An unusual case of 'uncompetitive activation' by ascorbic acid: purification and kinetic properties of a myrosinase from *Raphanus sativus* seedlings

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Myrosinase (thioglucoside glucohydrolase; EC 3.2.3.1) is a plant enzyme that hydrolyses glucosinolates, principally to isothiocyanates. Myrosinase was purified to homogeneity in good yield from 8-day-old seedlings of *Raphanus sativus* (daikon) using a four-step procedure involving chromatographies on anion exchange, hydrophobic Phenyl-Sepharose, gel filtration and concanavalin A–Sepharose. In order to stabilize the enzyme and to avoid excessive peak broadening during chromatography, 30% (v/v) glycerol was added to dialysis and chromatography buffers. The purified enzyme was eluted as a single peak from a gel-filtration sizing column with an apparent molecular mass of 120 kDa. The enzyme was resolved into two subunits with molecular masses of 61 and 62 kDa by SDS/PAGE. Ascorbic acid activated the purified enzyme more than 100-fold. The V_{max} and K_m values for the hydrolysis of allyl glucosinolate (sinigrin) were 2.06 μmol/min per mg of protein and 23 μM in the absence of ascorbate and 280 μmol/min per mg of protein and 250 μM in the presence of 500 μM ascorbate, respectively. As the ascorbate concentration was increased from 50 to 500 μM, the V_{max} and K_m values increased in parallel, and thus the V_{max}/K_m ratio remained constant. Similarly, raising the concentrations of sinigrin increased the concentration of ascorbic acid required for half-maximal activation (K_a). At a sinigrin concentration of 250 μM, the K_a for ascorbic acid was 55 μM. Sulphate, a reaction product, was a competitive inhibitor of activity, having a K_i of 60 mM with respect to sinigrin and of 27 mM with respect to ascorbate. Thus activation of myrosinase from *R. sativus* by ascorbic acid exemplifies an unusual and possibly unique example of linear 'uncompetitive activation' (i.e. a proportionate increase in V_{max} and K_m) of an enzyme. The enzyme also had β-glucosidase activity and hydrolysed *p*-nitrophenyl-β-D-glucopyranoside.

Key words: glucosinolate, isothiocyanate, sinigrin, β-thioglucoside glucohydrolase.

INTRODUCTION

The enzyme myrosinase (thioglucoside glucohydrolase; EC 3.2.3.1) is found in a variety of edible plants and catalyses the hydrolysis of secondary plant metabolites known as glucosinolates. Glucosinolates are β-thioglucoside *N*-hydroxysulphates, and the aglycone (or R group) is an alkyl, alkenyl, thioalkyl, thioalkenyl, aryl, arylalkyl or indolyl moiety. More than 100 glucosinolates have been isolated from 16 families of dicotyledonous plants, which also contain myrosinase [1]. This enzyme is normally physically segregated from the glucosinolates, but when plant cells are damaged (e.g. during food preparation, mastication or injury by predators such as insects) the enzyme is released and catalyses their hydrolysis to yield isothiocyanates principally, and also glucose and sulphate. The reaction involves an initial hydrolysis of the β-thioglucoside linkage with release of β-D-glucose [2]. The resultant thiohydroxamate intermediate (for which spectroscopic evidence has been obtained) releases sulphate and then undergoes a non-enzymic intramolecular Lossen rearrangement to give rise to isothiocyanates, as shown in Scheme 1.

Isothiocyanates display diverse and interesting biological properties. Some are hepatotoxic or goitrogenic, whereas others have anti-bacterial, anti-fungal, anti-protozoal, nematocidal and/or anti-carcinogenic activites (for reviews, see [1,3–7]). Recent interest has focused on the cancer-preventive properties of isothiocyanates [4,8–10], which have been ascribed to induction of phase-2 xenobiotic-metabolizing enzymes, e.g. glutathione transferases and NAD(P)H:quinone reductase, and inhibition of cytochromes P450-dependent activation of certain carcinogens.

Myrosinase has been purified and characterized from several sources, including white mustard (*Sinapis alba*) [11,12], cress (*Lepidium sativum*) [13], yellow mustard (*Brassica juncea*) [14], rape seed (*Brassica napus*) [15] and wasabi (*Wasabia japonica*) [16]. It has also been reported to occur in the vegetative tissue of *Raphanus sativus* (daikon or Japanese radish) [17,18]. The enzyme is a glycoprotein and usually exists as a dimer with subunit molecular masses of 60–75 kDa [11,19], although exceptions have been reported [14,16]. Recently, myrosinase from *S. alba* was crystalized and its X-ray structure determined, and it was found to be a zinc-containing enzyme [20].

An interesting and puzzling feature of most plant myrosinases is that they are strongly activated by ascorbic acid, as well as by several close structural relatives, but not by its oxidation product, dehydroascorbic acid [21,22]. The mechanism of this activation is clearly not dependent on the reductive properties of ascorbic acid and remains poorly understood. Ettlinger and co-workers [21] have suggested that a ternary complex between myrosinase, glucosinolate and ascorbic acid is formed and that the catalytic function of ascorbate depends on the generation of a singly charged enolate base that facilitates the addition of water.

In the present study, myrosinase was purified to homogeneity in high yield from a previously overlooked source, seedlings of *R. sativus*. The use of seedlings avoids the major disadvantages of seeds, i.e. their high lipid content, which complicates the purification procedure, and their highly variable myrosinase content.

Abbreviations used: DTT, dithiothreitol; NPG, *p*-nitrophenyl-β-D-glucopyranoside.

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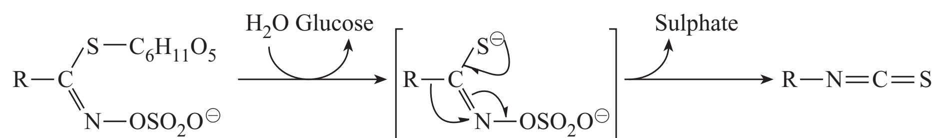

Scheme 1 Enzymic conversion of glucosinolates to isothiocyanates by myrosinase

An efficient procedure for obtaining purified myrosinase is needed for studies on the mechanism of the anti-carcinogenic effects of glucosinolates and isothiocyanates [10].

Highly purified daikon myrosinase described in this paper was almost totally inactive in the absence of added ascorbic acid. Activation by ascorbate showed the unique characteristics of linear 'uncompetitive activation', i.e. increases in both the K_m and V_{max} values for the glucosinolate substrate without changes in the V_{max}/K_m ratio. Although increases in both K_m and V_{max} of the enzyme from *S. alba* have been noted previously [23], to our knowledge this type of kinetic behaviour has not been characterized or designated previously as 'uncompetitive activation'.

EXPERIMENTAL

Materials

Sinigrin and methyl-α-D-mannopyranoside were from Sigma (St. Louis, MO, U.S.A.). L-Ascorbic acid, D-glucose, potassium sulphate and other reagents were from J. T. Baker (Phillipsburg, NJ, U.S.A.). Concanavalin A–Sepharose, Q-Sepharose Fast Flow and Phenyl-Sepharose Fast Flow were from Pharmacia (Piscataway, NJ, U.S.A.) and CentriPrep-10 concentrators were from Amicon (Beverly, MA, U.S.A.). *R. sativus* L. (daikon) seeds were from Dover Sales (Piedmont, OK, U.S.A.).

Growth of seedlings

Seeds were plated on sterile 1 % agar in trays at a density of about 20 seeds/cm² and allowed to grow at 25 °C by day/20 °C by night under cool-white fluorescent light on a 16:8 h light/dark cycle. After 7–8 days, seedling trays were frozen at −80 °C *in situ*, and aerial portions of the plants were collected and stored at −80 °C in 50-g portions. This rapid deep freezing prevented substantial myrosinase action on endogenous glucosinolates.

Myrosinase assay

Myrosinase activity was determined by measuring the rate of decrease in absorbance at 227 nm resulting from the hydrolysis of sinigrin [24]. The 1.0-ml reaction mixture contained (unless otherwise stated): 33 mM potassium phosphate buffer, pH 6.0, 150 μM sinigrin, 500 μM ascorbic acid and 1 mM EDTA. The reaction was initiated by the addition of 1–5 μl of enzyme and run for 3–5 min at 25 °C. Because of an initial lag of 0.5–1.0 min before the reaction velocity became linear, the rate was determined from the linear portion of the assay curve. Enzymic activity (1 unit) was defined as that which hydrolysed 1 μmol of sinigrin per min. A molar extinction coefficient of $\epsilon = 6780\ M^{-1} \cdot cm^{-1}$ [24] for sinigrin at 227 nm was used. Protein concentrations were determined by the bicinchoninic acid method [25] with BSA as a standard. Specific activities are expressed as units/mg of protein.

The β-glucosidase activity of myrosinase was assayed by measuring the liberation of *p*-nitrophenol from the substrate *p*-nitrophenyl-β-D-glucopyranoside (NPG) at 25 °C. The reaction mixture contained 50 mM Tris/phosphate buffer, pH 8.5, 1 mM EDTA, 500 μM ascorbate, 25 mM NPG and 0.005 % BSA in a volume of 1 ml. The reaction was initiated by the addition of enzyme and the increase in absorbance was monitored at 402 nm. A molar extinction coefficient for *p*-nitrophenol of $\epsilon = 17700\ M^{-1} \cdot cm^{-1}$ was used.

Velocity measurements used to derive kinetic parameters were, in most cases, based on multiple determinations. Agreement of the values with Michaelis–Menten predictions was checked by linear-regression calculations and found to be satisfactory. For example, for the data shown in Figure 4 (see below), the analysis for all data shown gave *r* values of > 0.995 and the slopes were all parallel within 95 % confidence limits.

Purification of myrosinase

Preparation of crude extracts

The small-scale crude extracts used in the initial activity screen were prepared in 0.5 M NaCl/20 mM potassium phosphate, pH 7.4, by grinding the plants in a glass homogenizer and centrifuging the homogenates at 1000 ***g*** for 3 min to remove particulate material.

The large-scale extracts ultimately used for enzyme purification were prepared by placing 50 g of frozen daikon seedlings (grown for 8 days) in a chilled (−20 °C) ceramic mortar and pulverizing under liquid nitrogen (total volume about 1 litre). The pulverized material was transferred to a glass beaker, allowed to warm to about 0 °C and then 100 ml of 10 mM imidazole/HCl (pH 6.0) containing 1 mM EDTA and 1 mM dithiothreitol (DTT) were added. This suspension was stirred for 30 min at 0 °C, and then centrifuged for 15 min at 10000 ***g*** at 4 °C. The supernatant fraction was maintained at 4 °C, filtered through glass wool and concentrated using CentriPrep-10 concentrators. The concentrate was dialysed exhaustively at 4 °C against 10 mM imidazole/HCl, pH 6.0, containing 1 mM EDTA, 1 mM DTT and 30 % (v/v) glycerol, which reduced the volume to about 50 ml.

Purification procedure

The dialysate was recentrifuged to remove insoluble material and applied to an open 2.5 cm × 9.0 cm column of Phenyl-Sepharose Fast Flow that had been equilibrated with the above imidazole/EDTA/DTT/glycerol buffer at 25 °C. The unadsorbed fraction (60 ml) was collected and then applied to a 2.5 cm × 7.5 cm Pharmacia FPLC column of Q-Sepharose Fast Flow, also at 25 °C, which had been equilibrated with the same imidazole/EDTA/DTT/glycerol buffer. After washing exhaustively with the starting buffer, the column was eluted with a 100-ml linear gradient of 10–100 mM imidazole/HCl, pH 6.0, containing EDTA, DTT and glycerol, as described above. Myrosinase activity was eluted at 80–100 mM imidazole, and the pooled main activity peak (30 ml) was concentrated to about 10 ml by means of CentriPrep-10 concentrators. The concentrated enzyme solution was dialysed against 20 mM Tris/HCl (pH 7.4) containing 0.5 M NaCl, 1 mM DTT and 30 % glycerol and applied

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to an open 1.2 cm × 0.7 cm column of concanavalin A–Sepharose at 4 °C. The column was washed with 10 ml of the same Tris/NaCl/DTT/glycerol buffer and then eluted with the same buffer, also containing 250 mM methyl-α-D-mannopyranoside. The flow rate was maintained at less than 2 ml per h. The active fractions were pooled (15–17 ml), concentrated to 2–3 ml in CentriPrep-10 concentrators and dialysed against 100 mM imidazole/HCl, pH 6.0, containing 1 mM EDTA, 1 mM DTT, 0.5 M NaCl and 50 % glycerol. This solution was diluted to 30 % glycerol and applied in 0.2-ml aliquots to a 1 cm × 30 cm Superose 12 column (Pharmacia) equilibrated with 10 mM imidazole/HCl, pH 6.0, containing 1 mM EDTA, 1 mM DTT, 0.5 M NaCl and 30 % glycerol. Activity was eluted at a constant flow rate of 0.1 ml/min. The purified enzyme was concentrated and then stored in small aliquots at −80 °C. Full activity has been retained for more than 2 years.

Analytical electrophoresis

Slab-gel electrophoresis was carried out at 25 °C in a Mini Protean apparatus (Bio-Rad, Hercules, CA, U.S.A.). For analysis of native enzyme, a separating gel was prepared with 7.5 % polyacrylamide and pre-run extensively with 75 mM Tris/HCl, pH 8.9, containing 1 mM mercaptoacetic acid. The stacking gel (2 % polyacrylamide in Tris/phosphate, pH 6.6) was polymerized using riboflavin. After electrophoresis at 4–8 mA for 100 min, one portion of the gel was stained with Coomassie Brilliant Blue R-250, and the other portion was incubated at room temperature for 5–10 min in 40 mM sodium acetate buffer, pH 6.0, containing 12 mM sinigrin, 0.5 mM ascorbic acid, 1 mM EDTA and 50 mM $BaCl_2$. The sulphate liberated by the enzymic reaction produced an easily visible precipitate of barium sulphate in the gel [15].

SDS/PAGE was carried out in the same apparatus by using a 10 % separating gel and a 5 % stacking gel, essentially according to the procedure of Laemmli [26], except that the separating and stacking gel buffers were Tris/phosphate. The proteins were visualized by staining with Coomassie Brilliant Blue R-250.

RESULTS

Preliminary screening of plant sources

In order to determine the most suitable source of seedlings for the preparation of myrosinase, crude extracts of 7-day-old seedlings of 18 different Cruciferae, including broccoli, daikon, Chinese cabbage, cress, mustard greens, mustard seed and arugula were screened initially for enzymic activity (results not shown). Daikon seedlings had the highest activity and, together with their low seed cost and low oil content, were chosen for the large-scale purification of myrosinase. Maximal specific activity in crude daikon extracts was attained at about 7–8 days after seeding (results not shown).

Table 1 Purification of the major species of myrosinase from 50 g of 8-day-old *R. sativus* (daikon) seedlings

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Initial extract	1789	1253	0.70	1.00	100
Phenyl-Sepharose	839	1093	1.34	1.91	87
Q-Sepharose	29.3	788	26.8	38.4	63
Concanavalin A–Sepharose	6.64	558	84.1	120	45
Superose 12	3.76	617	164	234	49

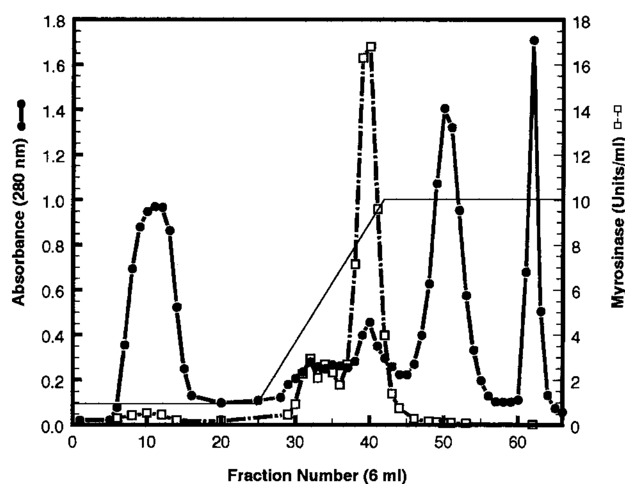

Figure 1 Typical chromatography of partially purified myrosinase on a Q-Sepharose Fast Flow ion-exchange column (25 mm × 75 mm)

The enzyme preparation applied to this column was the flow-through fraction obtained from the Phenyl-Sepharose column. The 43-ml sample applied contained 400 units of enzyme activity and 307 mg of protein (specific activity = 1.30 units/mg of protein). The enzyme was eluted with a gradient of imidazole/HCl from 10 to 100 mM, pH 6.0, also containing 1 mM EDTA, 1 mM DTT and 30% glycerol. The recovery of enzyme activity in the major peak (fractions 37–42) was 288 units at a specific activity of 26.7 units/mg of protein. Fractions of 5.0 ml were collected and the flow rate was 1.0 ml/min.

Purification and characterization of myrosinase

Myrosinase from 50 g of 8-day-old daikon seedlings was purified to homogeniety as described in the Experimental section. A typical purification procedure is summarized in Table 1. Myrosinase was purified approx. 230-fold to a final specific activity of 164 units/mg of protein with a yield of 49 %.

During purification, evidence was obtained for multiple forms of the enzyme. After chromatography on Phenyl-Sepharose Fast Flow, almost all of the myrosinase activity was recovered in the unadsorbed fraction. In subsequent ion-exchange chromatography on Q-Sepharose Fast Flow, a minor peak containing 22 % of the activity was eluted at 40–70 mM, in addition to the major peak (72 % of the activity) at 80–100 mM imidazole-HCl (Figure 1). The minor peak may have represented an additional form of the enzyme, which has been described previously for myrosinase from *S. alba* [11]. Only 4.5 % of the total enzyme activity eluted in the void volume.

Gel-filtration chromatography on Superose 12 showed a single protein peak, containing nearly 100 % of the applied activity, eluting at 1.63 void volumes. The apparent molecular mass of this species was 120 kDa. Its shape and location were virtually identical to that of the myrosinase activity peak, suggesting that at this stage the preparation was nearly homogeneous.

Purity of the preparation was also confirmed by electrophoresis on a 7.5 % native polyacrylamide gel. A single sharp band with a relative migration rate of 0.19 was detected after Coomassie Brilliant Blue staining. A single band was also detected at precisely this relative mobility on a duplicate gel stained for enzymic activity by the barium chloride method [15].

When the purified enzyme was subjected to SDS/PAGE and stained with Coomassie Brilliant Blue, two closely adjacent bands were observed; the more slowly migrating component

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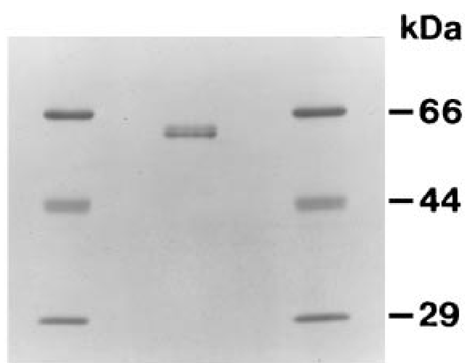

Figure 2 SDS/PAGE of 3 μg of purified myrosinase from 8-day-old daikon seedlings

Details are described in the Experimental section. Standards were BSA (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa).

(62 kDa) stained less intensely than the more rapidly migrating band (61 kDa; Figure 2).

Kinetic properties and activation by ascorbic acid

In the absence of ascorbic acid, the purified enzyme hydrolysed sinigrin only very slowly, with a maximum velocity (V_{max}) of 2.06 μmol/min per mg of protein, as determined by Lineweaver–Burk plots; the rate of hydrolysis was related linearly to enzyme concentration (Figure 3). The Michaelis–Menten constant (K_m) for sinigrin was 23 μM in the absence of ascorbic acid.

In the presence of ascorbic acid, enzymic activity was enhanced greatly. With increasing ascorbic acid concentration, the slopes of the Lineweaver–Burk plots remained constant and the *y* intercept decreased (Figure 4); thus ascorbic acid increased both V_{max} and K_m values. The V_{max} rose to 215 μmol/min per mg of protein and the K_m value to 250 μM, at a near-saturating concentration (500 μM) of ascorbic acid, when measured using an enzyme concentration of 0.093 μg/ml.

In view of this unusual kinetic behaviour, it became essential to establish that the measured reaction velocities were strictly proportional to enzyme concentrations, irrespective of ascorbic acid concentration. When the assay was repeated with a range of enzyme concentrations (0.024, 0.048, 0.096 and 0.143 μg/ml), the initial velocities under maximal activation by ascorbic acid (500 μM) were 8.9, 15.2, 25.6 and 40.0 nmol/min, respectively. Reaction velocity was therefore directly proportional to the enzyme concentration, with a calculated specific activity of 280 μmol/min per mg of protein. The double-reciprocal plots for different concentrations of the enzyme all had the same *x* intercept, indicating that the K_m value for sinigrin was also unaffected by enzyme concentration (Figure 5, with only the highest and lowest protein concentrations plotted). The reaction velocity was also proportional to enzyme concentration at ascorbic acid concentrations of 100 and 200 μM. Table 2 presents a summary of the kinetic constants for the enzyme.

The order of addition of the activator and the substrate to the enzymic reaction did not change the magnitude of the activation. When ascorbic acid was added to a reaction already in progress, the rate increased rapidly and reached the same level as that attained by the addition of enzyme to a reaction mixture containing both ascorbic acid and sinigrin. Prior incubation of the enzyme with 500 μM ascorbic acid for 5, 10 or 20 min in the

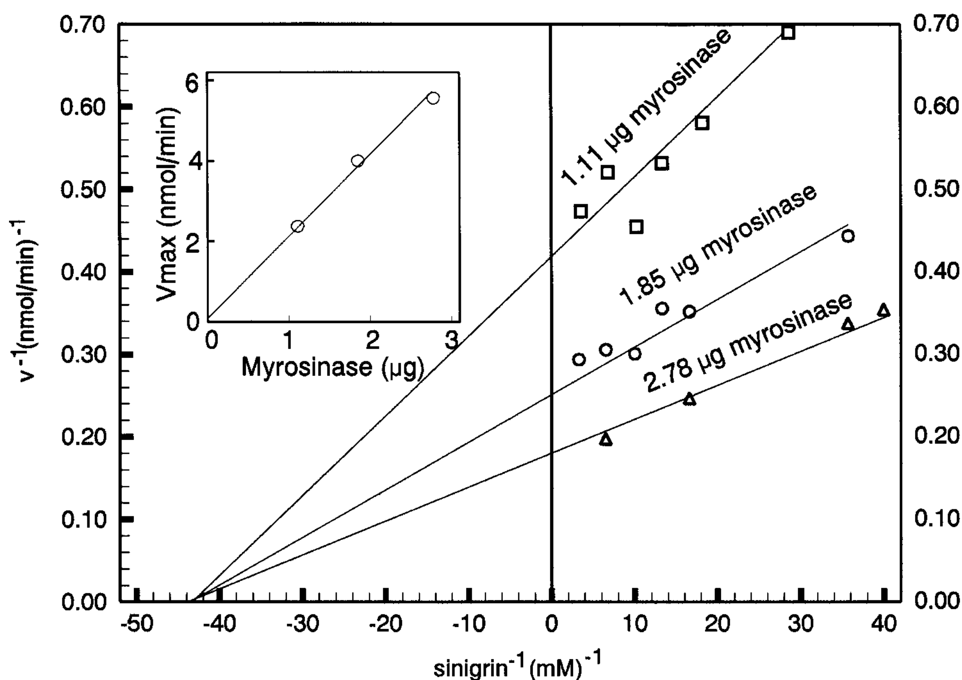

Figure 3 K_m and V_{max} values for sinigrin determined with purified myrosinase in the absence of added ascorbic acid

Double-reciprocal plots of initial velocity with respect to sinigrin concentration are shown. The quantities of purified myrosinase in the 1.0-ml assay system are indicated above each curve. Inset, V_{max} as a function of the amount of enzyme in the assay.

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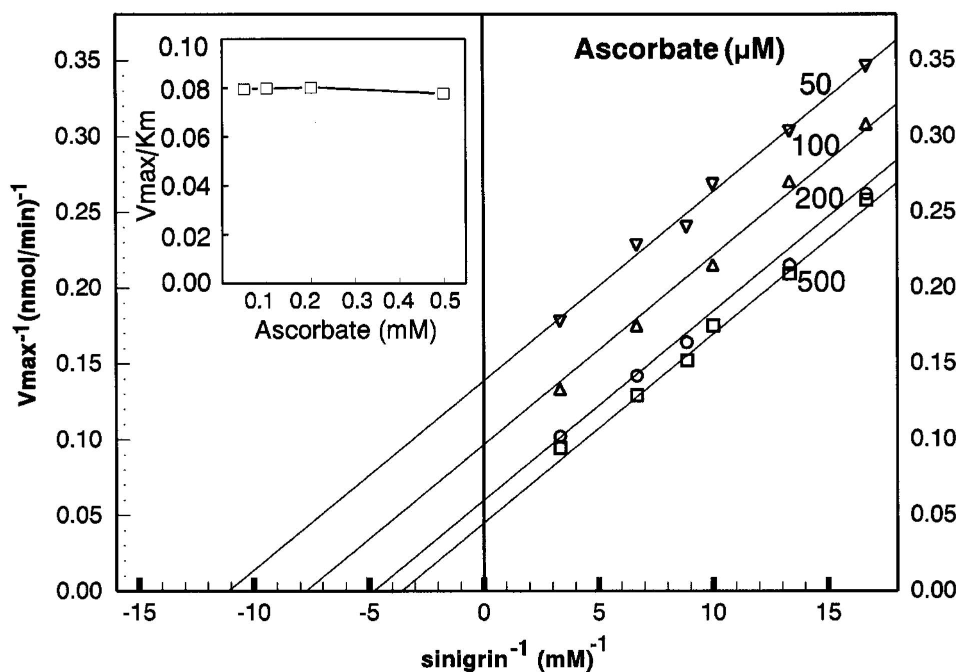

Figure 4 Effect of ascorbic acid concentration on the K_m and V_{max} values for sinigrin in the myrosinase reaction

The enzyme was incubated in a constant concentration of 0.093 µg/ml with 60, 75, 100, 113, 150 or 300 µM sinigrin in the presence of 50, 100, 200 or 500 µM ascorbic acid. Inset, V_{max}/K_m ratio as a function of ascorbic acid concentration.

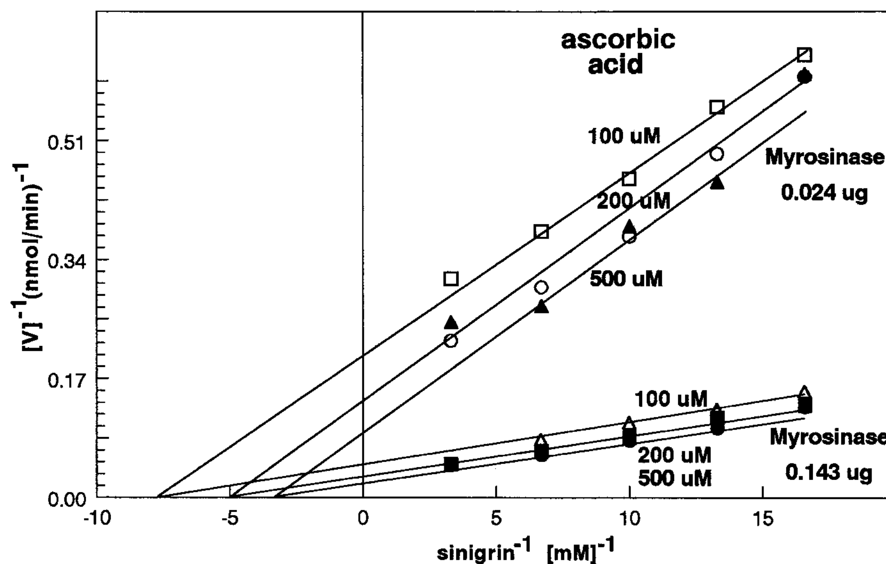

Figure 5 V_{max} under maximal activation by ascorbic acid as a function of enzyme concentration

Myrosinase (0.024, 0.048, 0.096 or 0.143 µg) was incubated in 1-ml assay solution containing 60, 75, 100, 150 or 300 µM sinigrin and 100, 200 or 500 µM ascorbic acid as indicated. For simplicity, only the data for the enzyme concentrations of 0.024 and 0.143 µg/ml are presented here.

absence of sinigrin resulted in similar levels of activation. Furthermore, the size of the shoulder of the reaction curve (i.e. the small time-lag that occurs before the reaction proceeds linearly) was not affected by prior incubation with the activator.

The results shown in Figure 4 were replotted in order to estimate the concentration of ascorbic acid required for half-maximum activation (K_a; Figure 6). K_a increased concomitantly with V_{max}, rising from 41 to 55 µM with sinigrin concentrations of 150 and 250 µM (the K_m for sinigrin), respectively (Figure 6, inset). The ascorbic acid concentration of 500 µM used in the routine assay was thus more than 10 times greater than the K_a value.

Ascorbic acid concentrations greater than 500 µM inhibited the enzyme; at 150 µM sinigrin, the reaction velocities with 0.75

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Table 2 Kinetic constants of purified daikon myrosinase in the absence and presence of ascorbic acid

The k_{cat} calculation is based on a mean subunit mass of 61.5 kDa and the assumption that there is one active site per subunit.

Treatment	K_m (μM)	V_{max} (μmol/min per mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
None	23	2.06	2.11	0.09
Ascorbate (500 μM)	250	280.0	287.0	1.14

and 1.0 mM ascorbic acid were 90 and 73 % of those with 500 μM ascorbic acid. Sinigrin could not be used at saturating concentrations because of the high initial absorbance of the reaction mixture in the direct spectrophotometric assay.

The purified enzyme was tested for β-glucosidase activity, in both the presence and absence of ascorbate, by using the chromogenic substrate NPG. Low activity was observed in the absence of ascorbate, and the rate was stimulated slightly more than 2-fold by 500 μM ascorbate (when the substrate concentration was 25 mM). However, the β-glucosidase rate at pH 8.5 was still only 1.3 % of the rate of sinigrin hydrolysis at pH 6.0. The K_m and V_{max} values for the chromogenic substrate were 14.0 mM and 1.7 μmol/min per mg of protein, respectively, in the absence of ascorbate. Both parameters increased several orders of magnitude in the presence of 500 μM ascorbate, indicating that ascorbate also behaves as an uncompetitive activator of the β-glucosidase activity of this myrosinase (results not shown). The enzyme was inactive with the thioglucoside analogue of NPG.

Product inhibition

The products of the myrosinase reaction with sinigrin as substrate, i.e. sulphate, glucose and allyl isothiocyanate, were tested for their ability to inhibit enzymic activity. Based on Dixon plots, sulphate inhibited activity competitively with respect to both sinigrin (Figure 7, upper panel) and ascorbate (Figure 7, lower panel), with K_i values of 60 and 27 mM, respectively. These values are the averages of two independent assays for each substrate, i.e. 55 and 65 mM, and 26 and 27 mM, respectively. Glucose was only a very weak inhibitor, with a K_i value above 1 M with respect to sinigrin (results not shown), and allyl isothiocyanate did not significantly inhibit activity at concentrations as high as 1 mM.

DISCUSSION

R. sativus seedlings (8-day-old) provided a convenient source for the purification of myrosinase because their high activity and low lipid content facilitated preparation of the initial extract. Although evidence was obtained for multiple forms of the enzyme, the myrosinase species purified here appears to be the major component of the enzymic activity that is extractable from vegetative tissue in a buffer without glycerol. It apparently belongs to the MA family of myrosinases described by Xue et al. [27].

The purified enzyme appears to be a glycoprotein, because it binds to concanavalin A–Sepharose in a manner similar to that described for other myrosinases [12,28]. Although the carbohydrate content of the purified enzyme was not determined, the sharpness of the bands observed on polyacrylamide gel electrophoresis suggests a low degree of protein glycosylation.

The native molecular mass of the purified enzyme was estimated to be 120 kDa, and it appeared to consist of two subunits, with molecular masses of 61 and 62 kDa. The difference in the apparent molecular masses of the two subunits may reflect differential glycosylation of two identical or similar polypeptides, because the larger peptide stained more weakly with Coomassie Brilliant Blue, which is known to stain glycoproteins less strongly

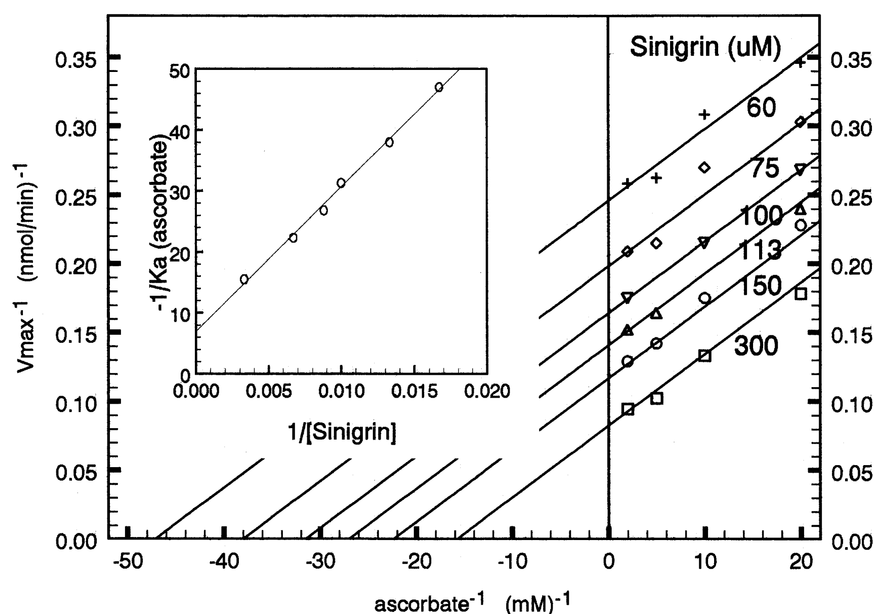

Figure 6 Effect of sinigrin concentration on the ascorbic acid concentration required for half-maximal activation of myrosinase (K_a)

The data shown in Figure 2 were replotted as double reciprocals of velocity with respect to ascorbic acid concentration. Inset, relationship between the K_a and sinigrin concentration.

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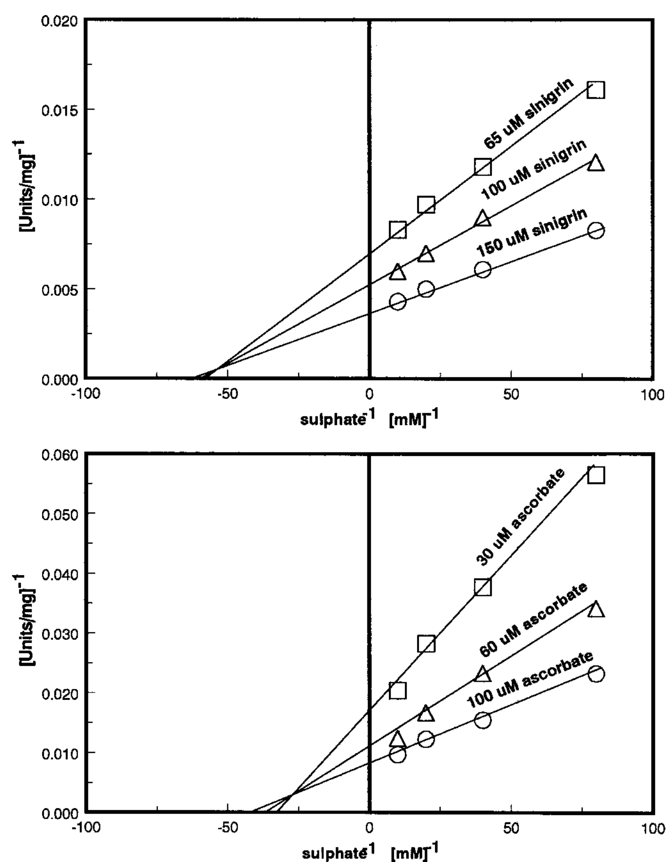

Figure 7 Inhibition of myrosinase by sulphate under standard assay conditions

Dixon plots of reciprocal initial velocity with respect to sulphate concentration with 65, 100 and 150 μM sinigrin and 500 μM ascorbate (upper panel) and concentrations of 30, 60 and 100 μM abscorbate and 150 μM sinigrin (lower panel).

than simple proteins. Myrosinases that have been purified from other plant sources, such as *S. alba* [11,19] and cress seedlings [13], have native and subunit molecular masses similar to those reported here for the daikon enzyme.

The purified enzyme has several interesting kinetic properties. Foremost among them is an almost absolute requirement for ascorbic acid. Ascorbate increased V_{max} from a basal unactivated level of 2.06 to a maximum of 280 μmol/min per mg of protein and simultaneously increased the K_m value for sinigrin from 23 to 250 μM. We have named this unusual phenomenon 'uncompetitive activation', in analogy to 'uncompetitive inhibition', in which an inhibitor decreases V_{max} and K_m values in parallel. Two examples of uncompetitive inhibition are the inhibition of mammalian glucose-6-phosphate dehydrogenase by dehydroepiandrosterone [29] and the inhibition of *myo*-inositol monophosphatase by lithium [30]. The rarity of this type of inhibition has been ascribed to its potentially catastrophic consequences for a metabolic system [31]. Graphically, uncompetitive inhibition is characterized by parallel lines in double-reciprocal plots of velocity with respect to substrate concentration at different inhibitor concentrations. Because both K_m and V_{max} are decreased by a constant amount $(1+[I]/K_i)$, the V_{max}/K_m ratio remains constant. Mechanistically, uncompetitive inhibition has been interpreted as requiring that the inhibitor binds only to the [ES] transition state and not to the free enzyme, and that the [ESI] complex is inactive [32]. By analogy, uncompetitive activation would imply that the activator (A) binds only to the [ES] complex, and that only the [ESA] complex and not the [ES] complex is active. However, in the absence of ascorbate, myrosinase does exhibit low activity, and the V_{max}/K_m ratio is much lower. At present, this anomaly is not understood, and additional experiments are needed to clarify this property.

Most plant myrosinases are activated to some degree by ascorbate. Wilkinson and colleagues [33] reported a range of ascorbate concentrations of 0.7–5.0 mM for maximal activation of myrosinase in partially purified extracts of six crucifers. The enzyme from *S. alba* retains significant activity in the absence of ascorbate and is only activated by about 2–3-fold, whereas the enzymes from several other *Brassica* species show an 8–12-fold activation by ascorbate [23]. The enhancement of myrosinase activity by ascorbate is apparently not due to its action as a reducing agent, since the 2-*O*-methyl derivative is also active, although to a lesser degree than ascorbic acid itself [21]. Other reducing agents, such as 2-mercaptoethanol and DTT, do not stimulate activity [16]. Ohtsuru and Hata [22] cited spectroscopic evidence suggesting that the myrosinase purified from *B. juncea* is conformationally altered by the addition of ascorbic acid. Ettlinger and co-workers, however, concluded that ascorbic acid probably functions as a base or nucleophile [21].

The daikon myrosinase also exhibited β-glucosidase activity, and this property has also been reported for myrosinases from other sources [34,35]. However, the effect of ascorbate on the β-glucosidase activity of other enzymes has been variable. Tsuruo and Hata [35] found that 1 mM ascorbate had no effect on the enzyme(s) from *B. juncea* seeds, whereas Durham and Poulton [34] reported a 2.6-fold stimulation of the activity from *Lepidium sativum* seedlings by the same concentration of ascorbate, and this is similar to the present observations with the daikon enzyme.

Of the products of the myrosinase reaction, only sulphate inhibited activity significantly at the concentrations tested, whereas glucose was not a significant inhibitor, consistent with the work of Botti and co-workers [36]. The sulphate group on the glucosinolate molecule appears to be required for binding to the enzyme, because a desulphoglucosinolate (desulphoglucocapparin) was neither a substrate nor an inhibitor of the ascorbate-activated *S. alba* enzyme [21].

In summary, highly purified myrosinase has been obtained in large quantity and in good yield from 8-day-old seedlings of *R. sativus*. Among the most interesting features of this enzyme is that its activity is linearly uncompetitively activated by ascorbic acid. More research will be required to elucidate the precise mechanism of this activation and its significance in the metabolism of glucosinolates in the intact plant.

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