Biochimica et Biophysica Acta, 327 (1973) 121–131

BBA 67044

STUDIES ON MYROSINASES

III. ENZYMATIC PROPERTIES OF MYROSINASES FROM *SINAPIS ALBA* AND *BRASSICA NAPUS* SEEDS

RUNE BJÖRKMAN AND BO LÖNNERDAL
Institute of Biochemistry, University of Uppsala, Box 531, S-751 21 Uppsala (Sweden)

(Received May 7th, 1973)

SUMMARY

A comparative study was performed on some enzymatic properties of myrosinase isoenzymes (thioglucoside glucohydrolases, EC 3.2.3.1) from white mustard seed *(Sinapis alba)* and rapeseed *(Brassica napus)*. The investigation comprised the determination of pH optima, temperature maxima and the enzymatic stabilities of the isoenzymes, when temperature, pH, buffer and time were varied. The effect of ascorbic acid on the kinetic constants K_m and V was found to be different for the isoenzymes.

Comparative studies were also made of the activities of the isoenzymes on various glucosinolates, and the myrosinase activities in a number of cultivars of *B. napus*, *Brassica campestris* and *S. alba*.

The results indicate that there are both physicochemical and enzymatic differences between myrosinase isoenzymes and that a large amount of variance exists. They are, however, likely to have the same genetic origin. No significant variation in substrate specificity was found among the isoenzymes.

INTRODUCTION

The existence of several myrosinase isoenzymes (thioglucoside glucohydrolases, EC 3.2.3.1) in plants has been demonstrated by gel electrophoresis by Vaughan *et al.*[1], MacGibbon and Allison[2], and Henderson and McEwen[3]. Ettlinger *et al.*[4] reported the separation of two myrosinases from *Sinapis alba*, one of which was activated by ascorbic acid and the other was not. Similar results were obtained by Vose[5]. Tsuruo *et al.*[6] separated two myrosinases from *Brassica juncea* by ion-exchange chromatography but these could not be distinguished by their activation with ascorbic acid.

Previous papers have described the separation, isolation and physicochemical characterization of myrosinase isoenzymes from white mustard seed *(S. alba)*[7] and rapeseed *(Brassica napus)*[8]. These seeds contain at least three and four isoenzymes,

respectively, which all catalyze the hydrolysis of sinigrin. They were found to be glycoproteins with slightly different molecular weights, isoelectric points and carbohydrate content. The present paper describes comparative studies of some of their enzymatic properties, including the effect of ascorbic acid.

MATERIALS

Myrosinase isoenzymes were prepared from white mustard seed *(S. alba)* and from rapeseed *(B. napus)* as described by Björkman and Janson[7] and Lönnerdal and Janson[8], respectively. Three isoenzymes, designated SA, SB, and SC, were separated from white mustard seed. The major component, SC, was obtained in a homogeneous form. From rapeseed, the separation of four isoenzymes, designated RA, RB, RC, and RD was achieved. Furthermore, the major component RC could be separated into three homogeneous components, denoted RC_1, RC_2, and RC_3. Glucosinalbin, glucotropaeolin (tetramethylammonium salts) and glucocapparin were obtained from Roth Laboratoriekemikalien, Karlsruhe, Germany. Glucocheirolin was purchased from Fluka AG, Buchs, Switzerland, and sinigrin from Koch-Light Laboratories Ltd, Bucks., England. Progoitrin was prepared as described by Björkman[9].

Ascorbic acid was obtained from Merck, Darmstadt, Germany, and ^{14}C-labelled ascorbic acid from NEN Chemicals GMBH, Frankfurt am Main, Germany.

The specific glucose reagent Glox was obtained from AB Kabi, Stockholm, Sweden.

METHODS

Assay methods

Myrosinase activities were determined by one of three methods:

(1) Determination of released glucose was performed with a specific glucose reagent, Glox containing glucoseoxidase, peroxidase and *O*-dianisidine. The enzyme assay was carried out as described previously[7]: 1 ml reaction solution, containing 0.5% sinigrin, 0.05 M citrate (pH 5.5) and enzyme was incubated at 40 °C for 30 min. After boiling for 5 min the amount of free glucose was determined. Unfortunately, the Glox reagent is inhibited by ascorbic acid. Assays in the presence of ascorbic acid were therefore performed by the following methods.

(2) Titrimetric determination of formed HSO_4^-. The assays were carried out in an automatic titration apparatus from Radiometer, Copenhagen, Denmark (titrator PMH-28, autoburett ABU 12, recorder SBR 2, titrator assembly TTA 3). The total reaction volume was 2 ml. The ionic strength was held constant with 0.2 M NaCl. Titration was performed with 0.05 M NaOH. On the alkaline side the titration was carried out under N_2.

(3) Spectrophotometric determination of consumed substrate. The decrease in absorbance at 227 nm was measured in a Beckman Acta C III ultraviolet spectrophotometer. The assays were performed at 23 °C in 0.5-cm cuvettes, containing 1.5 ml reaction solution of 0.05 M sodium acetate (pH 5.5), glucosinolate, and enzyme. In some experiments ascorbic acid was added. The reactions were followed for about 15 min on a recorder. Initial reaction rates were calculated from the chart. The sinigrin concentration was calculated according to Beer's law, using $\varepsilon = 7800$.

Stability

The purified myrosinases (SA, SB and SC) and the crude myrosinase preparations from mustard seed (SM) and rapeseed (RM) were stored in 0.05 M buffer solutions at various pH and temperatures for several months. The following 0.05 M buffers were used: citrate (pH 3.0, 4.5 and 6.0), imidazole–HCl (pH 6.0 and 7.5) and Tris–HCl (pH 7.5 and 9.0). The temperatures used were −20, 4, 25 and 37 °C. At certain time intervals (t = 0, 1, 3, 7, 9, 13, 17, 24, 38, 65, 93, 128 and 180 days) the myrosinase activities of all samples were assayed according to the Glox method. No ascorbic acid was present in these experiments.

Equilibrium dialysis

Six dialysis bags, each containing 3 ml of 0.33 mM purified myrosinase SC, were put in E-flasks, each containing 25 ml of a certain concentration of ^{14}C-labelled ascorbic acid ($1 \cdot 10^{-5}$, $2 \cdot 10^{-5}$, $5 \cdot 10^{-5}$, $1 \cdot 10^{-4}$, $2 \cdot 10^{-4}$ and $5 \cdot 10^{-4}$ M ascorbic acid was used). After shaking for two days at 4 °C, the radioactivity in the bags and in the outer solution was measured with a liquid scintillation counter (Nuclear Chicago, Mark I). The ascorbic acid concentrations were determined from a calibration curve. From the differences between the inner and outer bags the equilibrium constant and the number of ligands were calculated.

Preparation of seed extracts

Defatted, finely ground, meal (0.5 g) was extracted with 5 ml of 0.05 M sodium acetate (pH 5.5) for 30 min. After dialysis the extract was centrifuged. 15-, 25- and 50-μl samples were assayed spectrophotometrically with 0.12 mM sinigrin. 25-μl samples were also assayed in the presence of 1 mM ascorbic acid.

RESULTS

pH optima

The influence of pH on the activity of myrosinase SA, SB, SC, RA, RB and RC, as determined by the Glox method, is shown in Fig. 1. The pH dependence was also determined titrimetrically with a pH-stat. In these experiments a broad plateau from pH 4.5 to pH 9 was obtained.

Temperature maxima

The effect of temperature on the myrosinase activities was determined in 0.05 M citrate buffer at the pH optimum for each myrosinase by the Glox method. The reaction mixture, containing substrate, buffer and enzyme was incubated for 30 min at various temperatures. All the curves in Fig. 2 are essentially identical, having their maxima at about 60 °C. At higher temperatures the denaturation of the enzymes is predominant.

Stability

The effect of various pH values on the stability of the enzymes at 4 °C is shown in Fig. 3. At pH 3.0 the activity decreased rapidly. At pH 9.0 the purified enzymes seem to be rather stable, whereas the activity of RM decreased markedly. All the

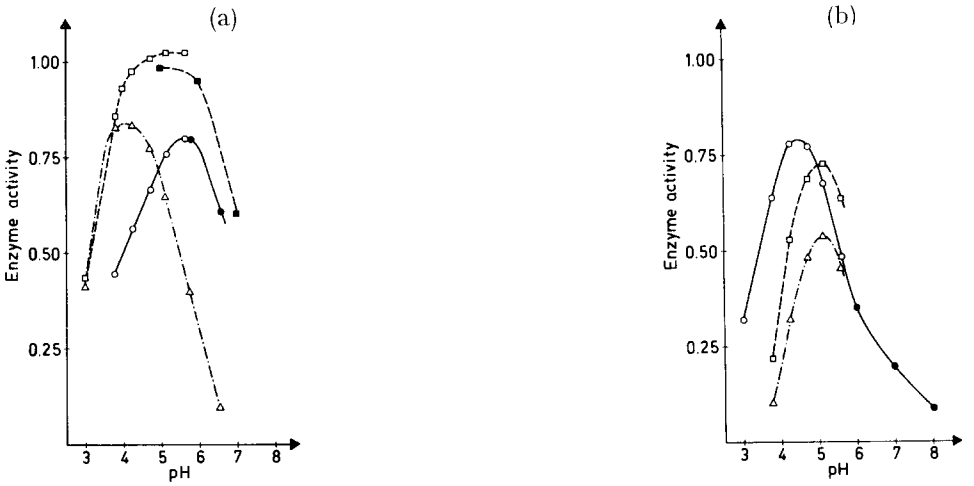

Fig. 1. Effect of pH on the enzymatic activity. Unfilled symbols: 0.05 M citrate buffers. Filled symbols: 0.05 M citrate-phosphate buffers except at pH 8 at which phosphate was used. The experiments were carried out at 40 °C according to the "Glox"-method, with sinigrin as substrate. △, myrosinase A; □, myrosinase B; ○, myrosinase C. (a) Myrosinases from *S. alba*; (b) myrosinases from *B. napus*.

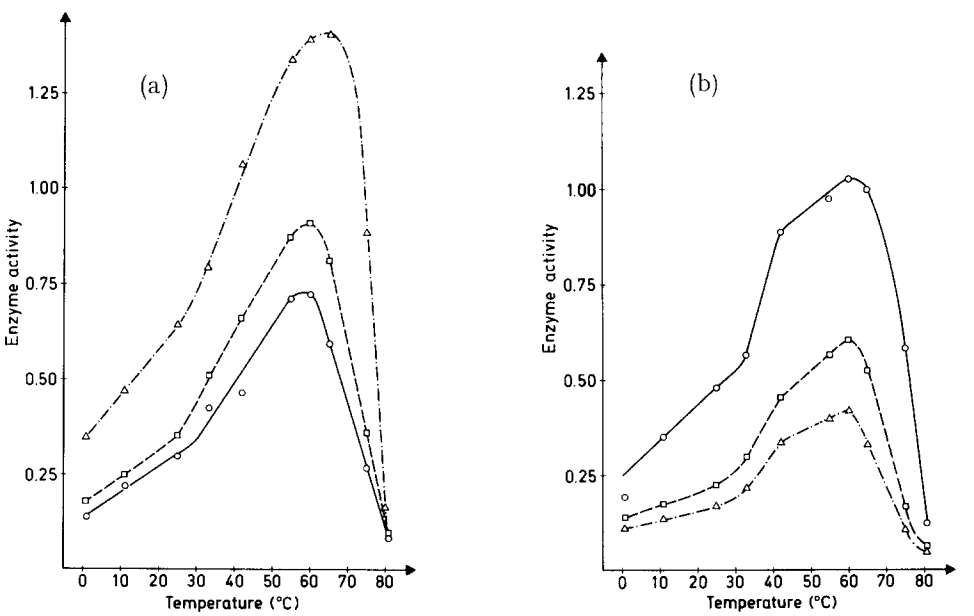

Fig. 2. Effect of temperature on the enzymatic activity. The assays were performed at the pH optimum for each myrosinase in citrate buffer for 30 min according to the Glox method, with sinigrin as substrate. △, myrosinase A; □, myrosinase B; ○, myrosinase C. (a) Myrosinases from *S. alba*. (b) myrosinases from *B. napus*.

samples had maximal stability at pH 6.0 and after 6 months the activity was unchanged.

Fig. 4 shows the stabilities at pH 6.0 at different temperatures. At 37 °C the fall in activity was very steep during the first two weeks. This was also true for RM

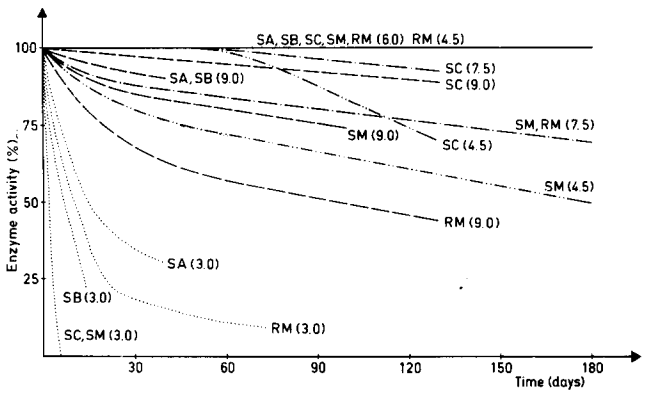

Fig. 3. Stability of myrosinases on storage at various pH at 4 °C (pH values in parenthesis). SA, SB and SC are purified isoenzymes from *S. alba*. SM and RM are meal extracts from *S. alba* and *B. napus* seed, respectively.

at 25 °C. It is noteworthy that the purified enzymes are more stable than the crude preparations. On the other hand, freezing completely destroyed the activity of the purified enzyme, but did not significantly change the activity of a crude extract or intact seeds.

A comparison between citrate and imidazole buffers showed that the latter is to be preferred. The best way of storing myrosinase solutions is to keep them in imidazole buffer at pH 6.0 at 4 °C.

Effect of ascorbic acid

Addition of limited amounts of ascorbic acid to the myrosinase–sinigrin mixture increased the rate of hydrolysis. When larger amounts were added the

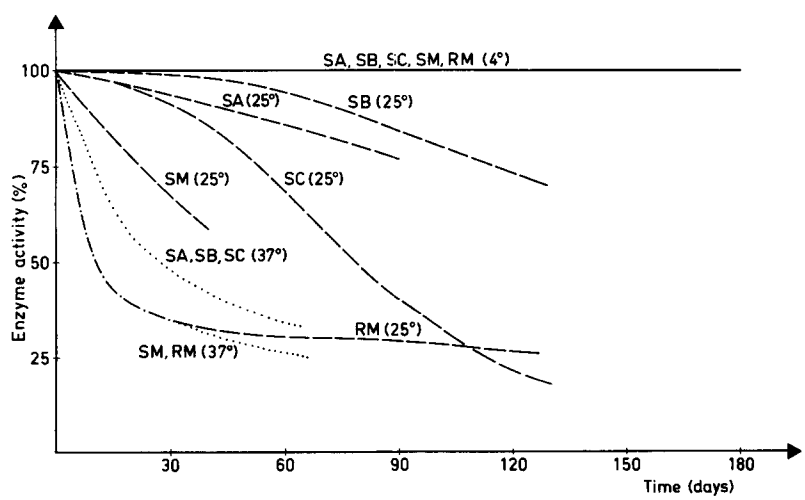

Fig. 4. Stability of myrosinases at various temperatures at pH 6.0. The symbols are explained under Fig. 3.

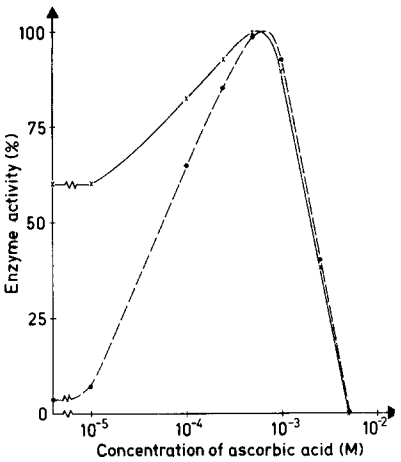

Fig. 5. Effect of ascorbic acid concentration on the activation of myrosinase. The assays were carried out spectrophotometrically at 23 °C with 0.12 mM sinigrin at pH 5.5 (acetate). ×, myrosinase C from *S. alba*; ●, myrosinase C from *B. napus*.

ascorbic acid functioned as an inhibitor. Fig. 5 shows the effect of various amounts of ascorbic acid on myrosinase SC and RC. The curves for the myrosinases SA, SB, RA, RB, and RD were very similar to that for myrosinase RC in Fig. 5, all showing a maximum at about 0.7 mM ascorbic acid. The assays were performed spectrophotometrically. When the ascorbic acid concentration was increased to 5–10 mM, the enzymatic activity was essentially absent. However, a very important difference between the various isoenzymes was their degree of activation by ascorbic acid. The change in V in the absence and presence of ascorbic acid was used as a measure of activation. All myrosinases showed Michaelis–Menten kinetics, both with and without the addition of ascorbic acid, but had different affinities (K_m) for the substrate (sinigrin). Fig. 6 shows Lineweaver–Burk plots of myrosinase SC. The K_m values of

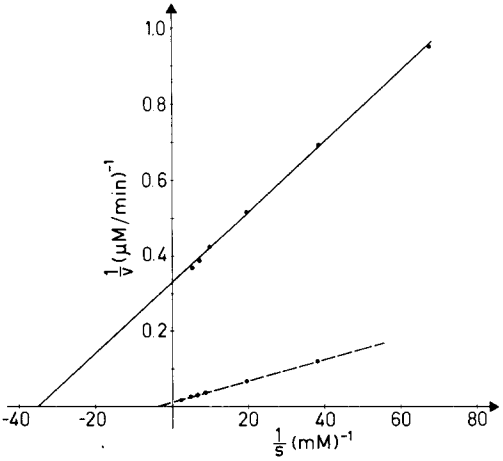

Fig. 6. Lineweaver–Burk plots of myrosinase C from *S. alba*. ———, without ascorbic acid; — — —, with 1 mM ascorbic acid. The assays were carried out spectrophotometrically at 23 °C with sinigrin as substrate, at pH 5.5 (acetate).

TABLE I

KINETIC DATA ON MYROSINASES FROM *S. alba* AND *B. napus*

Experiments performed at 23 °C in 0.05 M sodium acetate (pH 6.5) with sinigrin as substrate.

Myrosinase	*Apparent K_m (mM)*		*Activation factor with 1 mM ascorbic acid**
	No ascorbic acid	*1 mM ascorbic acid*	
SA		0.25	(40)
SB	0.06	0.3	20
SC	0.17	0.4	4
RA	0.05	0.4	20
RB	0.06	0.4	50
RC	0.03	0.3	40

* Activation expressed as the ratio between V in the presence and V in the absence of ascorbic acid.

the six isoenzymes and their activation with ascorbic acid are shown in Table I.

Equilibrium dialysis of the myrosinase (SC)–ascorbic acid complex indicated that an unusually large amount of ligand could associate with the protein. Unfortunately, the available amounts of isolated enzyme permitted only one set of experiments. From a Scatchard plot the equilibrium constant was determined to be 50 000 M^{-1}, shared by 22 ligands. The intercepts were obtained from a straight line, determined by least mean squares.

Activity on different glucosinolates

The enzymatic activities of the myrosinases SB, SC, RA and RB were compared for a number of glucosinolates. The glucosinolate concentrations and the reaction velocities were determined spectrophotometrically. For each type of glucosinolate the initial reaction rate was determined at two glucosinolate concentrations in the absence and at one concentration in the presence of ascorbic acid. It was found

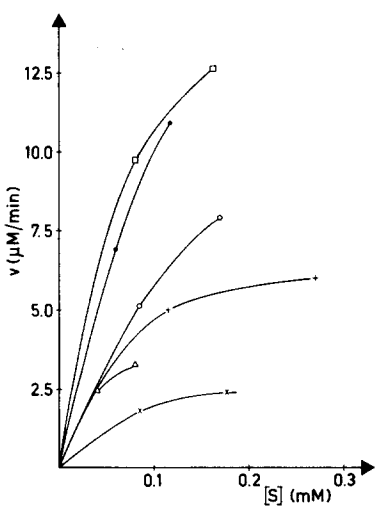

Fig. 7. Activity of myrosinase from *S. alba* on various glucosinolates (pH 5.5, 23 °C). □, glucotropaeolin; ●, sinigrin; ○, glucocheirolin; +, progoitrin; △, glucosinalbin; ×, glucocapparin.

that the different glucosinolates had different rates of hydrolysis. Fig. 7 presents the results with myrosinase SC. The curves for the other three myrosinases tested were practically identical to those in Fig. 7 (there was one exception: with myrosinase RB the activity with progoitrin was considerably higher and with myrosinase SB it was somewhat lower).

The degree of activation with 1 mM ascorbic acid is presented in Table II,

TABLE II

THE EFFECT OF 1 mM ASCORBIC ACID ON MYROSINASE ACTIVITIES WITH VARIOUS GLUCOSINOLATES

Glucosinolate	*Concn (mM)*	*Degree of activation with 1 mM ascorbic acid**			
		SB	*SC*	*RA*	*RB*
Glucotropaeolin	0.17	11	2.2	4	11
Sinigrin	0.12	9	1.8	4	11
Glucocheirolin	0.17	11	1.7	5	10
Progoitrin	0.27	11	2.6	7	6
Glucosinalbin	0.08	4	2.1	2	5
Glucocapparin	0.17	7	2.4	4	9

* The degree of activation presented here is not to be compared to the activation factor presented in Table I, which was calculated from concentrations at the V of the reactions. The figures above were calculated at the concentrations given in the table.

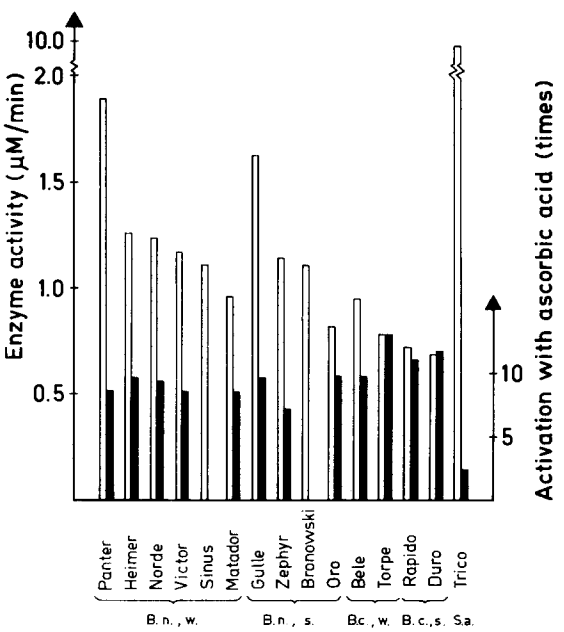

Fig. 8. Myrosinase activity (unfilled staples) and relative degree of ascorbic acid activation (filled staples) in seed extracts of *S. alba* and various cultivars of *B. napus* and *B. campestris*. The assays were carried out with 0.12 mM sinigrin at pH 5.5 at 23 °C. B.n., w., *B. napus*, winter type; B.n., s., *B. napus*, summer type; B.c., w., *B. campestris*, winter type; B.c., s., *B. campestris*, summer type; S.a., *S. alba*. Assays on the degree of activation of myrosinases in Sinus and Bronowski were not included in this investigation.

which shows that it is of the same magnitude for most of the glucosinolates. However, the hydrolysis of glucosinalbin seems to be somewhat different.

The results obtained in this investigation show that the relative substrate specificity is principally the same for all isoenzymes tested. Thus the myrosinases from different seeds are not specifically adapted to a particular set of glucosinolates.

Activity in various cultivars of rapeseed and white mustard

Screening experiments on the myrosinase activity in various cultivars showed that there are not only large differences between the species, but cultivars of the same species also differ. Fig. 8 shows the myrosinase activity and the activation with ascorbic acid of a number of cultivars. The most striking result is the high activity of white mustard, compared to rapeseed. The low activation with ascorbic acid is also characteristic for mustard seed. One also find that the average activity of *B. napus* is higher than that of *Brassica campestris*, but the activation with ascorbic acid behaves in the opposite manner. It should be pointed out that the activities not only depend on the origin of the seed, but are also to some extent dependent on the conditions under which the seeds are harvested and stored.

DISCUSSION

This investigation shows that the myrosinase isoenzymes from *S. alba* and *B. napus* are very similar in some properties, *i.e.* temperature maxima, stability, the effect of ascorbic acid and the pattern of activity with different glucosinolates. The previous papers[7,8] also show that, in the absence of ascorbic acid, the specific activities of completely purified myrosinases from *S. alba* and *B. napus* are almost the same.

The main differences between the isoenzymes are the pH optima, the degree of activation with ascorbic acid, some kinetic constants, and some physicochemical properties (molecular weight, isoelectric point, carbohydrate content *etc.* See refs 7 and 8).

The pH optima (pH 4–5.5, 0.05 M citrate buffer) are significantly lower than those reported by some investigators[6,10–13] which were within the pH range 6–9. However, diverse ionic strengths and buffers were used by the various investigators which complicates a direct comparison of the results. In myrosinase from *B. napus* Henderson and McEwen[3] reported an optimum at pH 5.5 in the presence of ascorbic acid. MacGibbon and Allison[14] found a myrosinase in aphid, having an optimum at pH 5.0 Other investigators[15–17] obtained no pronounced pH optima, but found a broad plateau between pH 4 and 9. This agrees with our results when the assays were carried out with a pH-stat and no buffers were used. The differences in the pH optima obtained by us and others may be due to differences in purity of the enzyme, type and concentration of buffer or substrate used.

Ettlinger *et al.*[4] and Vose[5] found two myrosinases in *S. alba*, one which was not activated at all (glucosinolase I) and one that required ascorbic acid for activity (glucosinolase II). These data and the isoelectric points reported by Vose are similar to those of myrosinase SC and SB, respectively, described in this paper. However, there are some differences: myrosinase C is activated about 4 times, while glucosinolase I is not activated at all. The differences found in K_m in myrosinase SB and SC were not found in glucosinolases II and I. Vose also reported differences in

temperature maxima not found in this investigation. The differences between the myrosinases reported in this paper and the myrosinases described by Vose and Ettlinger indicate that there is also a variation in myrosinase isoenzyme pattern, between diverse cultivars of *S. alba*.

The two isoenzymes in *B. juncea* separated by Tsuruo *et al.*[6] were reported to be identical in all respects investigated with the exception of their retention on a DEAE-cellulose ion-exchanger. They do not seem to be identical with any of the myrosinases found in *S. alba* and *B. napus*.

The enzyme mechanism of Tsuruo and Hata[18] postulates the presence of one site of action for the substrate and two sites for ascorbic acid. The substrate site has two moieties, one for the glycon and one for the aglycon part of the glucosinolate. The conformation of the glycon moiety is altered when the ascorbic acid site is occupied. Higher concentrations of ascorbic acid have an inhibiting effect on the enzyme, indicating that the second ascorbic acid site is the same as the substrate site. The data obtained in the present work can also accord with this hypothesis after some modification.

The increase in both K_m and V when ascorbic acid is added indicates that the stability of the enzyme–substrate complex is reduced, presumably by increasing the rate constant for formation of free enzyme and product. This change in stability is probably caused by conformational changes in the substrate site, which arise when the effector molecules are attached to a certain site or locus on the enzyme molecule.

The inhibitory effect of high ascorbic acid concentrations may be due to competitive binding at the substrate site. It may also be due to instability of the enzyme–substrate complex. When the effector concentration reaches a certain level, the rate constant for the reformation of free enzyme and substrate will exceed that for the formation of product.

ACKNOWLEDGEMENTS

The authors wish to thank Mrs Lise-Britt Wahlberg and Åsa Fast-Johansson for skilful technical assistance. We are also indebted to Dr Lars-Olof Andersson for valuable discussions. Financial support was given by the Swedish Board for Technical Development, AB Karlshamns Oljefabriker, Karlshamn, Sweden and Alfa Laval AB, Tumba, Sweden.

REFERENCES

1 Vaughan, J. G., Gordon, E. and Robinson, D. (1968) *Phytochemistry* 7, 1345–1348
2 MacGibbon, D. B. and Allison, R. M. (1970) *Phytochemistry* 9, 541–544
3 Henderson, H. M. and McEwen, T. J. (1972) *Phytochemistry* 11, 3127–3133
4 Ettlinger, M. G., Dateo Jr, G. P., Harrison, B. W., Mabry, T. M. and Thompson, C. P. (1961) *Proc. Natl. Acad. Sci. U.S.* 47, 1875–1880
5 Vose, J. R. (1972) *Phytochemistry* 11, 1649–1653
6 Tsuruo, I., Yoshida, M. and Hata, T. (1967) *Agr. Biol. Chem.* 31, 18–26
7 Björkman, R. and Janson, J.-C. (1972) *Biochim. Biophys. Acta* 276, 508–518
8 Lönnerdal, B. and Janson, J.-C. (1973) *Biochim. Biophys. Acta* 315, 421–429
9 Björkman, R. (1972) *Acta Chem. Scand.* 26, 1111–1116
10 Weis-Fogh, O. (1954) *Dansk Tidskr. Farm.* 28, 117–129
11 Reese, E. T., Clapp, R. C. and Mandels, M. (1958) *Arch. Biochem. Biophys.* 75, 228–242

12 Schwimmer, S. (1961) *Acta Chem. Scand.* 15, 535–544
13 Appelqvist, L.-Å. and Josefsson, E. (1967) *J. Sci. Food Agric.* 18, 510–519
14 McGibbon, D. B. and Allison, R. M. (1968) *N.Zld J. Sci.* 11, 440–446
15 Sandberg, M. and Holly, O. M. (1932) *J. Biol. Chem.* 96, 443–447
16 Ettlinger, M. G. and Thompson, C. P. (1962) *Final Report, Contract DA 19-129-QM-1689, W. M. Rice University, Texas, U.S.A.*, pp. 56–106
17 Daxenbichler, M. E., Van Etten, C. H. and Wolff, I. A. (1966) *Biochemistry* 5, 692–697
18 Tsuruo, I. and Hata, T. (1968) *Agr. Biol. Chem.* 32, 1425–1431