The myrosinase-glucosinolate interaction mechanism studied using some synthetic competitive inhibitors

Renato Iori[a], Patrick Rollin[b], Harald Streicher[c], Joachim Thiem[c], Sandro Palmieri[a,*]

[a]*Istituto Sperimentale per le Colture Industriali, MRAAF, via di Corticella, 133, I-40129 Bologna, Italy*
[b]*Institut de Chimie Organique et Analytique, URA 499, Université d'Orléans, B.P. 6759, F-45067 Orléans Cedex 2, France*
[c]*Institut für Organische Chemie der Universität Hamburg, Martin-Luther-King-Platz 6, D-20146 Hamburg, Germany*

Received 7 March 1996

Abstract Using synthetic deoxy-glucotropaeolins (6d-GTL, 4d-GTL, 3d-GTL, 2d-GTL) as substrates, myrosinase activity was studied in comparison to that determined on native glucotropaeolin (GTL) isolated from ripe *Lepidium sativum* seeds. When the deoxy substrates were used, in addition to an overall strong reaction rate decline, a significant decrease in the reaction rate was observed in going from 6d- to 2d-GTL. This finding allows us to propose a mechanism of catalysis which appears to be similar in many respects to that established for β-glucosidases. Finally, 2d-GTL was shown to be the first strong competitive inhibitor of myrosinase ever reported.

Key words: Myrosinase inhibitor; Deoxy-glucotropaeolin; Native glucotropaeolin

1. Introduction

Myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) isolated from *Sinapis alba* seeds is a glycopolypeptide containing various thiol groups and disulfide bridges together with ca. 18% carbohydrates, mainly hexoses. This enzyme consists of two identical subunits with a molecular weight of 71.7 kDa and it has a pI of 5.1 [1]. Myrosinase is present in all glucosinolate-containing plants, especially Cruciferae, although it was also found in microorganisms, mammalian tissues and fungi.

Glucosinolates (GLs) are sugar anionic thioesters containing a β-thioglucoside-type bond [2], which can be hydrolyzed by myrosinase to β-D-glucose, hydrogen sulfate ion, and a series of diverse aglucons such as isothiocyanates, nitriles, thiocyanates, thiones etc., depending on the substrate and the reaction conditions used.

Due to its biological, analytical, and technological role in food and feed quality, myrosinase is an important enzyme [3-5]. Recently, myrosinase has been demonstrated to be also capable of catalyzing the hydrolysis of some non-natural synthetic glucosinolates, which were modified either in the sugar moiety or in the aglucon [6,7]. These artificial substrates shared some important properties with the natural ones: (i) the negative charge; (ii) the β-configuration of the hexosidic unit, and finally (iii) the presence of all hydroxyl groups in the sugar moiety. With regard to the first two points, we established that these properties of the substrates are absolutely necessary to ensure the hydrolysis of these molecules [6,7]. The last point, however, raises the following question: can deoxy substrates be hydrolyzed and can some of them contribute to a better understanding of the enzyme-catalyzed GL hydrolysis mechanism?

In this paper, we discuss the importance of the hydroxyl groups of the sugar moiety of the substrate molecules with particular attention to that at C-2, the closest to the thioglucosidic bond, which is hydrolyzed during the myrosinase-catalyzed reaction. The results obtained using these artificial substrates permit us to propose a myrosinase-substrate interaction mechanism.

2. Materials and methods

2.1. Glucosinolates

Native glucotropaeolin (GTL) was extracted and purified from ripe seeds of *Lepidium sativum* L. [8,9], whereas the synthetic deoxyglucotropaeolins (deoxy-GTLs) (Fig. 1) were prepared as previously reported [10]. The deoxy-GTL concentration was determined using the same molar extinction coefficient of native GTL of 8870 M^{-1} cm^{-1} [8].

2.2. Myrosinase

Myrosinase was extracted and purified from ripe seeds of *Sinapis alba* [1]. The specific activity, determined spectrophotometrically at 227 nm using sinigrin as substrate [11], was 65 U mg^{-1} of protein. One myrosinase unit is defined as the amount of enzyme able to hydrolyze 1 μmol of sinigrin min^{-1} at pH 6.5 and 37°C.

2.3. Kinetic determination

Myrosinase activities on native GTL and deoxy-GTLs were determined by measuring the substrate hydrolysis following the linear absorbance decrease at 227 nm using 5 mm path length quartz cells on a Cary 219 recording spectrophotometer. A typical 1.5 ml assay reaction mixture contained 0.34 mM of substrate, 33 mM phosphate buffer pH 6.5 and 0.29 U of myrosinase. The buffered substrate was kept at 37°C for a sufficient time before enzyme addition. K_m and V_{max} values were determined by the Lineweaver-Burk plot using a suitable range of substrate concentrations (0.02–0.20 mM). K_i value for the 2d-GTL was calculated from the reciprocal plot 1/v versus 1/[S] [12]. In this case the assay mixture contained a fixed concentration of 0.025 mM of inhibitor and a GTL concentration ranging from 0.10 to 0.34 mM.

2.4. Activation energy determination

The activation energy values for the enzymatic hydrolysis reactions of GTL, 6d-GTL and 4d-GTL were calculated by plotting the log of V_{max} determined as indicated above at 25°C, 30°C and 37°C for each substrate, versus 1/T [13].

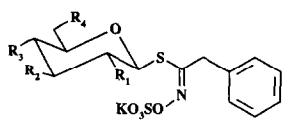
3. Results and discussion

3.1. Myrosinase activity and the active site conformation

All deoxy-GTLs are worse substrates for myrosinase than native GTL, thus demonstrating the importance of the presence and position of all hydroxyl groups in the sugar moiety (Table 1). This finding strongly suggests the achievement of a significant enzyme interaction with the sugar moiety of the

*Corresponding author. Fax: (39) (51) 6316851.

PII S0014-5793(96)00335-3



GTL R_1=R_2=R_3=R_4=OH
2d-GTL R_1=H; R_2=R_3=R_4=OH
3d-GTL R_1=OH; R_2=H; R_3=R_4=OH
4d-GTL R_1=R_2=OH; R_3=H; R_4=OH
6d-GTL R_1=R_2=R_3=OH; R_4=H

Fig. 1. Chemical structures of native GTL and synthetic deoxy-GTLs.

substrate, in addition to the electrostatic binding due to the presence of the negatively charged GLs sulfate group. Previous studies suggest that the active site of myrosinase has a conformation with two pockets, suitable for accommodating substrates such as GLs, which show a negatively charged head and a second glucon head group [6,14]. In this regard, the results of this study indicate that there are two important factors that can affect the kinetics of GL hydrolysis.

The first must be imputable to the favorable alignment of the sugar moiety in its specific binding site, presumably obtained by hydrogen bonding with the hydroxyl groups, with an increasing importance when switching from position 6 to 2, given that myrosinase activity decreases markedly from 6d-GTL to 2d-GTL.

The second factor is dependent on the polarization of the *S*-glucose bond due to the presence of the hydroxyl in position 2. This hydroxyl group appears to be not only more important than the others, but crucial for inducing the nucleophilic attack to the anomeric carbon, as has been demonstrated for the catalysis mechanism of β-glucosidases [15]. In fact, not only is 2d-GTL practically not hydrolyzed, it also strongly inhibits the hydrolysis of native GTL and, more generally speaking, of other GLs as well.

Table 1 also shows that the activation energies determined for GTL, 6d- and 4d-GTL are rather similar. This result unequivocally demonstrates that the hydroxyl group depletion in position 6 and 4 does not affect the reaction ΔG_O and therefore they appear to be only indirectly involved in the reaction mechanism. Presumably, these hydroxyl groups are important only as multiple sub-sites for binding glucose in the optimal position, particularly in the vicinity of the thioglucosidic bond, which has to be hydrolyzed. In fact, when they are lacking, only the enzyme-catalyzed reaction rate is strongly affected.

3.2. *Myrosinase inhibition*

The deoxy-GLs were designed and synthesized on the basis of previous studies on the myrosinase binding mechanism carried out with natural and synthetic substrates modified either on the aglucon part or in the sugar moiety [6,7].

The reciprocal plots shown in Fig. 2, done with and without 2d-GTL, confirm the behavior of this molecule as a competitive inhibitor of the myrosinase-catalyzed hydrolysis of native GTL with a calculated K_i of 1.9×10^{-5} M. This number is also validated by the Dixon plot determined at three substrate concentrations (data not shown). From Fig. 2, one can also determine the K_m value for GTL, which shows the same order of magnitude as the inhibition constant (Table 1). This result, in addition to indicating that myrosinase has a similar affinity towards both the traditional substrate(s) and the synthetic ligands, could be important for attesting that the first step of binding is equally efficient with both GTL and 2d-GTL, being of electrostatic type, due to the presence of an anionic charge in both ligands. In fact, inhibition experiments carried out using desulfo-GLs (0.1–0.3 mM), such as desulfosinigrin, readily prepared with the help of immobilized sulfatase purified from a commercial preparation of *Helix pomatia* extract [16], show a little effect only at high concentrations. We suppose that this weak inhibition (ca. 7%) is likely due to the interaction of myrosinase only with the sugar moiety of these molecules.

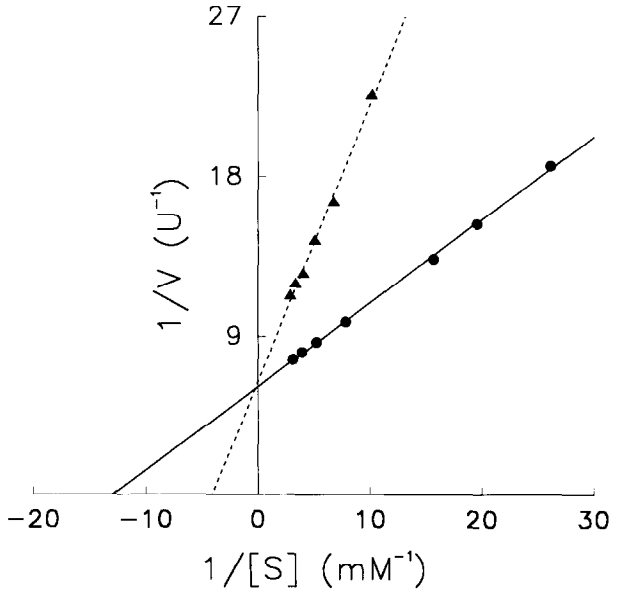


Fig. 2. The reciprocal plot of myrosinase-catalyzed hydrolysis of native GTL at pH 6.5 and 37°C obtained without (●) and in the presence of 0.025 mM of 2d-GTL (▲).

In addition, myrosinase is also inhibited by increasing the ionic strength, in particular using NaCl. In fact, 0.5 M NaCl reduces myrosinase activity to ca. 70% and a concentration of 2 M almost completely inhibits the enzyme (results not shown). This result indicates that the Cl^- anion, if present in sufficient concentration, interacts with the positive charge in the active site by replacing any other anions and those of

Table 1. Comparison of myrosinase activity determined on native GTL and deoxy-GTLs[a]

GL	K_{cat} (s^{-1})	Relative activity (%)	K_m (μM)	K_{cat}/K_m (s^{-1} μM^{-1})	Activation energy (cal mol^{-1})
GTL	65.6	100.0	75	0.87	10600
6d-GTL	9.4	14.3	68	0.14	10850
4d-GTL	3.1	4.7	33	0.09	10900
3d-GTL	1.6	2.4	n.d.[b]	n.d.	n.d.
2d-GTL	0.0	0.0	–	–	–

[a]All data are accurate to within ± 5%.
[b]n.d.=not determined due to the slow hydrolysis rate.

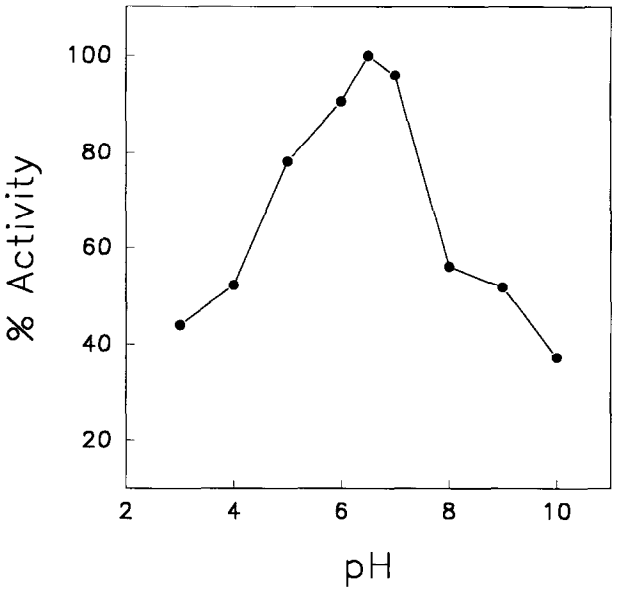


Fig. 3. Effect of pH on myrosinase activity.

the substrates in particular.

Finally, the fact that myrosinase is significantly less active with 6d-, 4d-, and 3d-GTL and that it is strongly inhibited by a GL modified in C-2 of the sugar moiety confirms the importance of the glycon binding site of the enzyme previously suggested [6].

3.3. *Proposed mechanism of GL myrosinase-catalyzed hydrolysis*

Although the results of this study give an account of a novel, strong competitive inhibitor of myrosinase, our aim in this paper is also to contribute to the understanding of GL hydrolytic mechanism. An elegant example of exploitation of competitive inhibitors for elucidating the enzyme catalytic mechanism was reported by Withers and co-workers using 2-deoxy-2-fluoroglucosides on β-glucosidases [15]. In addition, in a recent study on the substrate-assisted catalysis of *Agrobacterium* β-glucosidases, Wang and Withers suggest for myrosinases that the sulfate group of GLs itself provides the acid-base catalyst, thus showing a similar substrate-assisted catalysis version as in mutant β-glucosidases [17]. Unfortunately, our results obtained using deoxy-GTLs and also desulfo-GLs do not confirm the preceding hypothesis, but rather indicate that the sulfate group is necessary to bind the substrate or ligand to the active site of myrosinase.

The active site is configured to accommodate a bidentate substrate such as GLs with a negatively charged head and a second glycon group having a conformation with two pockets as formerly suggested [6,14]. The first pocket, adapted for accepting the charged group, likely contains a positive charge due to the presence of a lysyl or arginyl residue. In other respects, the second pocket is specific for a hexosidic unit with β-configuration, essentially β-D-glucose in native GLs, which can be allocated and correctly positioned at this site by four hydrogen bondings (Fig. 4).

As has been proposed for several β-glucosidases, the active center of myrosinase should contain both an acid and a nucleophilic anionic group, adequately represented by a histidyl residue and a carboxylate group (i.e. glutamyl or aspartyl residues) respectively. The presence of a histidyl residue in the active site was previously suggested by Ohtsuru and Hata [18] on the basis of the myrosinase inhibition observed using 10^{-3} M *p*-diazobenzene-sulfonic acid. The histidyl residue and the carboxylate anionic group would permit both a proton transfer from the enzyme to the substrate and the

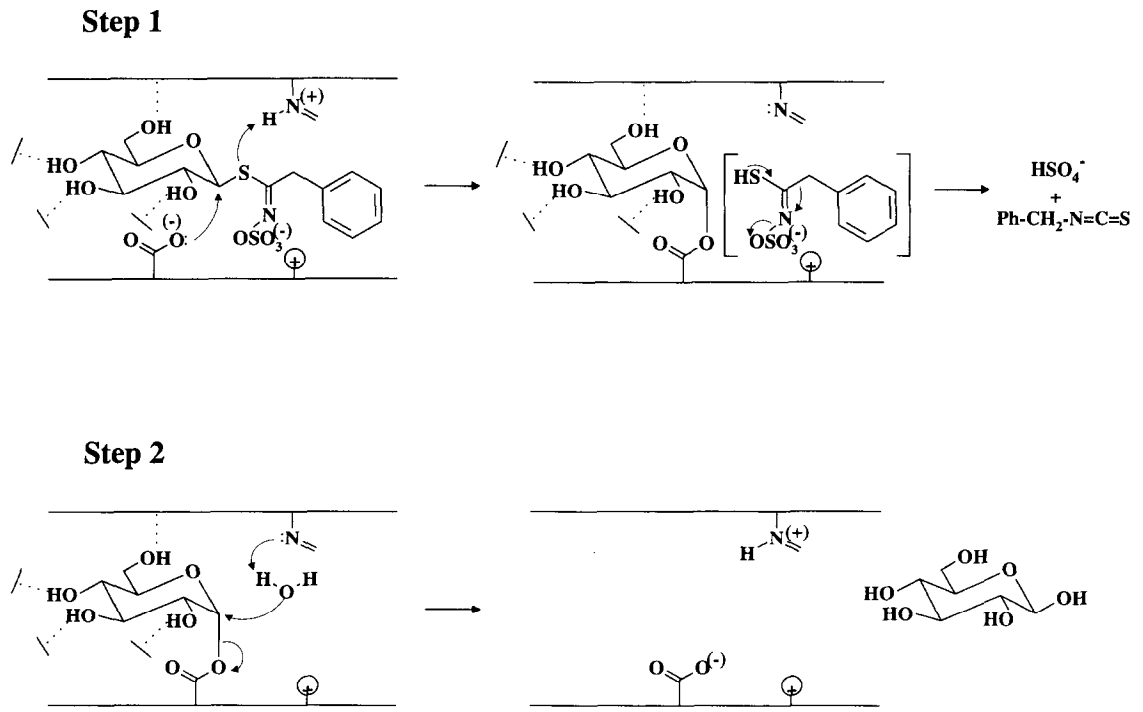


Fig. 4. Mechanism proposed for myrosinase-catalyzed glucosinolate hydrolysis.

nucleophilic attack on the anomeric carbon of the hexosidic group respectively.

Although the presence of these catalytically important limbs in the active center has not been directly determined, it can be reasonably assumed from the results of enzyme pH activity dependence, which shows a well-shaped curve with an optimal pH around 6.5 (Fig. 3). In this regard, previous studies on myrosinase structure, carried out by CD spectroscopy and fluorescence emission in the pH range 4.5–9.5 and 4.9–8.8 respectively, indicate that myrosinase does not change its spectral characteristics [1]. These findings allow one to postulate that the decrease of enzyme activity before and beyond the optimal pH cannot be ascribed to the conformational change of myrosinase structure. In fact, if one accepts the proposed active site arrangement, the myrosinase-catalyzed reaction should proceed efficiently only when one of these catalytically essential groups is protonated (histidyl) and the other is not (carboxylate). The theoretical pK_a of these ionizable groups appears to be sufficiently in agreement with the pH activity dependence data of myrosinase.

The results of this study and of previous experiments on the kinetics of this reaction using native [19,20] and synthetic GLs [6], lead us to propose a double-displacement mechanism also for the myrosinase-catalyzed GLs hydrolysis, similar to that already suggested for β-glucosidases [15]. In addition, as in the case of native β-glucosidases, the myrosinase mechanism should involve a first step acid-base catalyzed reaction with the formation of the glycosyl enzyme intermediate (Fig. 4) and the benzyl isothiocyanate, obtained by the enzyme-independent Lossen rearrangement of the transient thiohydroximate-*O*-sulfonate. Together with the benzyl isothiocyanate formation a sulfate anion and a proton were also released. Finally, the second step should be represented by the glycosyl enzyme intermediate hydrolysis with the release of a glucose molecule.

4. Conclusions

With this study, we demonstrate that 2-deoxy-glucotropaeolin is a strong competitive inhibitor of myrosinase isolated from *Sinapis alba*. The inhibitory effect is exclusively attributable to the lack of the hydroxyl group in C-2, which is crucial for the enzymatic hydrolysis of GLs. This finding is meaningful from both a practical and a theoretical point of view. In fact, the immobilization of 2d-GTL as a ligand on a suitable support could enable efficient isolation of large amounts of high-quality myrosinase, directly from different crude extracts by a highly specific affinity chromatography. This new chromatographic support, in principle easy to prepare, should be superior to the Con-A Sepharose that we proposed in 1986 and until now widely used for myrosinase isolation [21]. Con-A Sepharose in fact, although still advantageously used, is expensive and exhibits only a general specificity towards glycoproteins. Finally, the essential role of the C-2 hydroxyl group in the sugar moiety of the substrate appears to be helpful and of great interest for proposing a mechanism of myrosinase-catalyzed GL hydrolysis. This result, and those connected with the decreasing enzyme activity when we switch from 6d- to 2d-GTL as substrates, supports a thioglucosidic bond hydrolysis mechanism very similar to that described for the glucosidic bonds when β-glucosidases were used [15,22].

Acknowledgements: We are indebted with Prof. Severino Ronchi and Dr. Hugues Driguez for helpful discussion. This study was supported by the Italian Ministry of Agriculture, Food and Forestry Resources (MRAFF), target oriented project No. 17 'Resistenze genetiche delle piante agrarie agli stress biotici ed abiotici' and by the International Program of Scientific Co-operation 'Galileo'.

References

[1] Pessina, A., Thomas, R.M., Palmieri, S. and Luisi, P.L. (1990) Arch. Biochem. Biophys. 280, 383–389.
[2] Ettlinger, M.G. and Kjaer, A. (1968) Recent Adv. Phytochem. 1, 59–144.
[3] Fenwick, R.G., Heaney, R.K. and Mullin, W.J. (1983) CRC Crit. Rev. Food Sci. Nutr. 18, 123–201.
[4] Iori, R., Leoni, O. and Palmieri, S. (1983) Anal. Biochem. 134, 195–198.
[5] Palmieri, S. (1994) Proceedings Int. Euro Food Tox IV Conf., Olsztyn, Poland, 22–24 September 1994, pp. 40–45.
[6] Palmieri, S., Iori, R., Joseph, B. and Rollin, P. (1993) Colloque Glucosinolates '10 Années de Recherche' CETIOM Ardon, France, 13–14 January 1993, pp. 57–62.
[7] Iori, R., Joseph, B., Rollin, P. and Palmieri, S. (1994) GCIRC Bull. 10, 142–146.
[8] Thies, W. (1988) Fat Sci. Technol. 8, 311–314.
[9] Visentin, M., Tava, A., Iori, R. and Palmieri, S. (1992) J. Agric. Food Chem. 40, 1687–1691.
[10] Streicher, H., Latxague, L., Wiemann, T., Rollin, P. and Thiem, J. (1995) Carbohydr. Res. 278, 257–270.
[11] Palmieri, S., Leoni, O. and Iori, R. (1982) Anal. Biochem. 123, 320–324.
[12] Siegel, I.H. (1975) in: Biochemical Calculation, 2nd edn., John Wiley & Sons, Chichester, pp. 246–252.
[13] Siegel, I.H. (1975) in: Biochemical Calculation, 2nd edn., John Wiley & Sons, Chichester, pp. 278–280.
[14] Tsuruo, I. and Hata, T. (1968) Agric. Biol. Chem. 21, 1420–1424.
[15] Withers, S.G., Warren, R.A.J., Street, I.P., Rupitz, K., Kempton, J.B. and Aebersold, R. (1990) J. Am. Chem. Soc. 112, 5887–5889.
[16] Leoni, O., Iori, R., Haddoum, T., Marlier, M., Wathelet, J.-W., Rollin, P. and Palmieri, S., in preparation.
[17] Wang, W. and Withers, S. (1995) J. Am. Chem. Soc. 117, 10137–10138.
[18] Ohtsuru, M. and Hata, T. (1973) Agric. Biol. Chem. 37, 269–275.
[19] Björkman, R. and Lönnerdal, B. (1973) Biochim. Biophys. Acta 50, 121–131.
[20] Hochkoeppler, A. and Palmieri, S. (1992) Biotechnol. Prog. 8, 91–96.
[21] Palmieri, S., Iori, R. and Leoni, O. (1986) J. Agric. Food Chem. 34, 138–140.
[22] Clarke, A.J., Bray, M.R. and Strating, H. (1993) in: β-Glucosidases (Asim Esen, Ed.), ACS Symposium Series 533, pp. 42–55. American Chemical Society, Washington, DC.