Soluble and Total Myrosinase Activity in Defatted *Crambe abyssinica* Meal

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Crambe defatted meal contains 4−6% w/w of glucosinolates, with epiprogoitrin accounting for >90% of the total. This feature limits the use of the meal as feed due to the antinutritional properties of myrosinase−glucosinolate breakdown products. In this context, myrosinase activity assumes particular importance. In this study the total and soluble myrosinase activities have been evaluated directly on defatted meals of eight *Crambe abyssinica* varieties. The pH-stat method, which is the most suitable for assays in heterogeneous solid−water systems, was used. The total myrosinase activity in *C. abyssinica* varieties, determined using epiprogoitrin as substrate, ranged from 288 to 653 units g^{-1}. These activity values were up to 26 times higher than those obtained using other substrates, namely, sinigrin, glucosinalbin, glucotropaeolin, progoitrin, and glucoraphenin. Crambe myrosinase is unusual in that, unlike other Brassicaceae containing a typical main glucosinolate, it does not show the same specificity toward its natural substrates.

Keywords: *Crambe abyssinica; Brassicaceae; myrosinase; glucosinolate; epiprogoitrin; (5R)-5-vinyloxazolidine-2-thione*

INTRODUCTION

Myrosinases (thioglucoside glucohydrolase, EC 3.2.3.1) are found in a variety of edible plants, mostly Brassicaceae, and catalyze the hydrolysis of secondary plant metabolites recognized as glucosinolates (GLs) (**1**), anionic 1-thio-β-D-glycosides (*1*). Myrosinase-catalyzed hydrolysis of GLs leads to the formation of compounds with different functional groups, such as nitriles (**6**, **7**) isothiocyanates (**3**, **4**), or oxazolidinethiones (**5**) in addition to glucose and sulfate ions. The reaction starts with the hydrolysis of the β-thioglucoside bond and the release of a thiohydroximate-*O*-sulfonate (**2**). Following loss of the sulfate anion, GLs undergo a nonenzymatic Lössen rearrangement to give breakdown compounds, as shown in Figure 1. After this step, myrosinase (MYR) is still in the glycosylated form and the glucose is released only after its interaction with one water molecule, making it possible for MYR to return to its original configuration (*2*).

The GL breakdown products have, so far, mainly been studied for their antinutritional effects in animal feed. In recent years, these compounds have been considered valuable for their interesting biological and chemical properties. Some authors consider these molecules useful, not only for their activity against bacteria, fungi, nematodes, and tumor cell growth and in cancer prevention (*3*−*8*) but also because some of them could be used as important intermediates in chemical synthesis (*9*).

MYR occurs in all plants containing GLs, which have been investigated to study this enzyme activity. MYR has been isolated from several cruciferous plant sources, mainly from ripe seeds, namely, white mustard (*Sinapis alba*) (*10*−*12*), rapeseed (*Brassica napus*) (*13*), yellow mustard (*Brassica juncea*) (*14*), and wasabi (*Wasabi japonica*) (*15*), and seedlings, namely, watercress (*16*) (14) and daikon (*Raphanus sativus*) (*17*).

MYR is a glycopolypeptide containing various thiol groups, disulfide and salt bridges and, depending on the source, has multiple forms with different molecular weights (135−480 kDa), numbers of subunits (2−12), and a high percentage of carbohydrate (up to 22.5%), principally hexoses (*18*, *19*). The main MYR isoenzyme isolated from ripe seeds of *S. alba*, in water solution, consists of two identical subunits with a molecular weight of 71.7 kDa (*12*), containing 499 residues, stabilized by a Zn^{2+} ion bound on a twofold axis, with tetrahedral coordination. This MYR isoenzyme has 3 disulfide bridges per subunit and 21 carbohydrate residues distributed in 10 glycosylation sites on the surface (*20*). In addition, the mechanism of MYR-catalyzed hydrolysis of GLs has been studied using this isoenzyme and various types of GLs, desulfo-GLs, and some synthetic competitive inhibitors (*2*). The other isoenzymes found in ripe *S. alba* seeds have not been characterized in such detail, even though some of them have been isolated and partially characterized (*10*, *12*).

Much less is known about MYR of *Crambe abyssinica* (MYRc) than other MYRs of Brassicaceae mentioned above, although crambe is arousing interest due to the quality of its seed oil, the richest in erucic acid. Whereas this oil shows some physicochemical properties that are suitable for various industrial uses, the defatted meal does not attract the same market interest, although it contains ∼40% of good nutritional quality protein. The low appeal of this byproduct is due to the high content of GLs (4−6% w/w), mainly epiprogoitrin (ePRO), ∼90% of the total GLs (*21*, *22*). The presence of a large amount of ePRO, even though it has a moderate bioactivity, limits the use of the meal as feed due to the antinutritional property of the enzymatic breakdown products. In fact, (5*R*)-5-vinyloxazolidine-2-thione (VOT) (**5**) and nitriles (**6**, **7**) display antinutritional properties generally associated with endemic hypothyroidism and hepa-

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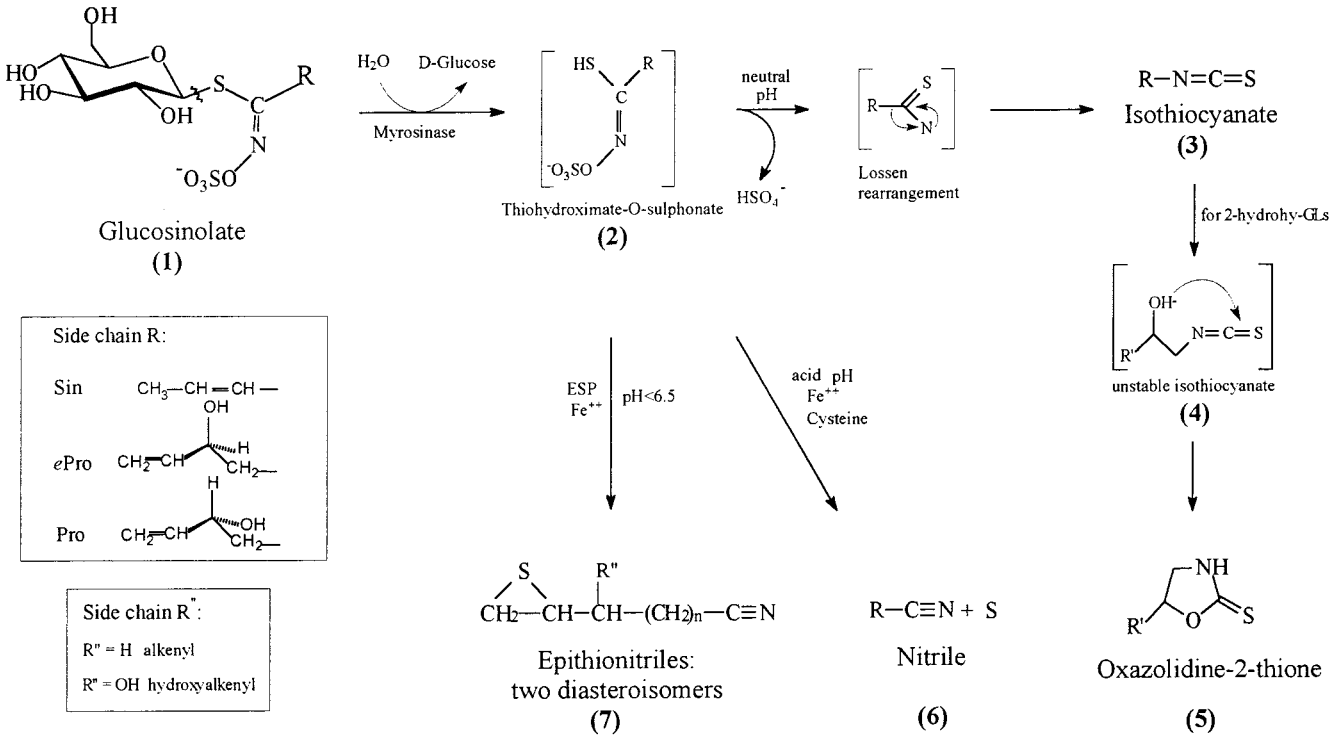

Figure 1. General scheme of glucosinolate hydrolysis.

toxicity in mammals (*23*). Recently, however, these compounds have been considered to be potentially useful for different innovative applications, owing to their molecular structure and biological activity (*9*, *24*).

For the above reasons, we decided to evaluate the total and soluble MYR activity in *C. abyssinica* defatted meal. Particular attention has been given to its activity and specificity toward some GLs of different origin already available in our laboratory, in homogeneous form and in suitable amounts. MYRc alone is responsible for the in vivo catalysis of ePRO hydrolysis, leading to the production of chiral and enantiomerically pure molecules with different chemical structures, which are interesting from chemical and biological points of view.

MATERIALS AND METHODS

Plant Material. The seeds of some crambe cultivars, namely, Belann, Belenzian, Meyer, and C-29, were obtained from the United States; Cebeco 9402 and Cebeco 9404 were from The Netherlands; Indy was from Austria, and Mario was a variety from our Institute. The seeds of *Brassica juncea* L. ISCI 20, *Iberis amara* L. ISCI 14, *Eruca sativa* Mill cv. Nemat, and *Sinapis alba* cv. Pira were also from our Institute. Seeds of *Brassica napus* spp. *oleifera* cv. Norin were obtained from Cerealtoscana SpA, Leghorn, Italy.

Glucosinolates. The GLs used were sinigrin (2-propenyl glucosinolate), progoitrin [(2*R*)-2-hydroxy-3-butenyl glucosinolate], epiprogoitrin [(2*S*)-2-hydroxy-3-butenyl glucosinolate], glucosinalbin (*p*-hydroxybenzyl glucosinolate), glucotropaeolin (benzyl glucosinolate), and glucoraphenin (4-methylsulfinyl-butenyl glucosinolate (see chemical structures and origins in Table 1). They were purified according to the method proposed by Thies (*26*) with some modifications as reported by Visentin et al. (*25*), starting from ripe seeds of some crucifers. The HPLC analyses of desulfo derivatives (*Official Journal of the European Community*, 1990), coupled with polarographic determinations of total GL content (*27*), showed that the GLs used in this study were almost homogeneous. Each GL was identified using NMR.

Analyses of Reaction Products. The GLC analyses of GL-DPs were carried out using a Hewlett-Packard GCD system model G1800A equipped with a 30 m × 0.25 mm capillary column HP-5MS, 0.25 μm. The flow rate of the carrier gas (He) was 1 mL min^{-1}, and 1 μL of sample was injected in splitless mode. The column temperature was 40 °C at the start and 220 °C at the end with an increase of 10 °C min^{-1}. The temperatures of the injector and detector were 250 and 280 °C, respectively.

Other GL-DPs were also analyzed by HPLC (*28*), using a Hewlett-Packard chromatograph model 1090L equipped with a diode array as detector and a 200 × 4.6 mm column HP ODS Hypersil C18, 5 μm.

Oilseed Meal Preparation. The seed samples were milled in a coffee grinder, and the meals were extracted overnight at room temperature with *n*-hexane (1:10 w/v) in a rotary shaker.

Soluble Enzyme Extraction. The crude extract of soluble enzyme was obtained by extracting 30 mg from each of the defatted meals with successive small volumes of a 1% NaCl solution up to a final volume of 4.5 mL.

Enzyme Assays. Myrosinase activities of entire crambe defatted meals (CDM) and of all other meals were determined using the pH-stat method (*29*). The assays were carried out by loading ~30 mg of defatted meal in 4.5 mL of 1% NaCl (total activity) or 4.5 mL of aqueous extract (soluble activity) into

Table 1. Origin and Structure of the Glucosinolates Tested

glucosinolate (trivial name)	systematic name	structure of the side chain R	mol wt	species of origin (ripe seeds)
sinigrin	2-propenyl	CH_2=$CHCH_2^-$	397.5	*Brassica juncea*
progoitrin	(2*R*)-2-hydroxy-3-butenyl	(2*R*)-CH_2=$CHCHOHCH_2^-$	427.5	*Brassica napus* cv. Jet neuf
epiprogoitrin	(2*S*)-2-hydroxy-3-butenyl	(2*S*)-CH_2=$CHCHOHCH_2^-$	427.5	*Crambe abyssinica*
glucosinalbin	*p*-hydroxybenzyl	*p*-$HOPhCH_2^-$	463.6	*Sinapis alba*
glucotropaeolin	benzyl	$PhCH_2^-$	447.6	*Lepidium sativum*
glucoraphenin	4-methylsulfinyl-3-butenyl	CH_3^-SOCH=$CH(CH_2)_2^-$	473.6	*Raphanus sativus*

Table 2. Total and Soluble Myrosinase Activity in Defatted Meal of Eight *C. abyssinica* Cultivars Coming from Different Countries and in *S. alba* Evaluated with Epiprogoitrin (ePRO) and Sinigrin (SIN) as Substrates

	myrosinase activity (units g^{-1})		
	soluble	total	
sample	EPRO	EPRO	SIN
C. abyssinica cv. Belann (USA)	31	486	71
C. abyssinica cv. Bellenzyan (USA)	50	543	83
C. abyssinica cv. Mayer (USA)	43	514	74
C. abyssinica cv. Mario (Italy)	9	401	57
C. abyssinica cv. C-29 (USA)	50	288	53
C. abyssinica cv. Cebeco 9402 (Netherlands)	56	600	92
C. abyssinica cv. Cebeco 9404 (Netherlands)	43	653	102
C. abyssinica cv. Indy (Austria)	31	379	72
S. alba cv. Maxi	32	101	109

the reaction cell at 37 °C. The reaction was started by adding 0.5 mL of a 0.5 M GL solution, after 8−10 min of conditioning, maintaining the starting pH constant. Enzyme activity was calculated by evaluating the slope of the straight line obtained by plotting the titrating nanomoles of NaOH used to maintain the pH constant versus time in minutes. One enzyme unit corresponds to 1 μmol of GLs transformed/min.

RESULTS AND DISCUSSION

Total MYR activity of CDM and of the other defatted meals was determined using the pH-stat method, which appears to be the most appropriate technique for heterogeneous solid−water systems. The use of this method made it possible to establish that MYR in CDM is mostly insoluble, presumably associated with some subcellular entities such as tonoplasts, plasmalemma, and endoplasmic reticulum (*32*). Table 2 shows that total MYRc activity in CDM of eight varieties was notably higher than that of the soluble enzyme. MYR activity was determined by adding pure GLs as substrates to the aqueous slurry after a ∼10 min conditioning at constant pH (6.5) and temperature (37 °C). The pH was maintained constant over the reaction course with the addition of suitable volumes of a titrated NaOH solution (Figure 2). We chose to work with native MYRc, as it is, in CDM, to avoid possible artifacts caused by the enzyme solubilization and purification technique. Nevertheless, experiments are in progress in our laboratory to establish the best solubilization and purification procedure for bound MYRc, without modification of the native enzyme characteristics, to study the molecular properties in detail.

When ePRO was the substrate, the main GL contained in crambe seeds, MYRc, appeared to be particularly active, ranging from 288 units/g (C-29) to 653 units/g (Cebeco 9404), whereas with sinigrin (SIN) the activity decreased ∼7 times, ranging from 53 units/g (C-29) to 102 units/g (Cebeco 9404). MYRc activity, determined using other exogenous GLs, showed lower values, as in the case of glucosinalbin (57 units/g), glucotropaeolin (47 units/g), glucoraphenin (23 units/g), and progoitrin (PRO) (59 units/g) (Table 3). The latter finding appears to be the most important because when PRO was used as substrate, as with other GLs, the MYRc-catalyzed reaction rate was ∼10 times lower than that determined with ePRO. From a stereochemical point of view, the only difference between ePRO and the correspondent PRO is the spatial location of the hydroxyl group in the side chain. Thereby a simple explanation of this different performance could be this structural diversity.

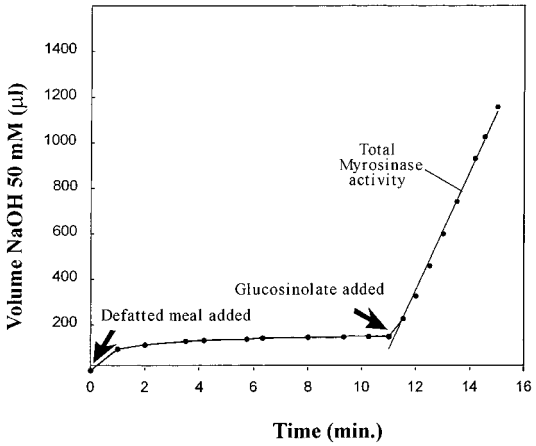

Figure 2. Typical pH-stat assay used to determine myrosinase activity in defatted cruciferous meal samples at constant pH 6.5.

Table 3. Total Activity of Crambe Myrosinase (Cebeco 9402 Defatted Meal) Using Different Substrates

substrate	total activity (units g^{-1})	substrate	total activity (units g^{-1})
epiprogoitrin	600	glucotropaeolin	47
sinigrin	92	progoitrin	59
glucosinalbin	57	glucoraphenin	23

These findings are remarkable and were not expected because the MYR activity contained in other Brassicaceae seeds toward different GLs is not so high. For instance, in white mustard (*S. alba)* defatted meal, the total MYR activity (∼32% in soluble form) was almost one-fifth of the average value of MYRc activity from eight CDM, determined with ePRO as substrate, and showed similar total activity both with ePRO and with SIN as substrate (Table 2). In addition, Figure 3 shows that only CDM contains a MYR that is so specific and active toward its main natural substrate (ePRO). The other defatted seed meals, obtained from *B. juncea, I. amara, E. sativa, S. alba,* and *B. napus*, did not show similar MYR activities with their main representative GLs, which are SIN, glucoiberin, glucoerucin glucosinalbin, and PRO, respectively. This finding is very interesting, especially if one considers the enzyme activity of *B. napus* seed meal (22 units g^{-1}). In this case, too, MYR did not show comparable performance to MYRc, with PRO or ePRO as substrate. For a better understanding of the singular behavior of MYRc, we determined the optimum pH and temperature of total MYRc in the native form, using ePRO and SIN as substrates.

The optimum pH for the total MYRc activity was determined at 37 °C. With both substrates, the pH-dependent enzyme activity shows wide curves, which define different optimum pH values around 6.5 and 7.5, respectively (Figure 4). These values are quite dissimilar from that previously reported by Tookey (*21*), who found the optimum pH between pH 8 and 9, using ePRO as substrate for a soluble MYRc. Different temperature enzyme activity profiles were also observed. These curves also displayed distinct optimum temperatures for the two substrates at 37 and 50 °C, respectively (Figure 5). It is not easy to interpret these findings; nevertheless, some attempts can be made to give a justification of this singular MYRc behavior. This performance, as well as confirming the high specificity of MYRc for ePRO, indicates that MYRc, in the presence of its

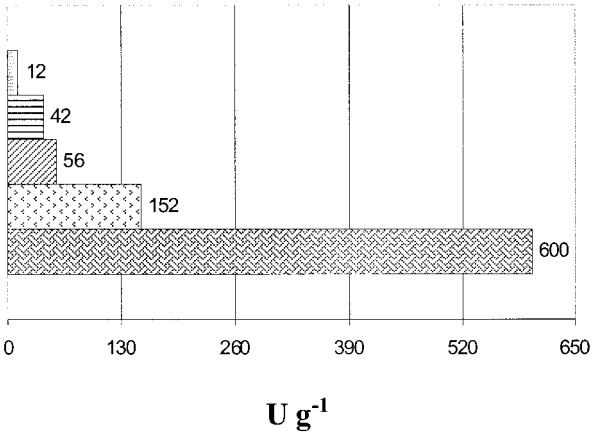

Figure 3. Myrosinase activity in different cruciferous defatted meals determined using the most representative endogenous glucosinolate as substrate (bars represent, top to bottom *B. juncea* with sinigrin; *I. amara* with glucoiberin; *E. sativa* with glucoerucin; *S. alba* with glucosinalbin; *C. abyssinica* with epiprogoitrin).

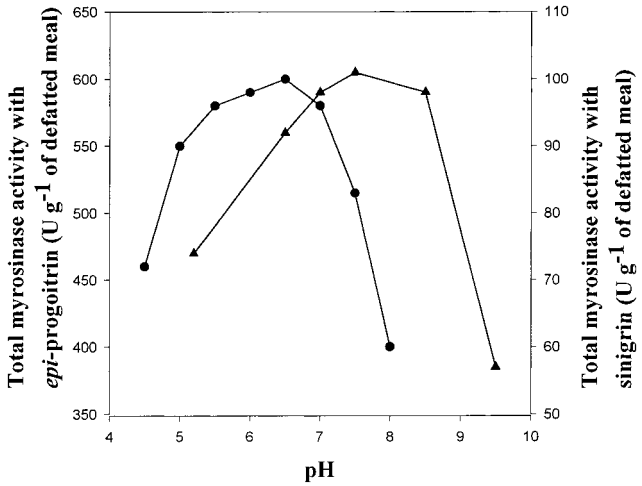

Figure 4. pH activity profiles of crambe myrosinase (Cebeco 9402 defatted meal) using epiprogoitrin and sinigrin as substrates at 37 °C.

natural substrate, could establish an additional stabilizing interaction with this GL in the active site, giving this special activity. In this case, one of the residues involved in the hydrophobic pocket of this enzyme, important for the docking of the substrate aglycon moiety, should be different from the other MYRs. In particular, it differs from *S. alba* MYR, for which the active site structure has been studied in detail (*20*). In principle, this explanation seems to be plausible because it gives the idea that MYRc at ~37 °C could assume an optimal conformation, allowing a better docking and stability of the *S*-hydroxylated substrate (ePRO) in the active site, in comparison to not only the nonhydroxylated substrate (SIN) but also the correspondent *R*-hydroxylated substrate (PRO).

The results of this study are very stimulating. They represent a first attempt to better comprehend how the MYRc−ePRO system would operate in vivo. In CDM, but presumably also in other plant tissues, there is "a special latent" mechanism that, when activated, is able to efficiently hydrolyze ePRO and produce four derived bioactive compounds, depending on the reaction conditions: pH, presence of Fe^{2+}, and myrosinase cofactors such as the "epithio specifier protein" (ESP). At pH values close to neutrality, the production of the corresponding, highly active, isothiocyanate is favored, as in the case of the enzymatic hydrolysis of the majority of GLs. Nevertheless, the 2-hydroxy-3-butenyl isothiocyanates derived from hydroxy GLs, such as PRO and ePRO, are unstable as they spontaneously rearrange to give (*S*) and (*R*) VOT, respectively, by a cyclization process. It is interesting to note that VOT, although it has a good chemical stability with a moderate reactivity, due to its amino and thiocarbonyl nucleophilic groups, showed a medium-low cytotoxic activity when tested in pure form in different contexts such as human cells, soil pathogenic fungi, and nematodes (*6*, *8*, *30*).

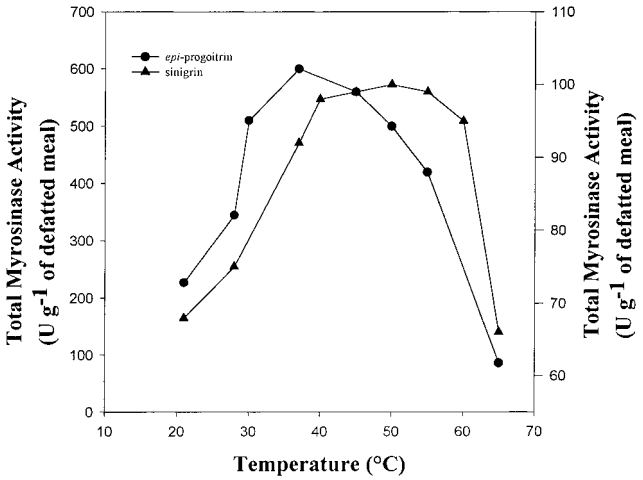

Figure 5. Temperature activity profiles of crambe myrosinase (Cebeco 9402 defatted meal) using epiprogoitrin and sinigrin as substrates at pH 6.5.

The other possible cytotoxic reaction products provided by MYRc-catalyzed hydrolysis of ePRO are the 2-hydroxy-3-butenylnitrile and two epithionitrile isomers. Both of these compounds are produced in mixture with VOT, in the presence of Fe^{2+} and at acidic pH values, although epithionitrile production also needs the presence of ESP, the epithio specific proteinic MYR cofactor, also contained in crambe seed cells.

Considering the strong activity and specificity of MYRc, as well as the variety of cytotoxic compounds that can be produced, the MYRc−ePRO system in crambe seeds appears to be extremely efficient. In fact, when primed, the MYRc−ePRO system can provide prompt and differentiated protective reactions against pathogenic attacks and, in particular, against seed-eating insects, without producing further injury to the attacked tissues. This "multivariate protective system" is activated following tissue wounding, making possible an interaction between MYRc and ePRO under different reaction conditions, determined by the contingent situation (pH, cofactors, etc.). In turn, the process triggers the production in situ of high concentrations of strongly active mixtures of 2-hydroxy-3-butenyl isothiocyanate (**3**, **4**), nitriles (**6**), and epithionitriles (**7**), which, during and after their protective action, must be rapidly "controlled" to avoid self-toxic effects on plant tissues. In particular, in the case of the isothiocyanate (**4**), probably the strongest cytotoxic degradation product of ePRO, this control could be performed by influencing the transformation rate of the isothiocyanate (**4**) into the much less active VOT (**5**) (cyclization). This spontaneous intramolecular reaction, due to a nucleophilic attack of hydroxyl oxygen on the isothiocyanate group carbon, could be controlled in crambe seeds or seedlings

by using the specific reaction "milieu", typical of these plant tissues.

CONCLUDING REMARKS

Many authors have reported that the main role of the GL−MYR(−ESP) system in Brassicaceae is to protect the tissues against biotic stresses. Nevertheless, there is little information that gives a realistic explanation for this phenomenon at a molecular level. In this study, we have made an effort in this direction, although much remains to be done, mainly from a biochemical point of view. First, we have demonstrated that in CDM, and presumably even in crambe seed, MYRc is mostly insoluble (>90%) and is very specific for ePRO, the main endogenous substrate. The properties of the soluble enzyme appear to be similar to those of the insoluble one; thus, both seem to be variable fractions of the total MYRc. Finally, because MYRc specificity is so high and a distinction can be made between the two progoitrin isomers, it is reasonable to think that this behavior could be ascribed to a better orientation of ePRO in the active MYRc site, which enhances the general stability of the enzyme−substrate complex. This aspect, which has to be confirmed with pure MYRc preparations, assumes significance in the crambe physiological defense mechanisms, which give natural resistance to flea beetle attacks, that destroy rapeseed when cultivated without insecticides (*31*).

ABBREVIATIONS USED

MYR, myrosinase; MYRc, crambe myrosinase; GL, glucosinolate; ePRO, epiprogoitrin; PRO, progoitrin; SIN, sinigrin; VOT, 5-vinyloxazolidine-2-thione; CDM, crambe defatted meal; ESP, epithio specifier protein.

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