

Enzymic Properties of Purified Myrosinase from *Lepidium sativum* Seedlings

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Z. Naturforsch. **45c**, 173–178 (1990); received September 9, 1989

Lepidium sativum, Thioglucoside Glucohydrolase, Myrosinase, Substrate Specificity, Glucosinolate Degradation

To establish the substrate specificity of the thioglucoside glucohydrolase myrosinase (EC 3.2.3.1), this enzyme was purified to homogeneity from light-grown cress (*Lepidium sativum* L.) seedlings by Sephadex gel filtration, Red Dye and anion exchange (FPLC Mono Q) chromatography, and preparative isoelectric focusing. Hydrolytic activity was shown toward only 4 of the 29 synthetic and natural O- and S-glycosides tested. Highest activity was displayed with the endogenous glucosinolates benzylglucosinolate (K_m , 295 μ M) and sinigrin (K_m , 300 μ M) at an optimum pH of 5.5 in sodium citrate buffer. The synthetic glycosides PNPG (K_m , 2.0 mM) and ONPG were poorer substrates at an optimum pH of 6.5 in potassium phosphate buffer. The enzyme was inactive with all other nitrophenyl glycosides tested including PNP- α -D-glucoside and PNP-thio- β -D-glucoside, suggesting a requirement for O- β -D-glucose as the glycone moiety within these substrates. PNPG hydrolysis was stimulated 2.6-fold by ascorbate (1 mM). The enzyme exhibited no metal ion requirement and was strongly inhibited by lead nitrate, mercury chloride, and ferric chloride at 1 mM concentration. The metal chelators DIECA, EDTA, *o*-phenanthroline, and 2,2'-dipyridyl were not inhibitory, but the thiol reagents PCMS, PCMB, and N-ethylmaleimide (at 1 mM) caused 50–80% inhibition of enzyme activity.

Introduction

Originally known as mustard oil glucosides, the glucosinolates are hydrophilic, non-volatile thioglucosides found within all crucifers including the important crops oilseed rape, cabbage, mustard and cress [1]. Glucosinolate-containing plants generally possess the thioglucoside glucohydrolase (EC 3.2.3.1) commonly referred to as myrosinase. Upon plant injury or during food processing, this enzyme catalyzes the rapid hydrolysis of glucosinolates to D-glucose and a multitude of physiologically active products (e.g. isothiocyanates, thiocyanates, organic nitriles and oxazolidine-2-thiones) [2]. The latter not only contribute to the distinctive flavor and aroma characteristic of crucifers but may also have undesirable effects in animal feedstuffs due to their pungency and goitrogenic activity [3, 4].

Abbreviations: EDTA, ethylenediamine tetraacetic acid; FPLC, fast protein liquid chromatography; IEF, isoelectric focusing; PCMB, *p*-chloromercuribenzoate; PCMS, *p*-chloromercuriphenyl sulfonic acid; ONPG, *o*-nitrophenyl- β -D-glucoside; PNP-, *p*-nitrophenyl-; PNPG, *p*-nitrophenyl- β -D-glucoside; SDS-PAGE, Na dodecylsulfate polyacrylamide gel electrophoresis.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/90/0300–0173 \$ 01.30/0

With glucosinolates (and their hydrolysis products) being implicated in interactions between crucifers and their potential herbivores, pathogens, competitors and symbionts [2], the need has become more acute for basic information about the pathways of glucosinolate metabolism and their regulation. For many cruciferous species, gathering this knowledge is hindered by the fact that a single plant may contain as many as 15 different glucosinolates [4]. Light-grown cress (*Lepidium sativum* L.) seedlings constitute a notable exception, since shortly after germination they contain significant levels (1.2 mg/g fresh weight) of a single glucosinolate, benzylglucosinolate, with lesser amounts of allylglucosinolate and 2-phenethylglucosinolate [5]. Due to its biochemical simplicity, cress has proven to be an excellent system for purifying and characterizing enzymes involved in glucosinolate metabolism. In 1988, we reported that cell-free extracts of cress seedlings catalyzed the sulfation of desulfobenzylglucosinolate and desulfoallylglucosinolate after endogenous myrosinase activity had been removed by Concanavalin A-Sepharose 4B chromatography [6]. More recently, we described the purification of cress myrosinase to apparent homogeneity and its potent competitive inhibition by the indolizidine alkaloid castanospermine [7].



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In past years, the use of synthetic rather than natural substrates during the purification and characterization of relatively crude but highly active β -glycosidase preparations has encouraged the widely accepted view that these enzymes lack aglycone specificity [8]. This viewpoint was recently challenged by the isolation of plant β -glycosidases having pronounced aglycone specificities toward particular cyanogenic glycosides, flavonoids, phenols, alkaloids, and steroids [9, 10]. Whether myrosinase displays such strict specificity towards either aglycone or sugar moieties of potential glycosidic substrates remains virtually unknown, since all studies to date have either utilized impure enzyme preparations [11–15] or have focused solely upon its reactivity towards different glucosinolates [12, 16].

In the present study, the substrate specificity of homogeneous cress myrosinase was probed by testing its activity towards a wide range of synthetic and naturally occurring S- and O-glycosides. In addition, we describe effects of metal ions, chelators and other reagents on myrosinase activity.

Materials and Methods

Plant materials

Lepidium sativum L. (No. 5089, Curled Cress) seeds were purchased from Park Seed Co. (Greenwood, SC). Seedlings were grown in artificial medium containing one part vermiculite and one part Jiffy Mix (W. R. Grace Co., Cambridge, MA) at 21 °C under continuous illumination and harvested after 5 days.

Chemicals and biochemicals

Chromogenic and fluorogenic substrates, sinigrin, bovine serum albumin, and thiol and chelating reagents were purchased from Sigma Chemical Co. Benzylglucosinolate was a generous gift from Dr. Schraudolf (University of Ulm, West Germany).

Enzyme purification

Cress myrosinase was extracted in imidazole buffer and purified to homogeneity as previously described [7] excepting that DEAE-cellulose chromatography was replaced by FPLC as follows. The unbound proteins obtained from Reactive

Red 120-Agarose chromatography were pooled and applied to a Pharmacia Mono Q HR 5/5 anion exchange column pre-equilibrated with 20 mM imidazole-HCl, pH 6.2 (buffer A). After washing extensively with buffer A to remove unbound proteins, bound proteins were eluted using a linear gradient (20 ml) of 40 mM to 225 mM NaCl in buffer A. Fractions (1 ml) were collected and assayed for myrosinase activity. Active fractions were pooled, desalted by chromatography on a Sephadex G-25 column (44 × 2.5 cm) equilibrated with buffer A, and subjected to isoelectric focusing using a Bio-Rad Rotofor Preparative IEF Cell and Pharmalyte ampholyte carriers (pH 4.5–5.4, 2% (v/v) final concentration) as described previously [7]. Focused fractions (approx. 2 ml) were assayed for myrosinase and α -mannosidase activities. Only myrosinase fractions lacking α -mannosidase activity were pooled for subsequent gel filtration on a Sephadex G-25 column (44 × 2.5 cm) with buffer A to remove the ampholyte carriers. Fractions (3 ml) containing purified myrosinase were pooled and stored at 4 °C in buffer A with 0.04% (w/v) sodium azide.

Enzyme assays

In common with some plant and fungal myrosinases [14, 17], the *L. sativum* enzyme showed activity towards PNPG and the glucosinolates benzylglucosinolate and sinigrin. These activities will henceforth be referred to as “PNPGase” and “thioglucosidase” activities, respectively. The simpler PNPGase assay was utilized during enzyme purification; this seemed justifiable after identical elution profiles were revealed by both assay procedures following isoelectric focusing and chromatography on DEAE-cellulose, FPLC Mono Q, and Concanavalin A-Sepharose 4B columns. Furthermore, the ratio of activities towards PNPG and sinigrin remained constant (1.1–1.4) throughout the purification.

Glycosidic activity towards glucosinolates and other non-chromogenic substrates was determined as follows. Assays contained 0.5 μ mol substrate, 50 μ mol sodium citrate buffer (pH 5.5), and 50–100 μ l enzyme preparation in a total volume of 0.5 ml. After incubation at 30 °C for 20–60 min, reactions were terminated by the addition of 0.04 ml 2.9 M HCl. Aliquots (0.5 ml) were re-

moved, and glucose production was measured by the Sigma glucose oxidase-peroxidase procedure as described elsewhere [18]. With exception of α -mannosidase assays, standard assays for chromogenic substrates contained 7 μ mol glycoside, 100 μ mol potassium phosphate buffer (pH 6.5), and 50 μ l enzyme preparation in a total volume of 1 ml. After incubation with the enzyme at 30 °C for 15–60 min, the reaction was terminated by addition of 2 ml 5% (w/v) sodium carbonate. The developed colors were measured at 400 nm. α -Mannosidase activity was assayed at pH 5.5 in sodium citrate buffer (100 mM concentration) as described above but replacing PNPG by PNP- α -D-mannoside (7 μ mol). The potential hydrolysis of 4-methylumbelliferyl sugars was monitored as described elsewhere [19].

All myrosinase assays were performed in duplicate, and the values shown represent the mean of both trials. Control incubations were routinely included in which active enzyme was replaced by enzyme preparations previously inactivated by boiling for 10 min.

Protein estimation

Protein estimation was performed according to Bradford [20], using crystalline bovine serum albumin as standard.

Results and Discussion

Enzyme purification

In the current study, myrosinase was purified to homogeneity from light-grown cress seedlings by Sephadex gel filtration, Red Dye and anion-exchange (FPLC Mono Q) chromatography, and preparative isoelectric focusing. As judged by silver-stained SDS-PAGE gels, homogeneity was

thereby achieved after an overall 13.7-fold purification of myrosinase with 36% recovery of activity (Table I). Mono Q FPLC replaced the DEAE-cellulose chromatographic step in our original purification protocol [7] due to its rapidity, superior resolution, and equivalent yields. Furthermore, since the pooled FPLC fractions could be subjected directly to isoelectric focusing after appropriate dilution, the need for gel filtration after anion-exchange chromatography [7] was obviated.

Kinetic properties

The purified myrosinase preparation retained >95% of its activity after 1 week and >90% after 1 month when stored at 4 °C in buffer A containing 0.04% (w/v) sodium azide. In contrast, all enzyme activity was lost when the purified preparation was stored overnight at –20 °C. Similar sensitivity to freezing was found with myrosinases from *Sinapis alba* and *Brassica napus* [16].

The pH optimum of the purified protein was determined by assaying hydrolytic activity toward sinigrin and PNPG in several buffer systems (Fig. 1). Thioglucosidase activity (toward sinigrin) was highest throughout the pH range 5.0–7.5 with a maximum in sodium acetate buffer at pH 5.5. At this pH, the rate of glucose production was linear with the amount of purified enzyme preparation added up to 3.6 μ g protein and in the range of 0–60 min incubation times. The purified enzyme exhibited PNPGase activity over a broad pH range (pH 5–9) with an optimum in potassium phosphate buffer at pH 6.5 (Fig. 1). The rate of *p*-nitrophenol formation was linear at this pH with respect to added amount of purified enzyme up to 4.5 μ g protein and in the range of 0–45 min incubation times.

Table I. Purification of myrosinase from cress seedlings.

Purification step	Volume [ml]	Protein [mg]	Activity ^a [U]	Specific Activity [U/mg]	Recovery [%]	Purification [-fold]
Sephadex G-25	38.0	77.5	478	6.2	100	1
Red Dye	51.0	19.7	497	25.2	104	4.1
FPLC Mono Q	47.5	6.18	470	76.1	98	12.3
IEF	19.2	2.30	210	91.3	44	14.7
Sephadex G-25	45.0	2.03	171	84.6	36	13.7

^a One unit of enzyme activity is defined as 1 μ mol *para*-nitrophenol formed h^{–1}.

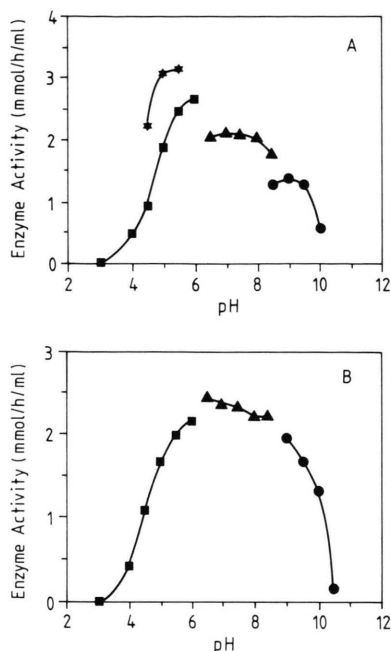


Fig. 1. Effect of pH on the hydrolysis of sinigrin (A) and PNPG (B) by cress myrosinase. The buffer systems used were: sodium citrate (★), potassium phosphate (▲), sodium acetate-HCl (■), and glycine-NaOH (●).

Although myrosinase has been purified from a limited number of plant and fungal species [17, 21], its substrate specificity has not been examined in depth. Focusing upon this point, the substrate specificity of homogeneous cress myrosinase was assessed by measuring its ability to cleave a variety of naturally occurring and synthetic glycosides. The glucose oxidase-peroxidase procedure was used to monitor the hydrolysis of glucosinolates and other non-chromogenic substrates, while the catabolism of chromogenic and fluorogenic substrates was followed spectrophotometrically. Of the 29 O- and S-glycosides tested, activity was observed in only four cases (Table II). Highest activity was recorded with the endogenous thioglucosides benzylglucosinolate and sinigrin, which were readily hydrolyzed to similar extents by this enzyme. These results agree with Björkman and Lönnerdal [16] who showed that myrosinase isoenzymes from *S. alba* and *B. napus* hydrolyzed all six glucosinolates tested at comparable rates. The fact that the only other substrates degraded by the cress enzyme were the synthetic glycosides PNPG and ONPG demonstrated its high degree of agly-

cone specificity. These compounds were hydrolyzed at 18% and 2%, respectively, of the rate observed with sinigrin. In contrast, the purified *Aspergillus sydowi* thioglucosidase exhibited lesser aglycone specificity, being active towards sinigrin, PNPG, salicin, arbutin, phenyl- β -D-glucoside and cellobiose [17]. Whether this wider substrate specificity reflects a major difference between fungal and plant myrosinases or contamination by other glycosidases remains in question.

Substrate	Relative activity ^a
Sinigrin	100
Benzylglucosinolate	102
<i>p</i> -Nitrophenol- β -D-glucoside	18
<i>o</i> -Nitrophenol- β -D-glucoside	2

^a Expressed as a percentage of sinigrase activity (100% = 70 μ mol/h/mg protein).

The commercially available *p*- and *o*-nitrophenylglycosides provided important information concerning how cress myrosinase responds to variations in the nature and linkage of the sugar moiety within these glycosidic substrates. While PNPG was hydrolyzed efficiently, activity was not observed with other β -linked PNP sugars. The necessity for an O- β -D-glucosidic linkage was further suggested by its inactivity toward PNP- α -glucoside and PNP-thio- β -D-glucoside. The failure of the purified enzyme to hydrolyze PNP- β -D-mannoside, PNP-N-acetylglucosaminide and PNP-phosphate demonstrated that the α -mannosidase, N-acetylhexosaminidase and phosphatase activities, which co-occur in cress seedling homogenates, had been successfully removed by this purification protocol.

The apparent K_m values of purified cress myrosinase were 300 μ M for sinigrin (pH 5.5), 295 μ M for benzylglucosinolate (pH 5.5), and 2.0 mM for PNPG (pH 6.5), as determined by the Lineweaver

and Burk method [22]. The corresponding V_{\max} values were 123 $\mu\text{mol/h/mg}$ protein (sinigrin), 204 $\mu\text{mol/h/mg}$ (benzylglucosinolate), and 86 $\mu\text{mol/h/mg}$ enzyme (PNPG), respectively. For other myrosinases, Michaelis constants ranged from 30–360 μM for sinigrin [16, 17] and equalled 2 mM for PNPG [14]. The foregoing values were determined in the absence of ascorbic acid, which increases both K_m and V_{\max} when sinigrin served as substrate [21]. Since the glucose oxidase-peroxidase assay used here is completely inhibited at ascorbate concentrations normally required for enzyme activation (0.1–1.0 mM), its effect upon the thioglucosidase activity of cress myrosinase could not be assessed. However, in contrast to a previous report [14], ascorbate (1 mM) increased PNPG hydrolysis 2.6-fold.

The effect of metal ions, metal chelators, and other reagents on myrosinase activity was investigated using the PNPGase assay. Enzyme activity was not stimulated by any metal ion tested when present at 1 mM concentration (Table III). Indeed, the enzyme was severely inhibited by lead nitrate, mercury chloride, and ferric chloride and moderately inhibited by copper sulfate and magnesium chloride. That the cress enzyme lacks a metal requirement was confirmed by the failure of the metal chelators EDTA (5 mM), diethyldithiocarbamate (5 mM), *o*-phenanthroline (1 mM) and 2,2'-dipyridyl (1 mM) to reduce enzyme activity. By comparison, several salts (*e.g.* Na_2SO_4 , K_2SO_4 , $(\text{NH}_4)_2\text{SO}_4$) increased the activity of yellow mus-

Table IV. Effect of sulfhydryl reagents and reducing compounds on myrosinase activity.

Compound	Concentration [mM]	% Activity ^a
PCMS	1.0	29
N-Ethylmaleimide	1.0	40
PCMB	1.0	48
Iodoacetamide	1.0	98
Iodoacetic acid	1.0	98
β -Mercaptoethanol	5.0	103
Dithiothreitol	5.0	144

^a Expressed as a percentage of PNPGase activity in the absence of thiol reagents.

tard myrosinase by 30–50% [23]. The *Wasabia japonica* myrosinase also was stimulated 20–50% by several monovalent and divalent cations but was strongly inhibited by 1 mM HgCl_2 (93%) and CuCl_2 (100%) and the metal chelators EDTA and *o*-phenanthroline (43% and 87% at 10 mM, respectively) [24].

Specific reagents which modify particular amino acids have implicated sulfhydryl groups as being essential for catalytic action of plant but not fungal myrosinases. The *B. juncea* myrosinase activity was completely inhibited by 0.1 mM PCMB [25, 26], but only 50% inhibition of the *W. japonica* enzyme was realized at 1 mM [24]. Likewise, purified cress myrosinase experienced approximately 50–70% loss of activity in the presence of 1 mM PCMB, PCMS or N-ethylmaleimide (Table IV). When myrosinase was pre-incubated with PCMS for 30 min, enzyme activity was inhibited by 80% of control values (data not shown). However, the reagents iodoacetamide and iodoacetic acid were not inhibitory, perhaps due to steric hindrance. In contrast to plant myrosinases, the fungal enzyme from *A. sydowi* was insensitive to PCMB and N-ethylmaleimide [27]. The reducing agent dithiothreitol caused 44% stimulation of cress myrosinase at 5 mM concentration, but β -mercaptoethanol was without effect.

Acknowledgements

We are grateful to the University of Iowa Graduate College for financial support. We also thank Dr. H. Schraudolf (University of Ulm) for his kind gift of benzylglucosinolate and Linda Donohoe for assistance in manuscript preparation.

Table III. Effect of metal ions on myrosinase activity.

Compound	Concentration [mM]	% Activity ^a
ZnCl_2	1.0	101
CoSO_4	1.0	99
NaCl	1.0	98
KCl	1.0	98
CaCl_2	1.0	97
MnCl_2	1.0	92
MgCl_2	1.0	69
CuSO_4	1.0	69
FeCl_3	1.0	26
$\text{Pb}(\text{NO}_3)_2$	1.0	1
HgCl_2	1.0	0

^a Expressed as a percentage of PNPGase activity in the absence of metal ion.

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