JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Isolation and Biochemical Characterization of a Basic Myrosinase from Ripe *Crambe abyssinica* Seeds, Highly Specific for *epi*-Progoitrin†

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On the basis of previous studies on the mechanism-based inhibition, activation, and active site structure of myrosinase(s) isolated from *Sinapis alba* and other cruciferous seeds, crambe myrosinase shows uncommon properties and behavior. For this reason homogeneous crambe myrosinase was isolated and investigated to establish the most important physicochemical features, including kinetic properties determined with the epimers progoitrin (*R*) and *epi*-progoitrin (*S*) as substrates, with and without ascorbate as an activator. The results of this study demonstrate that crambe myrosinase is highly specific for *epi*-progoitrin due to a better stabilization of the enzyme−substrate complex. This stabilization is caused by additional hydrogen bonding that only *epi*-progoitrin can set up between its hydroxyl group and a suitable residue in the hydrophobic pocket where the "docking" of the glucosinolates side chain takes place.

KEYWORDS: ***Crambe abyssinica*; Brassicaceae; myrosinase; glucosinolate; *epi*-progoitrin; (5*R*)-5-vinyloxazolidine-2-thione**

INTRODUCTION

Myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) catalyzes the cleavage of the *S*-glucose bond in a variety of plant anionic 1-thio-β-D-glucosides called glucosinolates via acid/base-catalyzed reaction with the release of aglycon and the formation of the glycosyl enzyme intermediate. Myrosinases are glycopolypeptides containing various thiol groups, disulfide, and salt bridges and, depending on the source, have multiple forms with different molecular weights (135−480 kDa), numbers of subunits (2−12), and a high percentage of carbohydrate (up to 22.5%), mostly hexoses (*1*, *2*). The main myrosinase isoenzyme isolated from ripe seeds of *Sinapis alba*, the typical source of this enzyme, consists of two identical subunits with a molecular weight of 71.7 kDa (*3*, *4*), containing 499 residues, stabilized by a Zn^{2+} ion bound on a twofold axis, with tetrahedral coordination. This myrosinase isoenzyme has three disulfide bridges per subunit and 21 carbohydrate residues distributed in 10 glycosylation sites on the surface (*5*). Myrosinases are typical enzymes of the Brassicaceae family that also contain their substrates, the glucosinolates, in variable concentrations and sites depending on plant organs and tissues. In undamaged tissues, enzyme and substrate(s) are confined in different sites of the cell. The myrosinase−glucosinolate system is always present in various arrangements and concentrations in all Brassicaceae organs. When activated following tissue damage, it plays a defensive role against plant pests (*6*−*8*).

Glucosinolates have a common structure with four main elements, that is, the thioglucosidic bond, the sulfate anion, the glucosidic residue, and a side aglycon chain of aliphatic, aromatic, or heteroaromatic type (**Figure 1**). At present ∼120 glucosinolates have been isolated and characterized (*9*). In their native form, glucosinolates have low biological activity, whereas their derived products (isothiocyanates, thiocyanates, nitriles, and epithionitriles), obtained by myrosinase-catalyzed hydrolysis, constitute an important group of bioactive molecules of vegetable origin. So far the enzymatic catalysis of glucosinolates has mainly been studied for its antinutritional effects in animal feed, although in recent years these compounds have been considered to be 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 (*10*−*13*) but also because some of them could be used as important intermediates in chemical synthesis (*14*). The mechanism of glucosinolate enzymatic hydrolysis has been studied using the main myrosinase isoenzyme isolated from white mustard (*Sinapis alba*) seeds and various types of glucosinolates, desulfoglucosinolates, and some synthetic competitive inhibitors (*15*, *16*).

Myrosinase has also been isolated from several other cruciferous plant sources, mainly from ripe seeds, namely, rapeseed

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† This study was carried out within the DICRA Project supported by the Commission of European Union (FAIR PL 98 4333).

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10.1021/jf020796g CCC: $25.00
Published on Web 03/28/2003

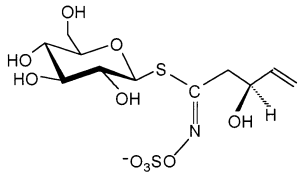


Figure 1. Structure of *epi*-progoitrin.

(*Brassica napus*) (*17*), yellow mustard (*Brassica juncea*) (*18*), wasabi (*Wasabi japonica*) (*19*), and seedlings of watercress (*Lepidium sativum*) (*20*) and daikon (*Raphanus sativus*) (*21*). Myrosinase of *Crambe abyssinica* (MYRc) is less known than myrosinases of other Brassicaceae mentioned above, even though Tookey in 1973 made a preliminary isolation and characterization of this enzyme (*22*). In crambe seeds, only myrosinase is responsible for the catalyzed hydrolysis of *epi*-progoitrin (*e*PRO), which leads to the production of four bioactive enatiomerically pure compounds (**1**, **2.1**, **2.2**, and **3**) depending on the reaction conditions, mainly pH, and the presence of the epithio specifier protein (ESP) (**Scheme 1**). The latter is an important myrosinase "satellite" protein (*23*−*25*), necessary to drive the reaction toward the production of two diastereomeric epithionitriles (**2.1** and **2.2**) with a high biological activity (*26*).

Although crambe has aroused interest in the past decade due to the quality of the extracted seed oil, the richest in erucic acid, no additional studies have been made since our initial report. For these reasons, we considered it important to evaluate the total and soluble myrosinase activity in *C. abyssinica* defatted meal of eight crambe varieties from different countries (*27*). In crambe seed, MYRc is mainly insoluble and the activity of the precipitate after centrifugation ranges from 83 to 98% of the total. Some of the molecular properties of MYRc in defatted crambe meal are unusual. MYRc shows an extraordinary specificity toward its natural substrate *e*PRO, with an activity up to 26 times higher than that obtained using some other glucosinolates as substrate, for example, glucoraphenin. In fact, in other Brassicaceae defatted meals, with a typical main glucosinolate like crambe, myrosinase did not show the same specificity toward its natural endogenous substrate. MYRc was so specific that it showed an extraordinarily distinct activity between the two isomeric substrates PRO and *e*PRO. MYRc displayed a moderate activity with PRO, similar to those observed when the majority of other glucosinolates were used as substrates (*27*).

The study of MYRc has important applications not only because it is responsible for the in vivo catalysis of *e*PRO hydrolysis but also because it produces chiral molecules with one (**1** or **3**) or two stereogenic centers (**2.1** or **2.2**) (**Scheme 1**). This is remarkable, from chemical and biological points of view, on the one hand for the feed and fine chemical industries and on the other hand for the knowledge of the physiological defense mechanism in crambe. To achieve a deeper understanding of this uncommon myrosinase, the main physicochemical features and its structure must be characterized. To do this, as with any protein, the first step is to set up an efficient isolation procedure to produce the enzyme in homogeneous form. Therefore, in this paper, we describe the MYRc isolation procedure and some of its main physicochemical properties. These include the kinetic parameters determined with the epimers PRO (*R*) and *e*PRO (*S*) as substrates, also using ascorbate as an activator. The results of this study not only explain how MYRc shows its high specificity toward its natural substrate *e*PRO but also provide an important initial step for more detailed X-ray structural studies, necessary to completely describe the active site mechanism of MYRc-catalyzed hydrolysis of *R* and *S* structured substrates.

MATERIALS AND METHODS

Plant Material. Crambe seeds (cv. Cebeco 9402) were a generous gift from Cebeco Handelsraad, Rotterdam, The Netherlands.

Chemicals and Equipment. Con A−Sepharose, SP-Sepharose HP, and prepacked Superdex 200 were supplied by Pharmacia Fine Chemicals (Uppsala, Sweden) as were the electrophoresis equipment, the PhastSystem, and the fast protein liquid chromatograph (FPLC) and related reagents. Hog pancreas lipase was from Fluka (catalog no. 62300), whereas all other enzymes were obtained from Sigma. All other reagents were of analytical grade. Spectrophotometric myrosinase activities were recorded with a Varian Cary model 219 connected to a PC equipped with special software to compute myrosinase activity data. Myrosinase activity was also determined by polarographic measurements, using a Gilson K-IC Oxygraph from Medical Electronics (Middleton, WI) equipped with a Clark type electrode. GlycoPRO deglycosylation kit (combination kit) was from Europa Bioproducts Ltd.

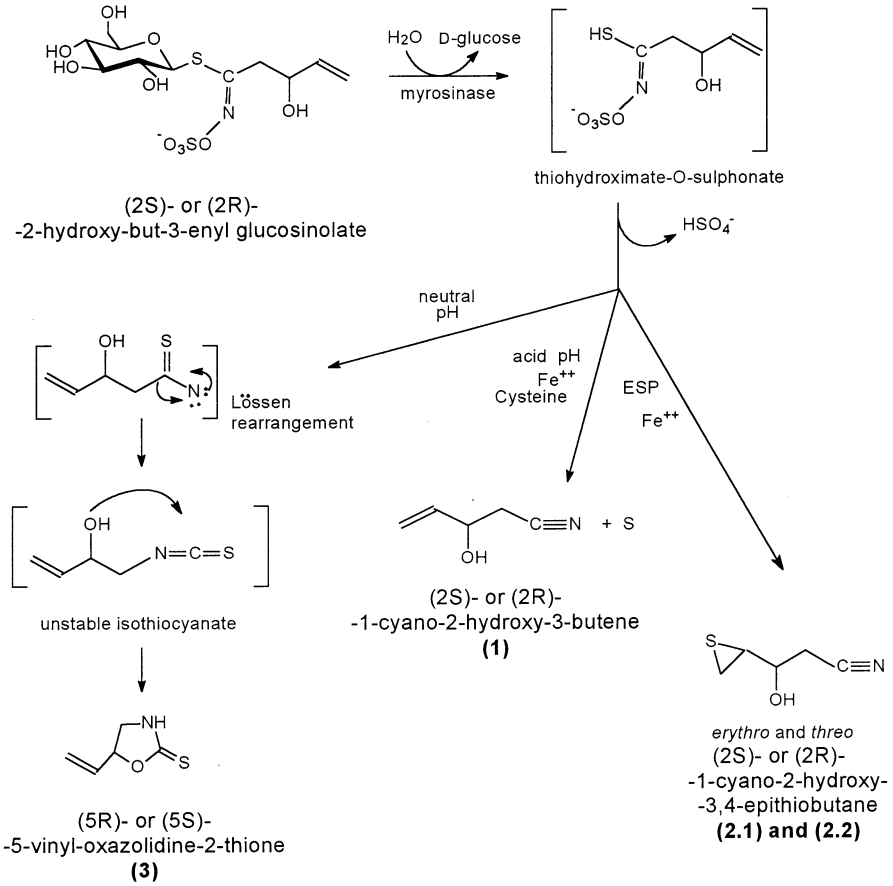
Glucosinolates. In this work we used sinigrin (2-propenyl glucosinolate), progoitrin [(2*R*)-2-hydroxy-3-butenyl glucosinolate] (PRO), *epi*-progoitrin [(2*S*)-2-hydroxy-3-butenyl glucosinolate] (*e*PRO), glucosinalbin (*p*-hydroxybenzyl glucosinolate), glucotropaeolin (benzyl glucosinolate), glucoraphenin (4-methylsulfinylbut-3-enyl glucosinolate), and glucobrassicin (indol-3-ylmethyl glucosinolate), which were purified starting from ripe seeds of *Brassica carinata*, *B. napus* cv. Jet Neuf, *C. abyssinica* cv. Belenzian, *S. alba* cv. Maxi, *L. sativum*, *R. sativum*, and leaves of *Isatis tinctoria* (accession Casolavalsenio), respectively, according to the method proposed by Thies (*28*) with some modifications reported by Visentin et al. (*29*) (**Table 1**). In particular, PRO was further purified from the glucosinolates mixture obtained from defatted rapeseed meal, where it predominates, following the method reported by Bjerg and Sörensen (*30*). The epimeric glucoisatisin mixture was a generous gift of Prof. Nicolas Fabre of the Laboratoire Pharmacofores Rédox, Phytochimie et Radiobiologie of the University Paul Sabatier, Toulouse, France. The HPLC analyses of desulfo derivatives (*31*), coupled with polarographic determinations of total glucosinolates content (*32*), showed that the glucosinolates used in this study were homogeneous. Each glucosinolate was also identified using NMR.

Enzyme Assays. In this study myrosinase activity was measured using different methods, depending on the type of myrosinase, substrate used, and kind of experiments. The total and the insoluble MYRc activities were determined using the pH-stat method (*27*, *33*). The activity of soluble MYRc was measured using different spectroscopic and polarographic methods. To determine the kinetic parameters of the purified myrosinase with different substrates, we used the spectrophotometric coupled enzyme assay (SCEA) described by Palmieri et al. (*33*), instead of the simpler direct spectrophotometric assay (DSA) (*33*−*35*), due to the spectroscopic characteristics of the hydroxylated substrate (viz. PRO and *e*PRO) degradation products, which strongly absorb at 240 nm. To avoid the possible influence of pH on hexokinase and 6-phosphate dehydrogenase, ancillary enzymes of myrosinase in the SCEA method, the optimum pH of MYRc was determined by the discontinuous method based on the incubation of MYRc with the substrates at 37 °C at different pH values. After an established incubation time, the reaction was stopped, and the glucose released was measured by the polarographic method (*33*). One myrosinase unit corresponds to 1 μmol of glucosinolates transformed per minute. The specific activities are expressed as units per milligram of soluble protein.

Enzyme pH Stability. MYRc was incubated at different pH values (from 4 to 11) at 37 °C. After 30 min of incubation, the activity was determined at pH 6.5 with *e*PRO and PRO. The values are reported as percentage of residual activity. These data were calculated on the basis of the maximum activity (100%) determined at optimal pH values.

Isolation and Purification. *Preparation of Crude Extract.* Ripe crambe seeds were milled in a coffee grinder, and the oil was extracted overnight at room temperature with *n*-hexane (1:10 w/v) in a rotary

Scheme 1. General Scheme of Myrosinase-Catalyzed Hydrolysis of Progoitrin and *epi*-Progoitrin



shaker. The crude extract was prepared by starting from a sample of 170 g of defatted crambe meal, which was added to 1.7 L of 0.5 M acetate buffer (pH 5.7), containing 17 g of hog pancreas lipase. The suspension was mixed with a magnetic stirrer and incubated at 40 °C for 30 h. The slurry was then centrifuged (15300*g*) at 4 °C. The centrifuged extract was dialyzed against distilled water, whereas the pellet was extracted again with a solution of 3 M LiCl (700 mL) overnight at room temperature. After centrifugation, the extract was dialyzed against distilled water. The dialyzed extracts were collected together and conditioned at pH 7.3 with 20 mM Tris-HCl, and a suitable amount of NaCl was added to reach a concentration of 0.5 M.

Purification Process. (1) Con A Affinity Chromatography. The dialyzed extract was recentrifuged and applied to a glass column (2.6 × 20 cm) filled with Con A−Sepharose to give a bed volume of ∼40 cm^3. The procedure used in this chromatographic step was essentially that reported by Palmieri et al. (*36*).

(2) Ion-Exchange Chromatography. The active fractions coming from Con A affinity chromatography were collected, dialyzed against 25 mM P buffer (pH 6.5), and applied to an SP-Sepharose H.P 16/10 column (Pharmacia) equilibrated with the same buffer. MYRc was eluted using a linear gradient of NaCl in the starting buffer, from 0.03 to 0.7 M in 200 mL with a flow rate of 1 mL min^{-1}, using a Pharmacia FPLC system equipped with a UVICORD SII detector (Pharmacia). Fractions containing MYRc activity were collected and dialyzed against 50 mM P buffer (pH 6.5), containing 0.15 M NaCl.

(3) Gel Filtration Chromatography. The dialyzed MYRc solution was loaded into an FPLC prepacked Superdex 200 HiLoad 26/60 gel filtration column (Pharmacia) equilibrated with 50 mM P buffer (pH 6.5), containing 0.15 M NaCl. The active fractions were collected and used for MYRc characterization. For gel filtration molecular weight determination, a prepacked Superdex 200 HR 10/30 equilibrated with the above buffer solution was used.

Electrophoresis. SDS-PAGE and IEF of the isolated enzyme were performed with a Phastsystem apparatus (Pharmacia) using homogeneous Phastgel 12.5% and Phast IEF 3−9, respectively. Pharmacia standards and protocols were used.

Protein Determination. Soluble protein concentrations were determined according to the Coomassie Brilliant Blue (G 250) method using BSA as standard reference (*37*).

Carbohydrate Analysis. The carbohydrate content of MYRc was determined using an enzymatic deglycosylation kit and calculating the difference in molecular weight between non-deglycosylated and deglycosylated MYRc with SDS-PAGE. We followed both the denaturing and the non-denaturing protocols available with the analysis kit. Nevertheless, the data reported in this paper were determined with only the non-denaturing protocol because when the other method was used, the protein precipitated, preventing the determination of carbohydrate content.

RESULTS AND DISCUSSION

Myrosinase Activity in Crambe Defatted Meal. This study indicates that the total myrosinase activity in defatted meal of some *C. abyssinica* varieties is mostly insoluble and specific for *e*PRO as a substrate. Although various glucosinolates, namely, sinigrin, glucoraphenin, glucosinalbin, glucotropaeolin, progoitrin, and *epi*-progoitrin, were used as substrates to determine the total myrosinase activity in these meals, *e*PRO was the most reactive. The high specificity of MYRc was further confirmed in this study using the pure enzyme (**Table 2**). We also established that soluble MYRc (determined after filtration of centrifuged enzymatic crude extract with a Millex-GV 0.22 μm sterile filter) ranged between 0.1 and 0.3% of the total activity. Due to this low percentage of soluble MYRc, we carried out several preliminary small-scale defatted meal extraction trials to find the best conditions to optimize enzyme yield. Different techniques, using diverse buffered extraction solutions contain-

Table 1. Origin and Structure of the Glucosinolates Tested

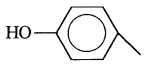
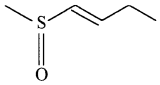
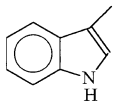
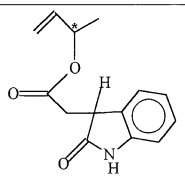
Glucosinolates systematic name	Structure of the side Chain (-R)	MW	Species and Origin
Sinigrin		397.5	*Brassica carinata*
Progoitrin (*R*) and *epi*-Progoitrin (*S*)		427.5	*Brassica napus* and *Crambe abyssinica*
Glucosinalbin		463.6	*Sinapis alba*
Glucotropaeolin		447.6	*Lepidium sativum*
Glucoraphenin		473.6	*Raphanus sativum*
Glucobrassicin		487.6	*Isatis tinctoria*
Glucoisatisin (*R*) and Glucoisatisin (*S*)		614.6	*Isatis tinctoria*

Table 2. Specific Activity of Crambe Myrosinase (MYRc) and White Mustard Myrosinase (MYRm) with Different Glucosinolates

glucosinolate	MYRc (units/mg)	MYRm (units/mg)
sinigrin	7 ± 0.1	69 ± 0.4
progoitrin	5 ± 0.2	61 ± 0.4
epi-progoitrin	59 ± 0.1	52 ± 0.3
glucosinalbin	5 ± 0.1	64 ± 0.4
glucotropaeolin	4 ± 0.2	77 ± 0.4
glucoraphenin	1 ± 0.2	60 ± 0.3
glucobrassicin	3 ± 0.1	59 ± 0.4
glucoiberin	1 ± 0.2	56 ± 0.3

ing variable salt concentrations (lithium and guanidinium chloride), detergents (Triton X-100 and SDS), enzymes (lipases, proteases, cellulases, and pectinases), and sonication, were examined. The most efficient technique was a two-step extraction procedure based on the initial incubation of meal with hog pancreas lipase at 40 °C and a second extraction of the residual meal with an aqueous solution containing 3 M LiCl. Using this method, ~5% of the total insoluble MYRc activity, originally contained in the starting meal, was solubilized and recovered after dialysis. Only lipase was effective in solubilizing MYRc and providing a stable enzyme yield, suggesting that myrosinase in crambe seed is presumably a membrane glycoprotein.

Purification and Characterization. A typical purification outline is reported in **Table 3**. Using this procedure, MYRc was purified to homogeneity. Partial purification with Con A-Sepharose affinity chromatography was carried out using the same procedure described in previous papers for the purification of myrosinase from white mustard seeds (MYRm) (*4*, *36*). This step made it possible to easily isolate and concentrate MYRc from a relatively high volume of dialyzed extract, increasing the specific activity up to 7.5 times. This result also shows that MYRc is a glycoprotein with carbohydrate chains distributed over the surface of the enzyme, responsible of the interaction with Con A and essential to isolate ~46% of total MYRc from the crude dialyzed extract. The yield of this purification step was, however, notably lower than what we normally find in MYRm isolation (~90%). In fact, the carbohydrate analysis of the purified MYRc, determined with approximation by defect, gave a maximum of 8% of protein glycosylation, much lower than that determined in MYRm (18−19%) (*3*, *5*).

Table 3. Purification of Myrosinase from Crambe Seeds (MYRc)

	specific activity (units/mg)	yield (%)	purification (factor)
crude dialyzed centrifuged extract	1–1.5	100	1
affinity chromatography Con A-Sepharose	8–11	46	5–7
ionic exchange chromatography SP-Sepharose HP	20–30	9	15–20
gel filtration Superdex 200	44–66	4	30–40

The second purification step was SP-Sepharose high-performance ion-exchange chromatography. This made it possible to increase the purity of the preparation by almost 3 times, even though in this case a high percentage of activity was lost. The chromatographic profile (not shown) showed a main peak containing the MYRc activity and three other smaller peaks of inactive components. Taking into account this result, it is reasonable to assume that this purification step removed a positive effector that is functional to MYRc. This might also explain the notably lower specific activity of MYRc as compared to that of MYRm. The two enzymes showed only a similar activity when *e*PRO was used as substrate. In the purification procedure, a first gel filtration chromatography showed two main peaks, the highest containing almost all of the loaded enzyme activity. A second gel filtration, carried out in the same conditions using the active fractions of the previous separation, gave a single peak corresponding to an apparent molecular mass for the holoenzyme of 470 kDa. This molecular mass value is uncommonly high when compared with those of other isolated myrosinases, which generally show molecular masses of ~150 kDa with two identical subunits (*2*). The only myrosinase with a comparable molecular mass is the myrosinase isolated from *W. japonica* (*19*), which is reported to be composed of 12 subunits of ~46 kDa each. The MYRc molecular masses, obtained by gel filtration in native and denaturing conditions, indicate that native MYRc is presumably composed of six identical subunits of ~75 kDa each. The results of the electrophoretic trials, SDS-PAGE, and IEF confirm not only the homogeneity of the MYRc preparation but also the molecular mass of a single subunit (75 kDa).

The p*I* of native MYRc is 8.1, a value not in common with other myrosinases. This result, together with the high molecular weight and number of subunits, indicates that MYRc exhibits unique properties as compared with those of other myrosinases. For this reason as well, MYRc merits additional study to define its molecular and structural characteristics. These studies are also important to establish whether the high molecular mass of native MYRc that we determined (see above) is also due to the particular shape of the protein and the carbohydrate content, as well as its distribution and configuration on the surface of the

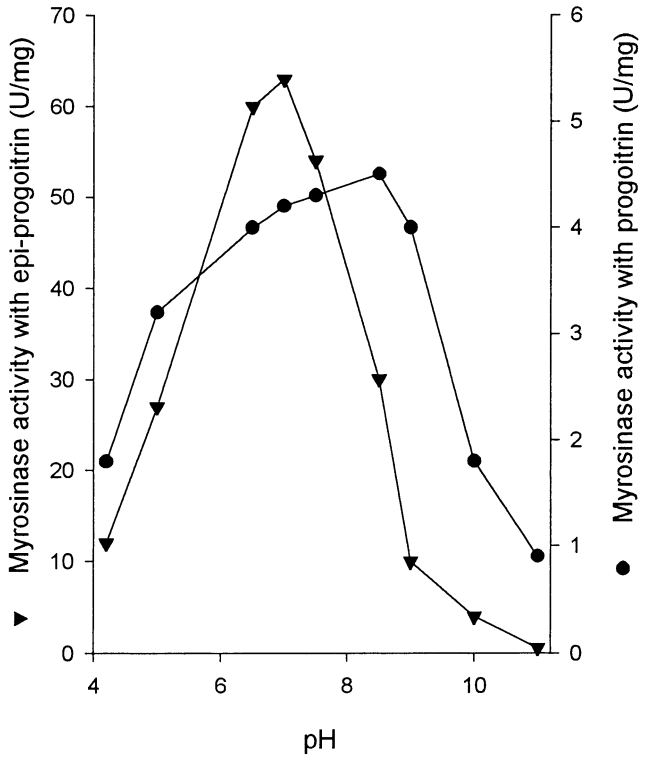


Figure 2. Crambe myrosinase pH activity profile using progoitrin (●) and *epi*-progoitrin (▼) as substrates. At optimum pH values, the specific activities were 6.3 and 54 units mg^{-1}, respectively.

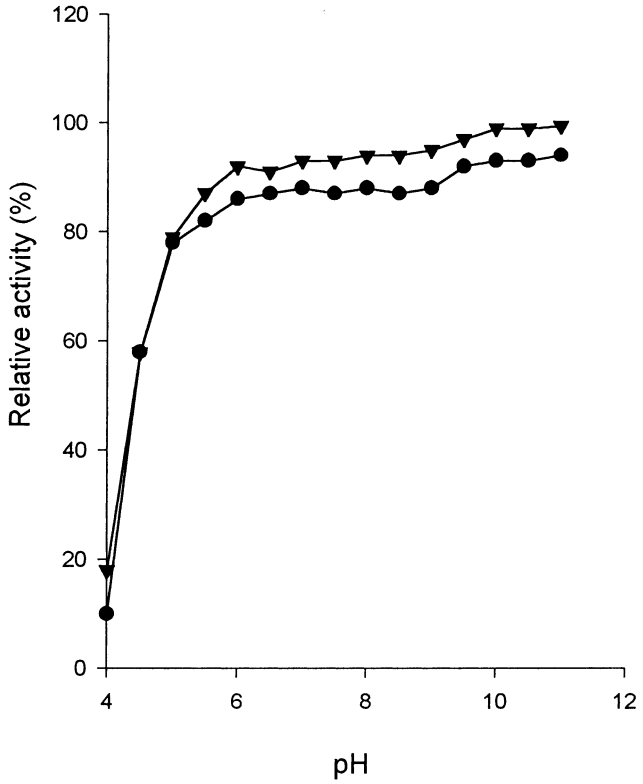


Figure 3. Crambe myrosinase pH stability using progoitrin (●) and *epi*-progoitrin (▼) as substrates. The samples were incubated at 37 °C for 30 min at the set pH values before the activity was determined.

hexameric protein. In this context, a binding protein may also be present. Moreover, the p*I* values of other isolated myrosinases range from 5.15 (*S. alba*) (*4*) to 4.6 (*B. juncea*) (*18*), whereas we observed a value of 8.1 for MYRc. This apparently means that MYRc is richer than other myrosinases in basic amino acids. This feature could also contribute to the high substrate specificity of MYRc toward *e*PRO.

Effect of pH. As reported by Finiguerra et al. (*27*), the trend of MYRc-catalyzed hydrolysis carried out at different pH values using defatted crambe meal was quite unusual depending on the substrate used. In particular, *e*PRO hydrolysis shows a special trend with maximum activity at pH 6.5, whereas sinigrin's maximum activity was always around pH 7.5. Using pure MYRc we obtained comparable results. With *e*PRO as substrate the optimum activity of MYRc was confirmed at pH 6.5. Using the epimer PRO, the optimum pH was 8.5, although we found that the activity remained near the maximum values for a wide pH range, essentially between 6.5 and 8.5 (**Figure 2**). Clearly this enzyme behavior determined by pH variation is closely related to both the ionization state of the prototropic groups present in the active site of MYRc and the diastereoisomeric structure of the two substrates. It is important to emphasize that the greatest effect of pH on MYRc-catalyzed hydrolysis of the two diastereoisomers was displayed within the pH range of 5.6−8.5, whereas outside these values the specificity of MYRc toward *e*PRO decreased notably. The reasons for this behavior are completely different. At acidic pH values (below pH 4), myrosinase lost its activity due to partial deactivation, whereas at basic pH values (over pH 8) it showed good stability, maintaining its original activity over an extended pH range (**Figure 3**). Nevertheless, despite this excellent stability the decrease in specificity beyond pH 8.5 indicates that at basic pH values MYRc could change conformation, thus also modifying the active site geometry. It would then become less "well-organized" for efficient docking and hydrolysis of the *S*-structured substrate (*e*PRO) but, at the same time, remain sufficiently efficient toward the *R*-substrate (PRO). In fact, at these pH values the hydrolysis rates with the two diastereoisomers tend to be similar.

As proposed in the previous study on MYRc (*27*), we think that at values around the optimal pH the highest activity that the enzyme showed toward its natural substrate (*e*PRO) may be due to an additional stabilizing interaction of this substrate on the active site. This stabilizing effect could be a consequence of an extra hydrogen bond between the hydroxyl group of the substrate and a suitable amino acid residue in the active site. In principle, this effect would be consistent with the chemical structure of both substrates, due to the presence of a hydroxyl group in C2. Nevertheless, we established that MYRc is highly specific for the *S*-structured substrate (*e*PRO) only; this finding would suggest a specific stereoisomeric feature as an explanation for its high specificity.

Effect of Temperature. MYRc also showed different temperature enzyme activity profiles when *e*PRO and PRO were used as substrates, although this difference is lower than that observed with crambe meal (*27*) (data not shown). In addition, we observed that up to these optimum temperature values, the difference between the activities obtained with these substrates remained almost constant. Increasing the temperature beyond the optimum, the negative effect of heat on MYRc was more evident with *e*PRO as substrate. It is not easy to explain this finding, even if tentatively it seems possible that heat could interfere with the assumed extra hydrogen bonding formation that only *e*PRO can realize in the MYRc active site with its hydroxyl group.

Catalysis, Kinetic Properties, and Activation by Ascorbic Acid. Despite what has been observed for some plant myrosi-

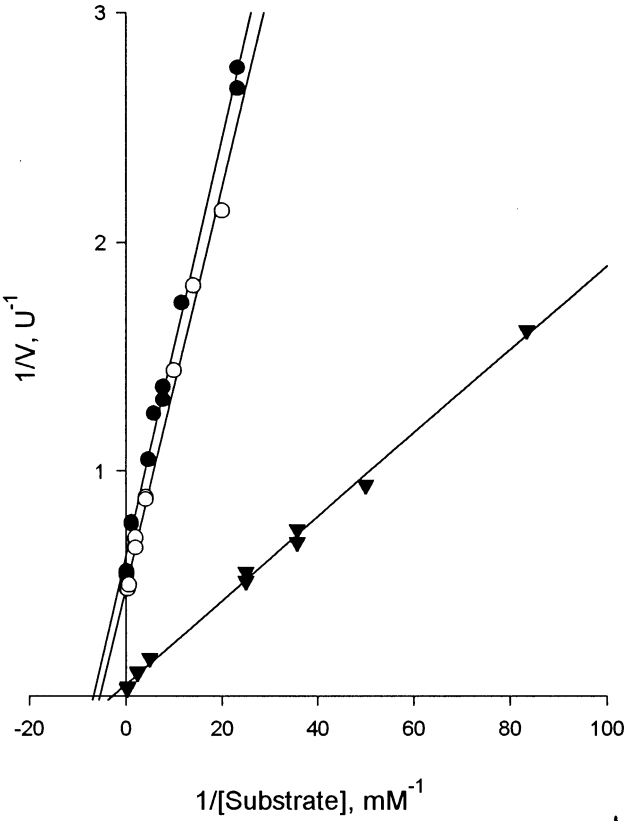


Figure 4. Double-reciprocal plots of crambe myrosinase-catalyzed hydrolysis of sinigrin (○), progoitrin (●), and *epi*-progoitrin (▼) as substrates at pH 6.5 and 37 °C.

Table 4. Kinetic Parameters of MYRc and MYRm without (A) and with (B) Ascorbic Acid

	K_m (μM)		V_{max} (units/mg)		V_{max}/K_m	
substrate	MYRc	MYRm	MYRc	MYRm	MYRc	MYRm
(A) Without Ascorbic Acid						
sinigrin	170	170[a]	8	60[a]	0.047	0.35
progoitrin	140	230	6.3	70	0.045	0.30
epi-progoitrin	240	220	54	75	0.22	0.34
(B) With Ascorbic Acid						
sinigrin	nd[d]	400[ac]	nd	240[a<c]	nd	0.60[c]
progoitrin	1100[b]	965[c]	133[b]	428[c]	0.12[b]	0.44[c]
epi-progoitrin	430[b]	752[c]	200[b]	512[c]	0.46[b]	0.68[c]

[a] Björkman, R.; Lönnerdal, B. *Biochim. Biophys. Acta* **1973**, *327*, 121–131. [b] 20 mM ascorbic acid. [c] 1 mM ascorbic acid. [d] Not determined.

nases, MYRc did not require ascorbic acid to be detectable. Thus, the steady-state kinetic study of this enzyme was carried out without this activator, used in other experiments, essentially to determine the activation level of the hydrolysis-catalyzed reaction trends with the two diastereoisomeric substrates.

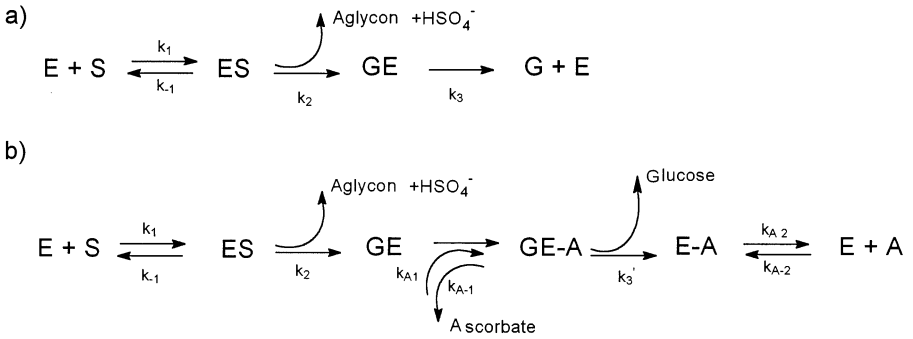
The Lineweaver−Burk plot (**Figure 4**) clearly shows that the slopes of PRO and sinigrin isotherms are very similar and much higher than that determined with *e*PRO. The reaction velocity of *e*PRO MYRc-catalyzed hydrolysis visibly stands out above the others, thus indicating a special behavior of MYRc with this substrate. The kinetic data, reported in **Table 4A**, show that the K_m of MYRc calculated for the three substrates is practically of the same magnitude, whereas the V_{max}, determined with *e*PRO as substrate, is on average almost 8 times higher than the V_{max} determined with the other two substrates. The kinetic properties of MYRc are intriguing, especially when this enzyme is compared with MYRm, the most studied myrosinase, often considered the reference enzyme. The data reported in **Table 4** lead to the following conclusions. Taking into account that MYRm displays a similar activity when *e*PRO, PRO, and other glucosinolates are used as substrates, the higher activity that MYRc shows with *e*PRO could be explained by assuming better stabilization of this substrate in the MYRc active site, which, however, should be structurally different from that of MYRm. If the above consideration is correct, at least one residue of the active site should be different and located in the hydrophobic pocket where docking of the glucosinolate side chain takes place. Because the only difference between PRO and *e*PRO is the side-chain spatial structure due to location of the hydroxyl group, it is reasonable to think that an additional stabilizing interaction between MYRc and its natural substrate (*e*PRO) occurs, for instance, through hydrogen bonding with the hydroxyl group of this glucosinolate and a suitable residue with a polar chain in the hydrophobic pocket. This mutation would decrease the general hydrophobicity of the pocket without jeopardizing the releasing capacity of the aglucons, which were formed during the myrosinase-catalyzed hydrolysis. Finally, a previous paper reported that the residues that constitute the hydrophobic pocket of MYRm, Ile^{257},Tyr^{330}, Phe^{331}, Phe^{371}, and Phe^{473}, are strongly conserved in nine other myrosinase sequences with the exception of Phe^{331}. The latter amino acid was substituted by a valine in *B. napus* and *Arabidopsis thaliana* and by a serine in the cyanogenic β-glucosidase of *Trifolium repens*, having physicochemical characteristics similar to those of myrosinase (*5*, *38*). Therefore, it is reasonable to think that the active site sequence of MYRc can also be modified and that the modification involves Phe^{331}.

Ascorbate Activation. The altered characteristics of the hydrophobic pocket in MYRc could also explain the different behavior of this enzyme with PRO and *e*PRO in the presence of ascorbate. In fact, Burgmeister et al. (*38*) demonstrated that, during ascorbate-activated catalysis, a portion of ascorbate is located in the hydrophobic pocket and in this way can positively affect the release of the aglycon part of glucosinolates.

It is well established that the myrosinase-catalyzed reaction, like those catalyzed by glycosyl hydrolases, proceeds with a three-step mechanism, the deglycosylation step being the rate-limiting one, which is activated by ascorbate (see **Scheme 2a**) (*38*). The latter, when it is present, interacts with glycosylmyrosinase, and MYRc activation increases up to a plateau for a wide range of ascorbate concentrations (data not shown), thus indicating a change in the rate-determining step of the general myrosinase-catalyzed reaction for both substrates (PRO and *e*PRO). Using *e*PRO as a substrate in the presence of ascorbate, the V_{max} of MYRc is still almost 2 times higher than that observed with PRO (**Table 4B**). This reveals a positive influence on k_1 and k_2, which apparently reflects a better stabilization of the MYRc−*e*PRO complex. Nevertheless, the question of whether a further stabilizing interaction occurs between the enzyme and its natural substrate or not remains open. To shed light on this, a final experiment was set up to compare the MYRc activity using the same epimeric mixture of glucoisatisins as substrate, isolated from woad (*Isatis tinctoria)* ripe seeds.

Epimeric Mixture of Glucoisatisins. Like PRO and *e*PRO, glucoisatisins contain an asymmetric carbon that provides *R* and *S* structures (*39*). In both cases, the hydroxyl group was esterified with a 2-oxoindolyl acetate, thus preventing further stabilization of the enzyme−substrate complex by hydrogen bonding as observed with PRO (see **Table 1**). With the epimeric mixture of glucoisatisins we obtained almost the same MYRc

Scheme 2. Reaction Mechanism of Myrosinase-Catalyzed Hydrolysis in the Absence (a) and Presence (b) of Ascorbic Acid[a]



[a] E, enzyme; S, substrate; G, glucose; GE, *g*lucosyl enzyme; A, ascorbate.

Table 5. Specific Activity of MYRc and MYRm Determined with Epimeric Mixtures of (*R*,*S*)-Glucoisatisins and (*R*,*S*)-Progoitrins

epimeric mixture	MYRc (units/mg)	MYRm (units/mg)
glucoisatisins	4 ± 0.2	72 ± 0.3
progoitrins[a]	34 ± 0.2	68 ± 0.3

[a] Expressly made by mixing progoitrin and *epi*-progoitrin 1:1

activity observed with pure PRO as substrate, measuring the reaction velocity with both the SCEA and the pH-stat method. Further trials carried out with MYRc and MYRm using a 1:1 mixture of PRO and *e*PRO as substrate demonstrated beyond doubt the special function of the hydroxyl group in the natural *e*PRO substrate in MYRc-catalyzed hydrolysis (**Table 5**). In fact, whereas with MYRm the activity data obtained with the two-glucosinolate mixtures were very similar, for MYRc these data were negatively affected by the esterification of the hydroxyl group in glucoisatisins mixture and by the presence of PRO in the other mixture.

Concluding Remarks. The glucosinolate−myrosinase system stands out as one of the most important enzymatic plant structures. Nevertheless, despite the significance of this enzymatic system and its modulation in vivo in the Brassicaceae defense against biotic stress, there is still little information about its action at the molecular level. In this study, working with pure MYRc and its purified natural substrates, we made a further attempt to fill this gap. In initial studies we demonstrated that in defatted crambe meal MYRc naturally contained in this material is mostly insoluble and specific for its native substrate, *e*PRO. We also found that the properties of soluble MYRc are very similar to those of the insoluble one, suggesting that these enzymatic forms are the same enzyme molecule in a different physical state. We have further established that, compared to other myrosinases, MYRc stands out for its unusual molecular properties (high molecular mass, high number of subunits, and alkaline p*I*) and maintains its high specificity toward *e*PRO. In addition, working with the epimeric mixture of *R* and *S* glucoisatisins, where the hydroxyl group in the side chain is esterified with an indolyl group, we have produced additional evidence suggesting that further hydrogen bonding between the hydroxyl group of *e*PRO and a "mutated" residue in the MYRc active site can occur. Determination of the three-dimensional structure of MYRc by X-ray crystallography will validate this hypothesis. This finding, however, does not fully explain the special kinetic behavior of MYRc. The effect of a third factor, for instance, an MYRc cofactor naturally present in crambe seed, is also possible. Its removal or deactivation would affect the reaction rate of MYRc-catalyzed hydrolysis and also explain the low purification yield of this enzyme and its low specific activity. Finally, we believe that this work not only answers some important biochemical questions of the MYRc−*e*PRO system but also highlights the importance of this enzymatic structure in crambe physiology and protection.

ABBREVIATIONS USED

MYRc, crambe myrosinase; MYRm, white mustard myrosinase; *e*PRO, *epi*-progoitrin; PRO, progoitrin.

ACKNOWLEDGMENT

We are grateful to Cebeco Handelsraad, Rotterdam, The Netherlands, for crambe seeds (cv. Cebeco 9402). We particularly thank Prof. Nicolas Fabre for the generous gift of the epimeric mixture of glucoisatisins.

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Received for review July 18, 2002. Revised manuscript received December 30, 2002. Accepted January 22, 2003.

JF020796G