5), which indicated that D-glucose is easily released, whereas the expected variation of the GLs chemical structure in aqueous solution at 227 nm, was difficult to detect during the first five-six minuts of enzymatic hydrolysis.

Therefore, because we had some doubts about the consistency of this hypothesis, given for instance, the low water solubility of the reaction products of the aromatic GLs, (after a few minutes a significant drift of the photometric baseline was observed, making thus a correct enzyme assay difficult) the hydrolysis was studied through solubilizing the enzymes and substrates in AOT-isooctane reverse micelles.

Reverse micelles (Fig 1), in this case, proved to be the only possible tool which allows the direct, accurante and real time study of the enzymatic hydrolysis of aromatic GLs by following the formation of the reaction products (7).

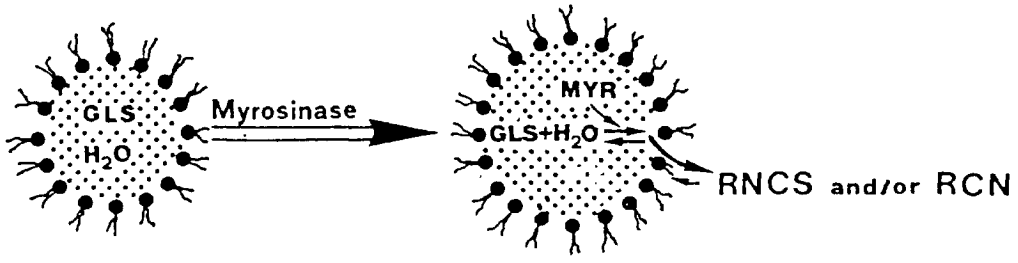

Fig. 1. Schematic representation of solubilization of myrosinase (MYR) and glucosinolates (GLS) in reverse micelles (cross section). GLS and MYR are extremely soluble in water while the product(s), the isothiocyanates (RNCS) and/or nitriles (RCN) are prevalently soluble in hydrocarbon solvents.

This system is highly dybnamic and thermodynamically stable and is constituted by small waterpools surrounded by a layer of surfactant molecules dispersed in the hydrocarbon solvent (8). The dimensions of the water droplets, in which the enzymes and, as in our case, the substrates can be solubilized, roughly represented in Fig.1, depend mainly on the raio of water molarity to surfactant molarity, which is the Wo. Using bis (2-ethylhexyl) sodium sulfosuccinate (AOT) as surfactant, if Wo increases from 5 to 40 the micelle diameter increases from about 4 to 15 nm (9).

This study was conducted comparing the enzymatic hydrolysis trend of glucotropaeolin (GTL) (7) and glucoviolin (GVL) which are natural and synthetic GLs respectively. This comparison is particularly interesting because these GLs show only a small difference due to the presence of one -CH2-group (the rotula) in the side chain of GTL.

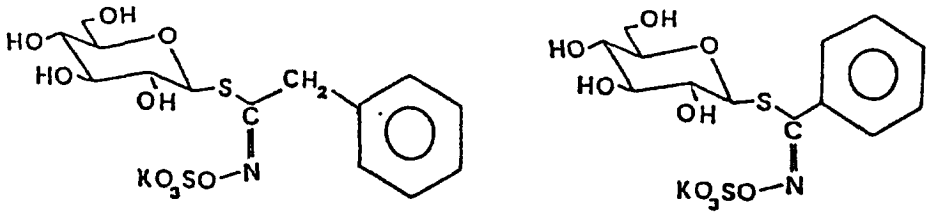

Fig. 2. Structures of natural glucotropaeolin (left) and synthetic glucoviolin (right) glucosinolates.

The enzymatic hydrolysis of these two GLs were conducted both with sulfatase and myrosinase, and, in this last case, the reaction conditions were performed for studying both isochtiocyanate and nitrile production. Nevertheless, only data regarding the hydrolysis by myrosinase at pH 7.5 are presented in this paper.

As reported in a previous paper (7), using GTL as substrate the absorbance at 227 nm decreases linearly with time up to ca. 90min. After this time, the spectrum of benzyl isothiocyanate (BITC) appears clearly. In fact, by calculating the BITC concentration at the end of the hydrolysis on the basis of its molar extinction coefficient (MEC) at 251 nm (ε =1634 $M^{-1}\,cm^{-1}$), one can establish that GTL is almost completely degraded. Whereas, as shown in Fig. 3, the spectrophotometric hydrolysis trend of GVL is qualitatively and quantitatively completely different from that of GTL. In fact, at 227 nm, which is the typical wavelength of the mximum absorbance of GLs, instead of a decrease of absorbance generally observed with other GLs, instead of a decrease of absorbance generally observed with other GLs, we detect a consistent increase in absorbance during the reaction time. This finding is due to the high molar extinction coefficient (ε = 38272 $M^{-1}\,cm^{-1}$) at 220 nm of phenyl isothiocyanate (PITC), which is justified by the conjugation between the electrons of the aromatic part and those of the isothiocyanate double bonds. On the other hand, if we consider the spectra both of the intact GVL (9.7×10^{-4}) and the standard phenyl isothiocyanate (3.5×10^{-5} M), shown in the above part of Fig.3, the complete different picture of the hydrolysis trend of this GL can be easily explained.

The results of this experiment allows the formulation of the following other important considerations :

1) the direct spectrophotometric assay for determining the myrosinase activity with GVL as substrate can be correctly performed in reverse micelles only at 279 nm, where the molar extinction coefficient of the two compounds are much different and therefore the absorbance of GVL contribution is very small. Moreover, this particularity offers the possibility to use an excess of substrate, which is an important condition for the precise steady state parameters determination.

2) comparing the spectra of standard PITC with that formed during the GVL hydrolysis (Fig.3), it is easy to note in the latter a valley at around 240 nm with an absorbance greater than that expected. For this reason, the following possibilities must be admitted : (i) GVL is not

completely degraded ; (ii) the intermediate, phenyl thiohydroximate-O-sulfate, which presumably has a MEC not much different from GVL, is not degraded completely to PITC ; (iii) the PITC, in our experimental conditions, is transformed spontaneously in other compounds that absorb in this wavelength range.

3) because no enzyme inhibition was observed using standard PITC in a typical myrosinase-catalyzed reaction, the first hypothesis should be excluded also considering the GL purity determined both by NMR and HPLC. On the other hand, the last two possibilities appear to be supported by the difference spectrum (seed dashed line in Fig.3) obtained by adding, in the reference cell, the theoretical concentration of PITC formed during the experiment, calculated on the basis fo its MEC at 279 nm. This spectrum shows, unequivocally, the presence of a compound different from both GVL and PITC.

This last finding is also supported both by the different myrosinase activity trends obtained with GTL and GVL, and by the percentage of GVL degradation, which is roughly less than half of that calculated for GTL (Fig.4).

In conclusion, although many attempts for explaining the mechanism of enzymatic hydrolysis of GVL has been done, these results are not still sufficient for establishing with certaintly this mechanims. We have, in fact, still too little experimental evidence to allow definite conclusions, which could be obtained with ohter aimed experiments, which are in progress in our laboratories.

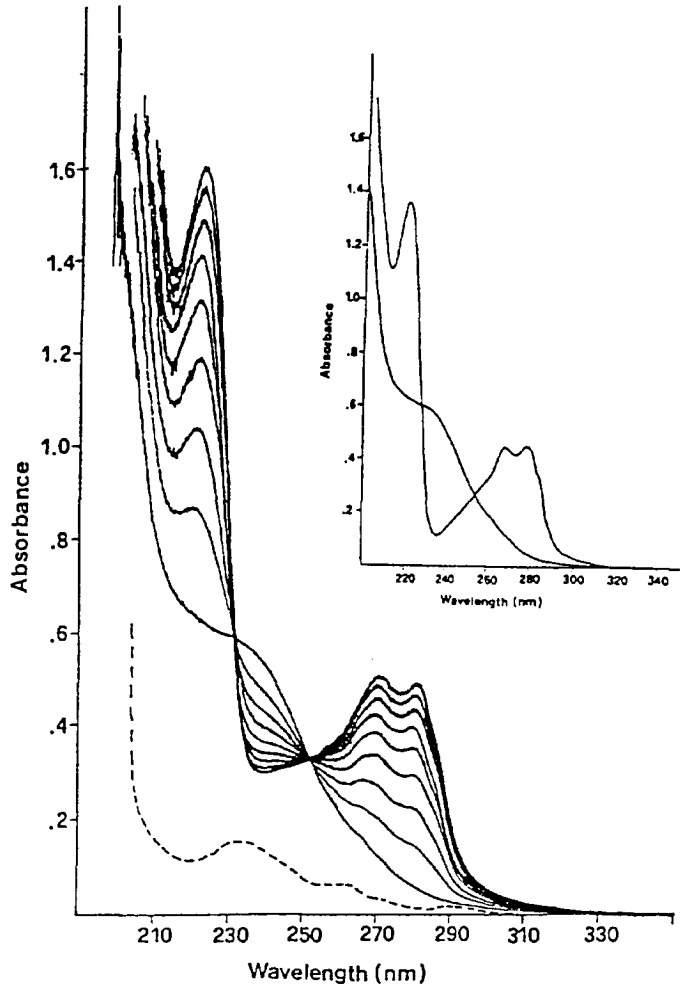

Fig. 3. UV spectral changes during myrosinase-catalyzed ß-glucoviolin hydrolysis in AOT reverse micelles: Reaction conditions were as follows: 100 mM AOT, Wo 39, pH 7.5 (50 mM TES, local concentration), 37 °C, 0.143 μg of myrosinase, final volume 400 μl. The spectra were recorded with a 1mm path-length cuvette at 10 min intervals after the first, which was recorded immediately after enzyme addition. The spectra reported inside show the intact glucoviolin (9.7×10^{-4} M) and standard phenyl isothiocyanate (3.5×10^{-5} M) in AOT reverse micelles. Dashed line represents the difference spectrum obtained at the end of reaction adding a suitable concentration of standard PITC in the reference cell.

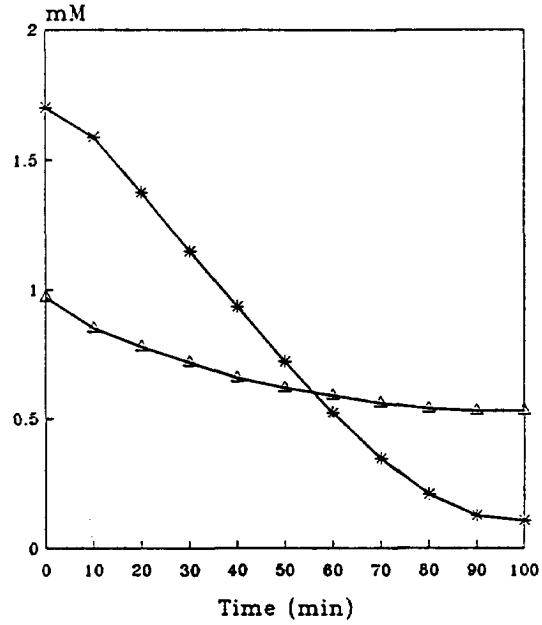

Fig. 4. Myrosinase activity trends determined in AOT reverse micelles with ß-glucotropaeolin (✱) with the direct determination of GLs hydrolysis at 227 nm and ß-glucoviolin (△) by determining the production of phenyl isothiocyanate at 279 nm.

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