INFLUENCE OF THE SUBSTRATE SUGAR STRUCTURE ON MYROSINASE ACTIVITY DEMONSTRATED BY USING NATIVE AND SYNTHETIC GLUCOTROPAEOLINS

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ABSTRACT

Some new results on the characterization of the active site of myrosinase (Myr) isolated from *Sinapis alba* with the help of natural and synthetic glucotropaeolins (GTLs) are reported. Focus is on the enzyme-substrate interactions for establishing the possible structure of the enzyme binding site(s). This study has been performed through synthesizing a series of deoxy-glucotropaeolins (6D-GTL, 4D-GTL, 3D-GTL, 2D-GTL) as modified substrates for studying the enzymatic activity in comparison to that determined on GTL isolated from *Lepidium sativum* seeds. All deoxy-GTLs are worse substrates of myrosinase than native GTL. Myrosinase activity decreases from 6D-GTL to 2D-GTL indicating that, in addition to the electrostatic interaction of the sulfate group, the formation of hydrogen bonds between the enzyme and the sugar moiety of GTLs is the rate limiting step of glucosinolate (GL) enzymatic hydrolysis. On the basis of these results, the hypothesis of a binding site divided in two pockets previously suggested seems to be more realistic, although other experiments are necessary for its verification.

INTRODUCTION

Although the molecular properties of myrosinase (β-thioglucoside glucohydrolase EC 3.2.3.1) has been studied from more than 40 years, some important aspects of biochemistry of the myrosinase-glucosinolates (Myr-GLs) system are still not completely clear. One of these is the relationship between the chemical structure of the substrates and the enzyme activity and, in particular, the properties of the active site(s) and the type of interaction(s) which allows the formation of the enzyme-substrate complex.

Previous studies (Tsuruo and Hata, 1968; Palmieri *et al.*, 1993; Iori *et al.* 1993) describe the active site of myrosinase to be presumably constituted by two pockets: an aglycon site and a glycon site. Although the aglycon site appears to be essential to bind tightly the substrates with a negative charge such as GLs, the role of the glycon site of myrosinase seems to be important too, both for selectivity and efficiency of the enzyme. In fact, there are evidences that both the desulfo-GLs and the GLs devoid of a β-

pyranosic structure of the sugar moiety do not hydrolyze thorough the myrosinase-catalyzed reaction (Palmieri *et al.*, 1993; Iori and Palmieri, 1994).

Therefore, with the aim of bringing a contribution in this field, we have studied the myrosinase hydrolytic catalysis of a series of synthetic deoxy-GTLs in comparison to the case of native GTL. This paper is a continuation of previous studies aimed at characterizing the active site of myrosinase.

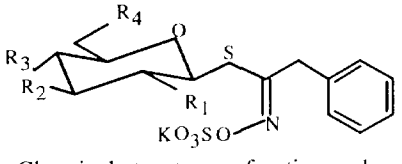
EXPERIMENTAL

Glucosinolates

Native GTL was extracted and purified from ripe seeds of *Lepidium sativum* L. using the method described by Visentin *et al.*,(1992), whereas the synthetic GTLs were modified only in the sugar moiety and produced as reported by Streicher *et al.*, (1995).

Myrosinase

Myrosinase was extracted and purified from ripe seeds of *Sinapis alba* following the procedure of Pessina *et al.*, (1990). The activity was determined spectrophotometrically at 227 nm as reported by Palmieri *et al.* (1982) but using a little excess of enzyme to determine, without any doubt, which deoxy-GTLs can hydrolyze. One myrosinase unit is defined as the amount of the enzyme able to hydrolyze 1μmole of sinigrin/min at pH 6.5 and 37°C. The GTLs concentration was determined using the molar extinction coefficient of native GTL of 8870 $M^{-1}cm^{-1}$ reported by Thies (1988).



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 and synthetic GTLs

From the results of Table I it is evident that all synthetic deoxy-GTLs are worse substrates for myrosinase than native GTL, thus demonstrating the importance of the presence of all hydroxyl groups in the sugar moiety. This finding makes realistic the possible production of a second type of interaction which leads to the hydrogen bonds formation in the glycon site. This phenomenon, which appears to be essential for the formation and stability of the enzyme-substrate complex, could take place simultaneously or just after the strong electrostatic interaction due to the presence of the negatively charged sulfate group. This interaction should be created in the micro-environment of the aglycon site of the enzyme, which presumably contains a high density of positive charges. In this regard, it is the case to emphasize that the uncharged desulfo-GLs, which can easily be obtained through the action of sulfatase, are unable to undergo hydrolysis by myrosinase, although a weak interaction with the glycon site of the enzyme still seems to be possible. This means that the anionic charge is absolutely necessary to bind the substrate to myrosinase, independently from the type of charged anion as shown by our preliminary results obtained with a synthetic phospho-GTL (Iori and Rollin, 1993; Lazar and Rollin, 1994). In addition, the results of Table I show that myrosinase activity remarkably decreases from 6D-GTL to 2D-GTL, thus indicating that the hydroxyl group in position 2 is much more important than the others. This

finding could be justified if one considers that this position is the closest to the S-glucose bond, which has to undergo hydrolysis in the enzymatic reaction.

Thereby one can reasonably assume that in this site a stronger interaction than in the other sites is necessary.

TABLE I. Comparison of Myrosinase Activity on Native GTL and deoxy-GTLs.

Glucosinolate	[GLs] mM	Myrosinase (U)	$\Delta mABSmin^{-1}cm^{-1}$	Relative activity
GTL	0.34	0.29	746.6	100.0
6D-GTL	0.34	0.29	108.7	14.6
4D-GTL	0.34	0.29	34.4	4.6
3D-GTL	0.34	0.29	16.3	2.2
2D-GTL	0.34	0.29	0.28	0.04

In conclusion, synthetic deoxy-GLs appear to be very useful tools for the characterization of the active site of myrosinase. In particular, with these compounds, we confirm the importance of the sugar moiety for substrates in which the hydroxyl groups are presumably involved in the production of hydrogen bonds in the glycon binding site of the molecule. On the basis of these results, a special role has to be ascribed to the hydroxyl in position 2. The latter is the most important, presumably due to its vicinity to the S-glucose bond, which is hydrolyzed during the myrosinase-catalyzed reaction.

Finally, it is also important to emphasize the possibility to use 2D-GTL and other 2D-GLs as possible myrosinase inhibitors both for further biochemical studies and for biotechnological purposes.

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