Mechanism-Based Inhibition and Stereochemistry of Glucosinolate Hydrolysis by Myrosinase[†]

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ABSTRACT: Myrosinase is a particular glucosidase which hydrolyzes a variety of plant 1-thio-β-D-glucosides known as the glucosinolates. This enzyme, which is the only glycosidase able to hydrolyze these naturally occurring thioglucosides, has been found previously to display strong sequence similarities with family 1 *O*-glycosidases. Myrosinase therefore offers the opportunity to compare the mechanism of enzymatic cleavage of *S*- vs *O*-glycosidic bonds. The stereochemistry of hydrolysis of sinigrin by *Sinapis alba* myrosinase was followed by ^{1}H NMR and the enzyme was found to operate with a mechanism retaining the anomeric configuration at the cleavage point exactly like the related *O*-glycosidases found in family 1. Myrosinase was readily inactivated by 2-deoxy-2-fluoroglucotropaeolin with inactivation kinetic parameters of $K_i = 0.9$ mM and $k_i = 0.083$ min^{-1}. Reactivation kinetic parameters were determined in buffer only, with $k_{react} = 0.015$ h^{-1} and $t_{1/2} = 46$ h, and also in the presence of acceptors of transglycosylation. No significant changes were observed in the presence of methyl β-D-glucoside, but with azide anion the half-life of reactivation was found to be reduced to $t_{1/2} = 20$ h. These results suggest that myrosinase inhibition by 2-deoxy-2-fluoroglucotropaeolin occurs via the accumulation of a long-life glucosyl-enzyme intermediate and that the catalytic machinery of the enzyme is composed of only one catalytic residue, a nucleophilic glutamate, while the acid catalyst residue found in the corresponding *O*-glycosidases is missing.

Glucosinolates constitute a large family of plant anionic 1-thio-β-D-glucosides found particularly in Cruciferae and which differ by substituents on the aglycon part of the substrate (Fenwick *et al*., 1983). Myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) is the only known glycosidase able to degrade these thioglucosides, resulting in the production of glucose, isothiocyanates, thiocyanates, and nitriles which cause thyroid and liver diseases to animals and humans during the ingestion of glucosinolate-rich food such as rape (*Brassica napus*) (McDanell *et al*., 1988).

Myrosinase from *Sinapis alba* is a dimeric glycoprotein with a molecular weight of 135 kDa and a p*I* of 5.1 (Pessina *et al*., 1990). The gene encoding the enzyme has been cloned and sequenced (Xue *et al*., 1992), and the deduced amino acid sequence of the protein has been shown to display strong similarities with family 1 of *O*-glycosidases, indicating that myrosinase probably shares the same $(\beta/\alpha)_8$ barrel fold (Henrissat *et al*., 1995). This suggests that like these *O*-glycosidases, myrosinase should operate with overall retention of the anomeric configuration, a molecular mechanism of hydrolysis involving a double displacement reaction through a glucosyl-enzyme covalent intermediate (McCarter & Withers, 1994). Detailed analysis of residue conservation has suggested that only one of the two residues constituting the usual catalytic machinery of family 1 *O*-glycosidases, namely, the nucleophilic Glu-426 of myrosinase, is present while the acid/base glutamic acid is replaced by a glutamine (Henrissat *et al*., 1995; Wang & Withers, 1995).

The aim of this work was to evaluate the predictions based on sequence analysis about the sterochemical course and the mechanism of the reaction catalyzed by myrosinase using ^{1}H NMR and kinetic studies *via* mechanism-based inhibition.

MATERIALS AND METHODS

General. *Sinapis alba* myrosinase was a generous gift from Professor S. Palmieri (Pessina *et al*., 1990). Sinigrin (allyl glucosinolate, Figure 1a) and 4-nitrophenyl β-D-glucopyranoside (PNPG)[1] were from Sigma. 2-Deoxy-2-fluoroglucotropaeolin (2-FGTL, Figure 1b) was synthesized from 2-deoxy-2-fluoroglucose *via* a route which will be published elsewhere (S. Cottaz, P. Rollin, and H. Driguez, unpublished results).

NMR Determination of Reaction Stereochemistry. Myrosinase (2.5 μg) was dissolved in 1 mL of 99.8% D_2O. After lyophilization, the enzyme was resuspended in 0.1 mL of 99.95% D_2O. Similarly, sinigrin (1.7 mg) was exchanged three times by lyophilization from 0.4 mL of 99.8% D_2O, redissolved in 0.4 mL of 99.95% D_2O, and placed in a 5-mm NMR tube. The stereochemistry of the hydrolysis reaction was determined by ^{1}H NMR performed at 303 K in a Bruker AC 300 spectrometer equipped with a ^{1}H/^{13}C 5-mm probe and operating at 300 MHz. After the spectrum of the substrate was recorded, 0.1 mL of D_2O-exchanged myrosinase was added to the tube, which was immediately placed back in the spectrometer. The first spectrum was recorded 10 min after the addition of the enzyme, and then spectra were recorded every 30 min for 2 h.

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[1] Abbreviations: PNPG, 4-nitrophenyl β-D-glucopyranoside; 2-FGTL, 2-deoxy-2-fluoroglucotropaeolin.

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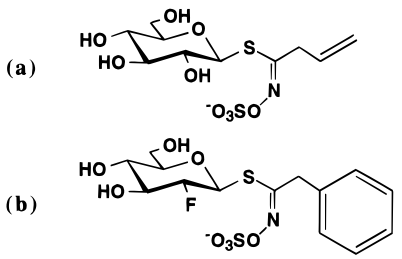

FIGURE 1: (a) Sinigrin (allyl glucosinolate); (b) 2-deoxy-2-fluoroglucotropaeolin (2-FGTL).

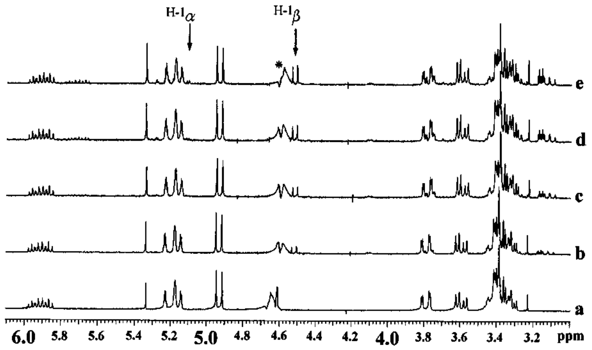

FIGURE 2: Time course of hydrolysis of sinigrin catalyzed by myrosinase, followed by ^{1}H NMR. (a) Spectrum recorded before addition of the enzyme; (b−e) spectra recorded 10, 30, 60, and 120 min after addition of the enzyme. H-1$_\alpha$ and H-1$_\beta$ indicate the resonances of H-1 of α- and β-D-glucose, respectively. An asterisk indicates the residual peak of HDO.

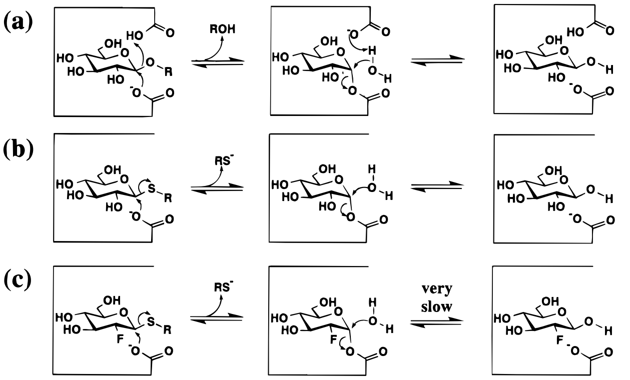

FIGURE 3: Postulated molecular mechanism for (a) retaining β-glucosidase with a *O*-β-D-glucoside; (b) myrosinase with a glucosinolate; (c) myrosinase with a 2-deoxy-2-fluoroglucosinolate.

Enzyme Kinetics. All kinetics were performed at 39 °C, pH 7, in a 0.35 mM sodium phosphate buffer. Myrosinase activity was determined by using either sinigrin (Palmieri *et al.*, 1982) or PNPG (Botti *et al.*, 1995) as substrates, by monitoring the variation of absorbance at 227 and 430 nm, respectively ($\epsilon = 6784$ M^{-1}·cm^{-1} for sinigrin, $\epsilon = 7002$ M^{-1}·cm^{-1} for 4-nitrophenol). Time-dependent inactivation and reactivation of myrosinase were determined as described previously by Withers and co-workers for other glycosidases [Miao *et al.* (1994) and references cited therein]. The inactivation of myrosinase by 2-FGTL was followed by incubation of the enzyme (13.5 μg·mL^{-1}) under the above conditions in the presence of different concentrations (0, 0.125, 0.25, 0.5, and 1 mM) of 2-FGTL. Residual enzyme activity was monitored at appropriate intervals of time by withdrawal of an aliquot (0.1 mL) from the inactivation mixture, addition to a solution of sinigrin (1.4 mL, 0.1 mM) in the sodium phosphate buffer, and measurements of the decrease of absorbance. Hydrolysis of sinigrin was found to be pseudo-first-order; the inactivation rate constant (k_i) and the equilibrium constant for the inhibitor (K_i) were determined by fitting the observed rate constant (k_{obs}) to

$$k_{obs} = k_i[I]/(K_i + [I])$$

where I = 2-FGTL. The reactivation of inhibited myrosinase (2.7 mg·mL^{-1}) was studied after inactivation by 2.6 mM 2-FGTL for 18 h at 40 °C, followed by several ultrafiltrations on Nanosep 30 kDa (Pall Filtron Corp.) to remove the excess inactivator. Reactivation was monitored by withdrawing aliquots (20 μL) from the solution of inactivated enzyme (0.27 mg·mL^{-1}) at appropriate intervals of time, and the samples were assayed as above. First-order rate constants (k_{react}) were determined by fitting the recovered activity *vs* time to a first-order reaction equation.

RESULTS AND DISCUSSION

Stereochemistry of the Hydrolysis Reaction Catalyzed by Myrosinase. Figure 2a shows the ^{1}H NMR spectrum of the substrate sinigrin before addition of myrosinase. The HDO signal at 4.60 ppm was decreased by selective irradiation at the appropriate frequency. Spectra recorded 10, 30, 60, and 120 min after the addition of the enzyme are shown in Figure 2b−e. After 10 min of reaction, a doublet centered at 4.51 ppm ($J = 8$ Hz) assignable to H-1 of free β-glucose appeared, together with peaks between 3.08 and 3.18 ppm, assignable to allyl isothiocyanate. After 60 min of reaction, the doublet centered at 4.51 ppm has grown, and at the same time, a smaller doublet centered at 5.10 ppm ($J = 3.7$ Hz) corresponding to H-1 of free α-glucose has appeared. After 2 h the ratio between the β- and the α-anomer was 1/0.20, compared to the ratio of 1/0.57 for glucose at mutarotation equilibrium. The presence of α-glucose is therefore due to the mutarotation of the β-glucose initially produced. β-Glucose being the anomer produced during hydrolysis of sinigrin, this demonstrates that *S. alba* myrosinase operates with overall retention of the anomeric configuration, as predicted from the sequence similarity between myrosinase and family 1 *O*-glycosidases (Henrissat *et al.*, 1995). Not only the stereochemical course of the reaction but also the molecular mechanism of myrosinase has been predicted to be analogous to the β-glucosidases, which belong to the same family (Figure 3a) (Henrissat *et al.*, 1995; Iori *et al.*, 1996). This mechanism involves a covalent glycosyl-enzyme intermediate which should be trapped using a suitable 2-fluoroglucosinolate inhibitor by analogy with the work of Withers and co-workers (Figure 3b,c) (Withers & Street, 1988; Street *et al.*, 1992).

Inactivation of S. alba Myrosinase. 2-FGTL has been synthesized as a potential enzyme-activated inhibitor because the corresponding substrate, glucotropaeolin, has a better affinity for myrosinase with a K_m of 75 μM (Iori *et al.*, 1996) than sinigrin with a K_m of 156 μM (Palmieri *et al.*, 1982). Incubation of myrosinase with 2-FGTL resulted in time-dependent inactivation of the enzyme according to pseudo-first-order kinetics (Figure 4a). Pseudo-first-order rate constants (k_{obs}) were determined at each inactivator concentration, allowing the determination of the inactivation parameters $k_i = 0.083$ min^{-1} and $K_i = 0.9$ mM (Figure 4b).

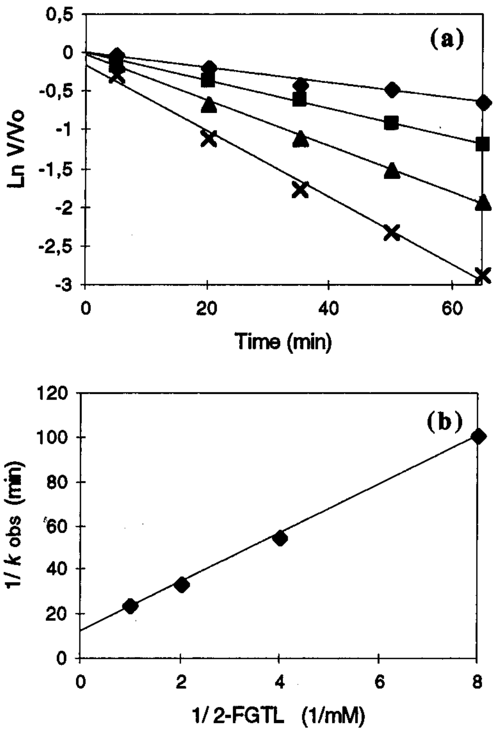

FIGURE 4: Inactivation of *S. alba* myrosinase by 2-FGTL. (a) Semilogarithmic plot of residual activity *vs* time at the following inactivator concentrations: (◆) 0.125 mM; (■) 0.25 mM; (▲) 0.5 mM; (×) 1 mM. (b) Replot of first-order rate constants from panel a *vs* 2-FGTL concentrations.

These results suggest that inactivation of myrosinase is a consequence of accumulation of a long-life 2-deoxy-2-fluoroglucosyl-enzyme covalent intermediate similar to those reported for several β-glycosidases by Withers and co-workers (Withers *et al.*, 1987; Miao *et al.*, 1994; Ziser *et al.*, 1995).

In order to confirm that inactivation of myrosinase arises through stabilization and trapping of the glucosyl-enzyme intermediate, the reactivation of the inhibited enzyme was studied as described below.

Reactivation of Inactivated Myrosinase. The catalytic competence of inactivated myrosinase was determined after removal of the excess inhibitor from a solution of labeled enzyme. The reactivation was monitored by assaying the recovery of activity resulting from the regeneration of the free enzyme. Incubation in buffer only at 40 °C resulted in the spontaneous reactivation of the enzyme according to pseudo-first-order kinetics (Figure 5). By fitting of the data to a first-order rate equation, the reactivation rate constant of $k_{\text{react}} = 0.015\ \text{h}^{-1}$ could be calculated, corresponding to a half-life ($t_{1/2}$) of 46 h. The effects of nucleophiles other than water in the reactivation process were also studied. It has been demonstrated with other enzymes that reactivation can be considerably accelerated by transglycosylation to a sugar acceptor [Miao *et al.* (1994) and other examples cited therein]. By incubating the reactivation mixture in the presence of 200 mM methyl β-D-glucoside, no significant increase in the reactivation rate was observed (Figure 5). This result is in agreement with other observations, indicating that, contrary to most β-glycosidases acting with retention of configuration, myrosinase does not catalyze transglycosylation on glycosyl acceptors (Botti *et al.*, 1995). By incubating the labeled enzyme in the presence of 200 mM sodium azide, the half-life of reactivation is reduced by a factor of 2.3, with $t_{1/2} = 20$ h and $k_{\text{react}} = 0.035\ \text{h}^{-1}$ (Figure 5). Since anions such as azide have been reported to be excellent competitors with water for reaction with glycosyl-enzyme intermediates (MacLeod *et al.*, 1994; Huber & Chivers, 1993), this suggests that myrosinase can tranglycosylate to acceptors that do not require activation by a catalytic amino acid. This suggestion confirms the sequence analysis of myrosinase predicting that this enzyme holds only one of the two usual catalytic residues, namely, the glutamic acid acting as the nucleophile, while the acid/base catalytic residue is replaced by a glutamine (Henrissat *et al.*, 1995). Two related explanations have been proposed for the lack of the latter in myrosinase: (i) the aglycon of glucosinolates being a sufficiently good leaving group, the nucleophile alone would be sufficient to catalyze the hydrolysis reaction (Henrissat et al., 1995) or (ii) the sulfate group carried by the aglycon of glucosinolates would act as the acid catalyst and provide substrate-assisted aglycon departure (Wang & Withers, 1995). The half-life of the 2-deoxy-2-fluoroglucosyl-myrosinase intermediate (46 h) appears sufficiently long to allow the determination of the three-dimensional structure of the covalent intermediate by X-ray crystallography and will perhaps help examining the validity of these hypotheses. This work is currently under progress.

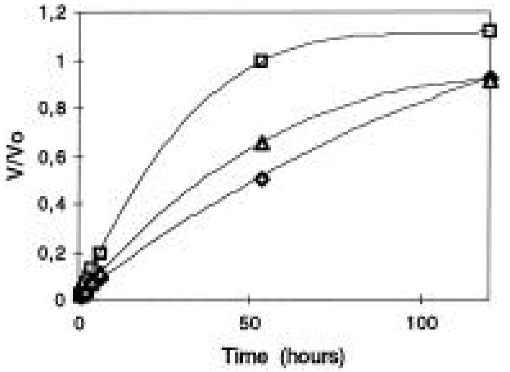

FIGURE 5: Reactivation of 2-fluoroglucosyl myrosinase by various nucleophiles: (◇) buffer only; (△) 200 mM methyl β-D-glucoside; (□) 200 mM sodium azide. Plot shows recovered activity *vs* time.

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