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THE FRACTIONATION OF TWO GLUCOSINOLASES FROM *SINAPIS ALBA* SEED BY ISOELECTRIC FOCUSSING

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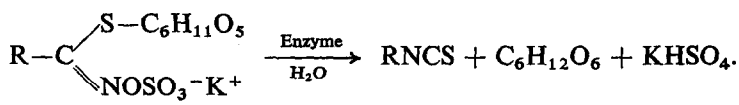
Abstract—Two distinct glucosinolases were isolated from mature seeds of yellow mustard by isoelectric focussing. One had an absolute requirement for ascorbic acid, had an isoelectric point of pH 5·55 (20°), and was inhibited by temperatures above 45°. The other glucosinolase did not require ascorbic acid, had an isoelectric point of pH 5·0 (20°), and showed a temperature optimum of about 75°. Both glucosinolases liberated *p*OH benzyl isothiocyanate, glucose and sulphate from *p*OH benzylglucosinolate. They had identical K_m values and did not differ in response to various pH values.

INTRODUCTION

MUSTARD oil glucosides are hydrolysed by glucosinolases (myrosinases) at neutral pH values to the isothiocyanate, glucose and sulphate. Ascorbic acid is a cofactor for this reaction.[1]



$$R-C(S-C_6H_{11}O_5)(NOSO_3^-K^+) \xrightarrow[H_2O]{\text{Enzyme}} RNCS + C_6H_{12}O_6 + KHSO_4.$$

There has been some controversy concerning the nature of the glucosinolase enzyme, especially regarding whether there is one enzyme or two. Gaines and Goering[2] developed the two enzyme concept of glucosinolase to involve a thioglucosidase or glucosidase and a sulphatase. An alternative proposal was put forward by Ettlinger's group,[3,4] with the concept of a cleavage of the thioglucosidic linkage by the glucosinolase, followed by a Lossen rearrangement resulting in the release of sulphate and liberation of the isothiocyanate. Tsuruo *et al.*[5] fractionated a mustard glucosinolase into two proteins using TEAE-cellulose chromatography, but could find no difference between the two glucosinolases. There was no difference in response to ascorbic acid and both had glucosidase and sulphatase activities.

Recent studies[6,7] on mustard oil glucosinolases have utilized the techniques of starch gel and acrylamide gel electrophoresis, and a number of glucosinolase isoenzymes have

[1] Z. NAGASHIMA and M. UCHIYAMA, *Bull. Agric. Chem. Soc. Japan* **23**, 555 (1959).
[2] R. D. GAINES and K. J. GOERING, *Biochem. Biophys. Res. Commun.* **2**, 207 (1960).
[3] M. G. ETTLINGER and A. LUNDEEN, *J. Am. Chem. Soc.* **78**, 4172 (1956); *ibid.* **79**, 1764 (1957).
[4] M. G. ETTLINGER, G. P. DATEO, JR., B. W. HARRISON, T. J. MABRY and C. P. THOMPSON, *Proc. Natl. Acad. Sci.* **47**, 1875 (1961).
[5] I. TSURUO, M. YOSHIDA and T. HATA, *Agric. Biol. Chem.* **31**, 18 (1967).
[6] J. G. VAUGHAN, E. GORDON and D. ROBINSON, *Phytochem.* **7**, 1345 (1968).
[7] D. B. MACGIBBON and R. M. ALLISON, *Phytochem.* **9**, 541 (1970).

been demonstrated. However, a disadvantage of gel electrophoresis is the small sample size and the problem of eluting the enzyme from the gels. In order to overcome these problems, electrofocussing techniques[8] were utilized in this work and the nature of the major glucosinolase isoenzymes of yellow mustard was clarified.

RESULTS AND DISCUSSION

When the fractions collected from the electrofocussing column were assayed for glucosinolases, only one peak of activity was detected. However, when the assay was conducted in the presence of 0·15 mM ascorbic acid, a second peak of glucosinolase activity was found located at pH 5·55 on the column. The details of the elution pattern off the electrofocussing column are given in Fig. 1. Good resolution was obtained between the two glucosinolases, and when isolated could be re-subjected to electrofocussing without any interconversion from one to the other. It is therefore suggested that these two fractions, I and II, represent distinct forms of the mustard glucosinolases.

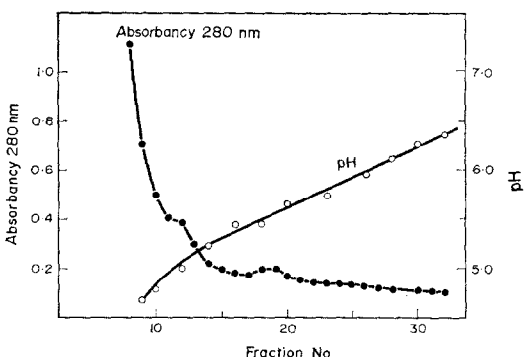


FIG. 1a. ELECTROFOCUSSING OF YELLOW MUSTARD GLUCOSINOLASES.

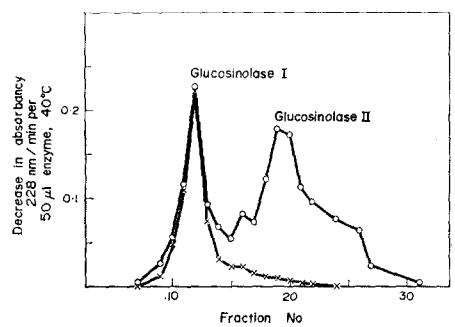


FIG. 1b. ELECTROFOCUSSING OF YELLOW MUSTARD GLUCOSINOLASES.

Procedure for electrofocussing in Experimental and ref. 8. Column was operated at 500 V for 24 hr at 20°. 1 ml fractions were then eluted off the column and assayed for glucosinolase activity, protein and pH. Assays marked × were made in the absence of ascorbic acid, those marked ○ included 0·15 mM ascorbate in the reaction medium.

The purification procedure of the glucosinolase is summarized in Table 1 and shows that the pooled glucosinolases I and II, were isolated with a 68-fold increase in specific activity with a 26% recovery. When the crude enzyme was fractionated by discontinuous acrylamide gel electrophoresis, three distinct bands exhibiting sulphate formation were observed (Fig. 2, No. 5). A fourth band of activity was noted (Fig. 2, No. 6) when the gels were incubated in the presence of ascorbic acid (1 mg/ml) in addition to the substrate, allyl glucosinolate and $BaCl_2$.

The glucosinolases from the electrofocussing column were dialysed and concentrated to a small volume (using Aquacide at 4°), and subjected to gel electrophoresis. The results

[8] O. VESTERBERG, in *Methods in Enzymology* (edited by W. B. JAKOBY), Vol. XXII, p. 382, Academic Press, New York (1971).

TABLE 1. PURIFICATION OF THE GLUCOSINOLASES FROM *S. alba*

Step	Total vol. (ml)	Total protein (mg)	Total activity	Specific activity*	Recovery (%)
I Crude enzyme	200	736	6256	8·5	100
II 35–55% $(NH_4)_2$ SO_4	25	55	50	1·0	1
III 55–75% $(NH_4)_2$ SO_4	33	129	3767	29·2	60·2
IV Isoelectric focussing peak I	5	4·25	1071	252	17·1
V Isoelectric focussing peak II	5	1·75	581	332	9·3

* μg sinigrin hydrolyzed/mg. protein/min. Assay by measuring decrease in absorbancy at 228 nm.

are given in Fig. 2. Gels 1 and 2 are from the glucosinolase I, and show the presence of two bands. The presence of ascorbic acid had no effect on the intensity of these bands of activity. Gels 3 and 4 are from glucosinolase II. The only distinct band was noted when the gels were incubated in the presence of ascorbic acid (No. 4).

The difference between the two glucosinolases in their response to ascorbic acid is given in Fig. 3. Ascorbic acid had only a slight effect on the glucosinolase I, which exhibited very high activity in the absence of the cofactor. On the other hand, glucosinolase II was greatly

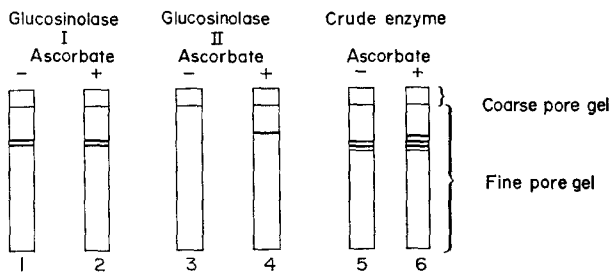


FIG. 2. ACRYLAMIDE GEL ELECTROPHORESIS OF MUSTARD GLUCOSINOLASES.
Disc electrophoresis by method of Davis.[16] 1 mg enzyme added per gel. Electrophoresis at 3 ma/gel for 3 hr at 4°. Glucosinolase assay by submerging rimmed gels in a reaction solution containing 4 mg/ml allyl glucosinolate and 8 mg/ml $BaCl_2$ (with or without 1 mg/ml ascorbate as shown above) at 40°. White bands of precipitated $BaSO_4$ occurred wherever glucosinolase was present (normally within 5 min).

stimulated by ascorbic acid. In the absence of cofactor there was only a trace of activity of this glucosinolase. Levels of ascorbic acid above 0·5 mM showed no further stimulation of enzyme activity. D-Arabo ascorbic acid was found to be only 7% as effective as ascorbic acid as a cofactor for this reaction.

A second major difference between the two glucosinolases was shown in their response to increased reaction temperatures. The results in Fig. 4 indicate that glucosinolase I is a thermo-stable enzyme with a temperature optimum around 75° above which temperature severe denaturation of the enzyme occurs, with resultant loss in activity. The activity of the ascorbate-activated glucosinolase II showed a temperature optimum of about 45°, above which there is inhibition of activity.

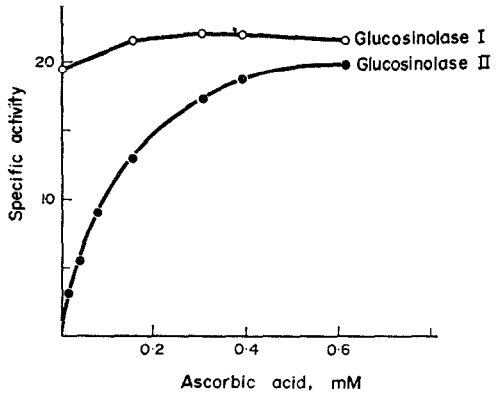


FIG. 3. EFFECT OF ASCORBIC ACID CONCENTRATION ON THE ACTIVITY OF GLUCOSINOLASE I AND II.

Reaction at 40° in 2·5 ml phosphate buffer 0·05 M at pH 6·5. Various amounts of ascorbic acid added as shown. Specific activity given as μg sinigrin hydrolysed per min per 50 μl enzyme.

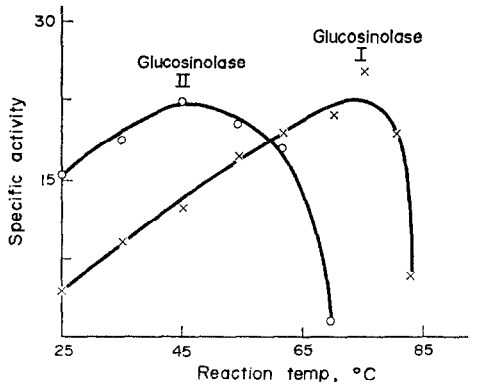


FIG. 4. EFFECT OF REACTION TEMPERATURE ON THE RATE OF REACTION OF GLUCOSINOLASE I AND II.

Specific activity is given as μg sinigrin hydrolysed per min per 10 μg protein. In order to obtain activity with glucosinolase II, ascorbic acid (0·2 mM) was included in the reaction mixture.

The reaction products of these two glucosinolases appeared to be identical. At neutral pH values the main product of the glucosinolase reaction with *p*-hydroxyybenzyl glucosinolate is the *p*-hydroxybenzyl isothiocyanate (R_f 0·30).

The Michaelis–Menton kinetics of the two glucosinolases were also studied. No difference in K_m could be detected between the two glucosinolases with a figure of $8{\cdot}0 \times 10^{-5}$ M applying to both fractions.

Two distinct glucosinolases have also been noted by Tsuruo *et al.*[5] They used a TEAE-cellulose column, and eluted two separate glucosinolases from an enzyme prepared from yellow mustard. However, they were unable to detect any variance in effect of ascorbic acid on the two glucosinolases. In the current work, the two glucosinolases were also successfully fractionated on both DEAE- and TEAE-cellulose columns using the same methods reported by Tsuruo *et al.*[5]

The first glucosinolase eluted off both columns was found to be highly activated by ascorbic acid, while the second enzyme was slightly inhibited by ascorbic acid. The best resolution was obtained with the TEAE-cellulose column. In accordance with the results of Tsuruo *et al.* both the glucosinolases exhibited thioglucosidase activity and gave rise to sulphate formation, although in the absence of ascorbic acid, the glucosinolase II exhibited very low thioglucosidase activity.

The present work demonstrates that electrofocussing techniques can be utilized to obtain highly active preparations of glucosinolases. The isolation of an ascorbate-dependent glucosinolase confirms the results of Ettlinger *et al.*[4] who first suggested the presence of this enzyme. Utilization of these techniques could now provide a useful system for fully elucidating the mechanism by which ascorbic acid acts a cofactor in the complex glucosinolase reaction.

EXPERIMENTAL

Enzyme purification. A crude glucosinolase preparation was made from yellow mustard seeds (*Sinapis alba* variety Kirby) by a modification of the method of Youngs.[9] A 100-g sample of mustard seed was ground

[9] C. G. YOUNGS, Private communication (1970).

in cold 30% acetone in a Waring Blender for 5 min. The slurry was then centrifuged at 12 000 *g* for 10 min at 0°, and the supernatants pooled. Cold acetone was then added to bring the final concentration of acetone to 70%. The gummy protein precipitate was removed and dissolved in 50 ml cold distilled water, and then freeze-dried to give a fluffy-white crude enzyme preparation (2–3 g).

A 55–75% $(NH_4)_2SO_4$ fraction of this crude enzyme was found to contain the bulk of the glucosinolase activity (Table 1). This fraction was dissolved in 33 ml distilled water and dialysed overnight at 4° against distilled water. The dialysate was then centrifuged at 12 000 *g* for 10 min, and the clear supernatant was mixed with the Ampholine carrier ampholyte solution (1%, w/v) pH 5–8 and applied in a sucrose density gradient to the electrofocussing column. The apparatus was arranged so that the anode was at the bottom of the column, and the cathode was at the top. The column was cooled by tap-water and was maintained at 20°. A pH gradient was formed in the column by applying a voltage of 500 V DC for 24 hr. Under these conditions the proteins in the sample migrated to the point where they were electrically neutral, i.e. where the pH = pI. After completion of the electrofocussing, 1 ml fractions were collected off the column and assayed for enzyme activity, protein content and pH.

Assay for glucosinolase activity. Glucosinolase activity was routinely assayed by the method of Schwimmer[10] by following the decrease in absorbancy of an allyl glucosinolate (sinigrin) solution at 228 nm using a Unicam SP1800 recording spectrophotometer with a water-jacketted cuvette-holder. Protein concentration was measured both by the absorbancy at 280 nm and by the Lowry procedure,[11] with bovine serum albumen as standard. Glucosinolase activity was also assayed by the release of sulphate utilizing the benzidine precipitation method of Dodgson and Spencer.[12] Glucose was measured by the dinitrosalicylic acid method.[13]

Column chromatography of the glucosinolases was made on 1·5 × 15 cm columns of DEAE- and TEAE-cellulose.[14] Enzymes were eluted with 0·02 M Tris–HCl buffer pH 8·5 with increasing concentrations of NaCl.

Products of the glucosinolase reaction were identified by TLC. The enzyme reaction was stopped by addition of 1 ml 1 N HCl, and the phenolic products extracted with Et_2O. The extract was then dried and evaporated under reduced pressure. Aliquots were spotted on 0·3 mm MN-300 cellulose TLC plates, and developed with a 2% w/v formic acid. Dried plates were sprayed with either diazotized sulphanilic acid for detection of phenols, or iodine-azide reagent[15] with a starch spray to fix the background, for sulphur containing compounds.

Disc acrylamide gel electrophoresis as described by Davis,[16] was used to study the two glucosinolases obtained following electrofocussing. Enzyme activity was assayed on the gels by the method of MacGibbon and Allison.[7]

[10] S. SCHWIMMER, *Acta Chem. Scand.* **15,** 535 (1961).
[11] O. H. LOWRY, N. J. ROSENBROUGH, A. L. FARR and R. T. RANDALL, *J. Biol. Chem.* **193,** 265 (1951).
[12] K. S. DODGSON and B. SPENCER, *Biochem. J.* **55,** 436 (1953).
[13] J. B. SUMNER, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. I, p. 149, Academic Press, New York (1955).
[14] E. A. PETERSON and H. A. SOBER, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. V, p. 6, Academic Press, New York (1962).
[15] F. FEIGL, *Spot Tests in Organic Analysis*, 5th Edn., p. 88, Elsevier, Amsterdam (1956).
[16] B. J. DAVIS, *Ann. N.Y. Acad. Sci.* **121,** 404 (1964).

Key Word Index—Sinapis alba; Cruciferae; mustard, glucosinolase; isoenzymes; isoelectric focussing.