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EFFECT OF ASCORBIC ACID ON THIOGLUCOSIDASES FROM DIFFERENT CRUCIFERS

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Key Word Index—*Sinapis alba*; *Crambe abyssinica*; *Brassica* spp.; Cruciferae; thioglucosidase; ascorbic acid activation; isoenzymes.

Abstract—Thioglucosidase activity was demonstrated in partially-purified preparations from several Cruciferae oilseeds, both in the presence and absence of ascorbic acid. The amount of activation by ascorbic acid differed among the enzyme preparations from different species. Buffer composition and pH were found to significantly affect enzyme activity, the turret rape enzyme showing a second optimum at pH 7·1 in the presence of ascorbic acid and sodium phosphate buffer. Disc electrophoresis on polyacrylamide gel revealed distinct isoenzyme patterns from crude extracts of all nine species or varieties studied. Some differences in the patterns were noted from electrophoresis of partially-purified preparations. Ascorbic acid was found to affect isoenzyme patterns and the rate of development of equivalent isoenzymes from yellow mustard and from turret rape.

INTRODUCTION

MEALS which remain after the extraction of edible oil from Cruciferae oilseeds possess a high protein quality and may be used as supplements in livestock and poultry feeds.[1–3] Extensive utilization of these potentially valuable meals has been hampered by the presence of thioglucosides. These compounds are readily hydrolysed by thioglucosidase (thioglucoside glucohydrolase, E.C. 3.2.3.1.) to yield a number of goitrogenic, growth-inhibitory and toxic substances, including isothiocyanates, oxazolidinethiones and several nitriles.

In view of the importance of thioglucosidase activity in unheated oilseed meals, a greater understanding of enzyme action is desirable if a high-quality product is to be obtained at minimum cost. The greater amount of research on this enzyme to date has been carried out using *Sinapis alba*,[4,5] one variety of *Brassica juncea*,[6–10] and *Aspergillus sydowi*.[11] Activation by ascorbic acid has been demonstrated by several workers since the initial observation by Nagashima and Uchiyama.[12] Tsuruo *et al.*[6] demonstrated the presence of two enzyme species, both activated by ascorbic acid. Vaughan and Gordon[13]

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suggested that thioglucosidase from *S. alba* and from *B. juncea* may exist in two serologically different forms, but the enzyme was shown to be similar in the two species except for a higher concentration in *S. alba*. MacGibbon and Allison,[14] using disc electrophoresis on polyacrylamide gel, obtained isoenzyme patterns from several plant species, in which up to four thioglucosidase isoenzymes were resolved in any one species.

It is the purpose of the present paper to report upon the effect of ascorbic acid on thioglucosidases from several oilseeds of the Cruciferae family of practical or potential importance, and to relate this effect to isoenzymes.

RESULTS

Standard Method

Absorption spectra of sinigrin and of the reaction product, allyl isothiocyanate, revealed that at equal molar concentrations, the absorbance at 227·5 nm (absorption maximum of sinigrin, ϵ 6900) due to allyl isothiocyanate (ϵ 565) is 8·2% of that of sinigrin. This is slightly higher than the equivalent value of 7·2% reported by Schwimmer,[5] but confirms the validity of his technique allowing the decrease in absorbance at 227·5 nm to represent the rate of reaction equivalent to sinigrin disappearance. Also, we confirm that the decrease in absorbance at 227·5 nm is linear with respect to time, until almost 50% of the sinigrin has been degraded. The final absorbance attained during the reaction was equal to that expected for the amount of allyl isothiocyanate formed.

TABLE 1. EFFECT OF ASCORBIC ACID ON THE ACTIVITIES OF VARIOUS ENZYME PREPARATIONS

Source material	Method of purification: precipitation by	Enzyme activity* Control (i)	+ AA† (ii)	Ratio (ii)/(i)
S. alba	Ethanol	94·0	243·0	2·6
	Acetone	65·2	200·0	3·1
B. napus cv. Turret	Ethanol	6·3	52·4	8·3
	Acetone	3·0	18·1	6·0
	Ammonium sulphate	6·2	48·0	7·7
B. napus cv. Bronowski	Ethanol	2·6	25·4	9·8
B. campestris cv. Echo	Ammonium sulphate	4·5	47·6	10·6

The standard reaction mixture composition was: sinigrin, 0·188 mM; enzyme preparation, 0·15 mg; dissolved in 3·0 ml citric acid–Na_2HPO_4 buffer, 3·2 mM, pH 5·7. The disappearance of sinigrin was monitored at 37° at 227·5 nm over a 10–20 min reaction period.

* Enzyme activity is expressed as μM sinigrin degraded per minute per μM protein.

† + AA = presence of 0·375 mM ascorbic acid.

Effect of Ascorbic Acid on overall Thioglucosidase Activity

Reaction rates of the *S. alba* enzyme were estimated in the presence of different concentrations of L-ascorbic acid up to 0·550 mM. Activity was demonstrated in the absence of ascorbic acid, but the enzyme was activated by increasing concentration of the acid until maximum activation was first attained in the presence of 0·375 mM ascorbic acid. The

[14] D. B. MACGIBBON and R. M. ALLISON, *Phytochem.* **9**, 541 (1970).

relationship between activity and ascorbic acid concentration was similar to that for a first-order Michaelis–Menten curve. Activation by 0·375 mM ascorbic acid was shown to differ proportionally among the various enzyme preparations.

Table 1 shows that the activating effect is more pronounced on the rapeseed preparations than on the yellow mustard preparations. The data in Table 1 also indicate that the *S. alba* enzyme is considerably more active than the *Brassica* enzymes, in our experimental conditions.

Effect of pH with Special Reference to Ascorbic Acid Activation

The effect of pH on enzyme reaction rate was determined for *S. alba* and for *B. napus* cv. Turret. Figure 1 shows that *B. napus* enzyme activity in the presence of ascorbic acid is greater in sodium phosphate buffer than in citric acid–Na_2HPO_4 buffer over the pH range 6·0–7·2: moreover a second pH optimum occurs at 7·1 when sodium phosphate buffer is used. For comparison, activity was determined with sodium phosphate buffer in the absence of ascorbic acid, within the pH range 6·2–7·9, and shown to be low and constant.

The effect of ascorbic acid was determined for the *S. alba* enzyme, employing sodium phosphate buffer over the pH range 5·7–7·5. Considerable activation by ascorbic acid was shown over the entire pH range tested, with a slight second pH optimum at 7·1.

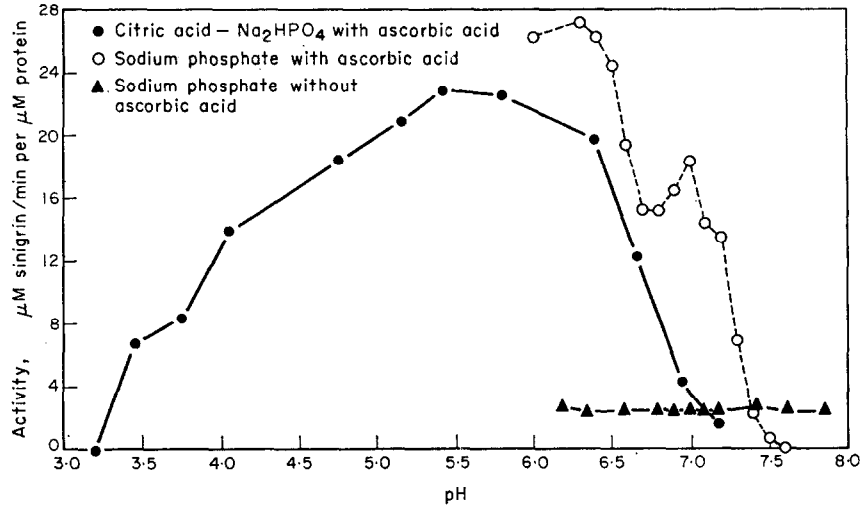

FIG. 1. THE EFFECT OF pH ON *B. napus* CV. TURRET THIOGLUCOSIDASE ACTIVITY.
Enzyme activity was determined in the presence of 2 mM citric acid–4 mM Na_2HPO_4 buffer plus 0·375 mM ascorbic acid; in the presence of 4 mM sodium phosphate buffer plus 0·375 mM ascorbic acid; in the presence of 4 mM sodium phosphate buffer without ascorbic acid. The enzyme was partially purified by acetone precipitation. The experimental conditions otherwise are described in the text and in the legend to Table 1.

Isoenzyme Patterns

Seed crude extracts. Electrophoresis of crude extracts from the nine oilseeds revealed isoenzyme patterns which are shown in Fig. 2. The amount of barium sulphate precipitate observed for *S. alba* greatly exceeded that shown for other species. The band patterns for *S. alba*, *B. campestris* and *B. napus* cv. Turret resembled those reported by MacGibbon and Allison.[14]

Comparison of enzyme preparations. Several partially-purified enzyme preparations were subjected to disc electrophoresis on polyacrylamide gel in similar conditions to those for the whole seed studies. The isoenzyme patterns are shown in Fig. 3. It was possible to locate active isoenzymes in the *Crambe abyssinica* and the two *B. juncea* preparations, even though activity could not be detected by the spectrophotometric method. This may indicate the sensitivity of the technique or may be due to pretreatment of the sample before the electrophoretic run.

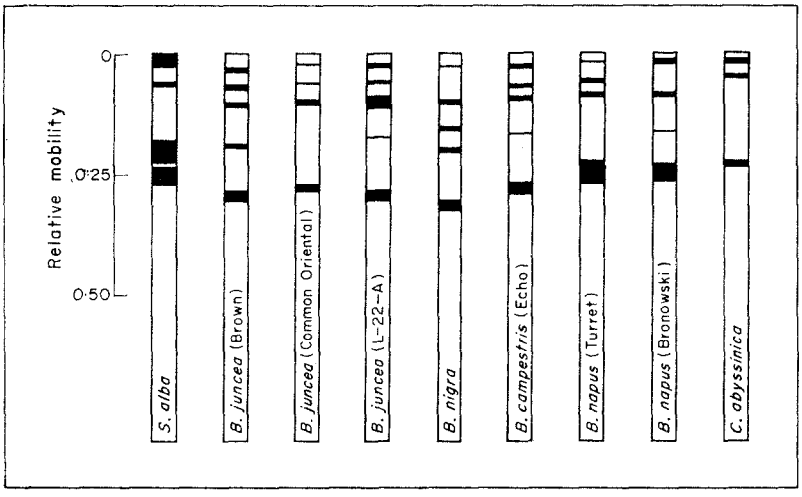

FIG. 2. ISOENZYME BANDS FROM NINE VARIETIES OF CRUCIFERAE OILSEEDS.
Crude extracts were prepared according to the method of MacGibbon and Allison.[14] The conditions of electrophoresis and of band pattern development are described in the text. Band widths in the line drawings are based on peak heights from densitometer tracings of the band patterns.

Some differences in isoenzyme band patterns were observed between the crude extracts and the partially-purified preparations, notably two bands in the R_m 0·25 region for the turret rape and crambe preparations, in place of one band from the crude extracts. Also, in the crambe preparation two faint bands at R_m 0·35 and 0·41 were observed, but bands in this relative mobility range were not observed for any of the other preparations.

Effect of ascorbic acid on specific isoenzymes. Due to the variation in ascorbic acid activation in different species, experiments were conducted to determine if ascorbic acid preferentially activated specific isoenzymes. As ascorbic acid was included in the standard developing solution, a control solution was prepared in which ascorbic acid was omitted. Gels, on which partially-purified enzyme preparations from *S. alba* and from *B. napus* cv. Turret had been electrophoretically separated, were developed in the two solutions. At regular time intervals, the gels were scanned by densitometry.

It was observed over an 8 hr period that the slower-moving bands in the turret rape preparations failed to appear when ascorbic acid was absent. These bands appeared 45 min after commencement of development when ascorbic acid was present. Activation of the turret rape enzyme was also shown by the greater R_m 0·25 peak height attained in the presence of ascorbic acid. For the yellow mustard enzyme preparation, the R_m 0·25 peak height was unaffected by ascorbic acid, but there was greater activity within the R_m 0·15–0·25 region in the presence of the acid.

Figure 4 was constructed by comparing the rate of development of the R_m 0·25 isoenzyme band for the two species in the presence and absence of ascorbic acid. Comparisons were based on peak height, in all cases the height after 8 hr development being taken as 100% development. It was found that ascorbic acid had no significant effect on the rate of

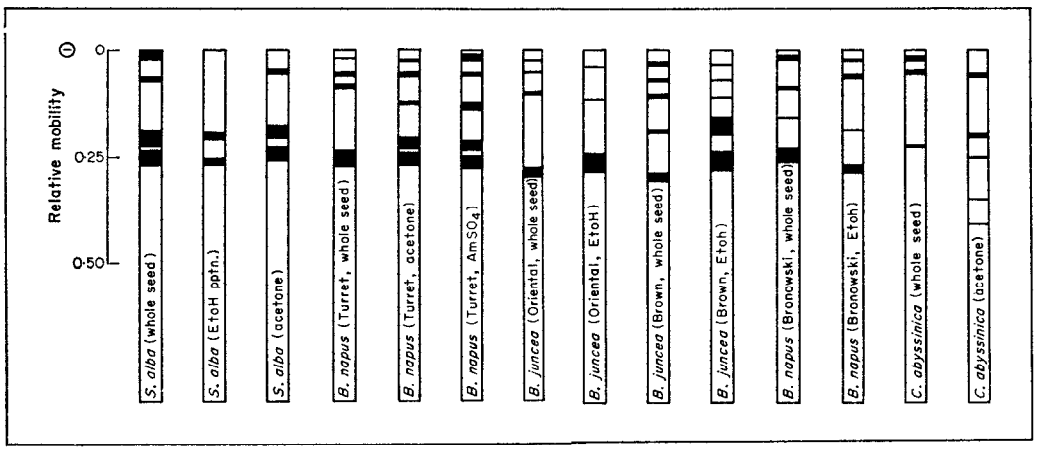

FIG. 3. ISOENZYME BANDS FROM PARTIALLY PURIFIED ENZYME PREPARATIONS.
The preparations were treated according to the method of MacGibbon and Allison.[14] The conditions of electrophoresis and of band pattern development are described in the text. Band widths in the line drawings are based on peak heights from densitometer tracings of the band patterns.

development of this isoenzyme band for *S. alba* over the 8 hr reaction period. For turret rape, however, development of this band was more rapid in the presence of ascorbic acid. This compound was therefore shown not only to affect overall isoenzyme band patterns, but also the rate of development of equivalent isoenzymes in the two species studied, as reflected in the different responses to ascorbic acid.

DISCUSSION

The results described here indicate differences among the thioglucosidases from several species or varieties of Cruciferae oilseeds. It was found that the *S. alba* enzyme was much more active than that occurring in the other species studied. The low activity in the *Brassica* and crambe preparations may reflect low reactivity towards sinigrin, varying degrees of extractability, sensitivity towards the method of preparation, or the presence of naturally-occurring inhibitors. Tookey and Wolff[15] reported that the crambe enzyme was relatively insoluble in water or in 0·035 M 2-mercaptoethanol, suggesting that only about 10% may be dissolved upon simple extraction.

Electrophoresis of crude extracts revealed band patterns in which 3–5 thioglucosidase isoenzymes were resolved in all nine species or varieties examined. The patterns may have chemotaxonomic value for Cruciferae species.[14] However, changes in isoenzyme patterns were demonstrated in partially-purified preparations compared to those from corresponding crude extracts. Isoenzyme patterns appear to depend in part upon the method of enzyme

[15] H. L. TOOKEY and I. A. WOLFF, *Can. J. Biochem.* **48**, 1024 (1970).

preparation, and should therefore be interpreted with caution. Bands which appear from partially-purified preparations but which are not observed from crude extracts, as in the case of turret rape and crambe, may arise from the dissociation of high molecular weight aggregates, or from some other type of alteration. Further investigation is necessary to characterize such isoenzyme bands.

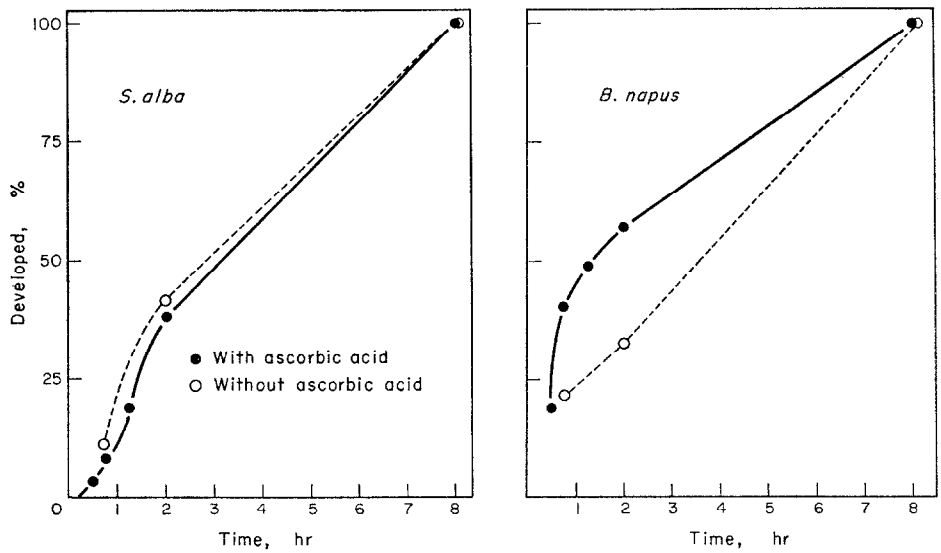

FIG. 4. COMPARATIVE RATES OF DEVELOPMENT OF THE ISOENZYME AT R_m 0·25 OF *S. alba* AND OF *B. napus* CV. TURRET.
The enzyme activity bands were developed in the standard developing solution with and without ascorbic acid. The peak height of the R_m 0·25 isoenzyme band was measured at the times indicated. The measurements were calculated in percentages with the peak height at 8 hr being taken as 100% development.

We have demonstrated varying response to ascorbic acid among thioglucosidases from the different species. Activity was shown both in the presence and in the absence of ascorbic acid. Differences in the degree of activation of *Brassica* preparations compared with *S. alba* preparations, the effect of pH and buffer composition, and the differing responses of isoenzymes to ascorbic acid, all suggest dissimilar enzymic molecular configurations among the species studied.

The effect of pH and specific buffer composition on enzyme activity appears to be a key factor. The second pH optimum at 7·1 shown for the *B. napus* enzyme supports the theory of Tsuruo and Hata[8] that the binding of ascorbic acid affects the sensitivity of the active site towards anions. Such a change in the active site may also account for inactivation at pH levels above 6·8 in citrate–phosphate buffer, and above 7·3 in sodium phosphate buffer. However the *S. alba* enzyme was not significantly inactivated at similar alkaline pH in the presence of ascorbic acid and sodium phosphate buffer.

There was not only variation in response to ascorbic acid among the different species examined, but also between equivalent isoenzymes. It was shown for turret rape and yellow mustard that isoenzymes with comparable mobility did not respond in the same way to ascorbic acid. This result suggests that there may be fundamental differences between the two species with regard to the ascorbic acid activation mechanism.

Previous workers have provided evidence to suggest that thioglucosidase possesses allosteric properties. Tsuruo and Hata[7,9,10] suggested the presence of a substrate site and an effector site for ascorbic acid on the surface of the enzyme protein. Moreover, the substrate site was thought to consist of two areas, for the glycon and aglycon moieties of the thioglucoside, the later area being affected by the binding of ascorbic acid to the enzyme. Ascorbic acid would induce a change in the conformational state of the enzyme, accompanied by an increase in affinity for the substrate.[16–18]

Our results demonstrate the occurrence of 3–5 isoenzyme bands within any one plant species, but further investigation is required to characterize these bands, for instance through molecular weight determination. The differing responses to ascorbic acid may be accounted for by enzyme subunit association–dissociation, which may give rise to active enzyme forms, among the plant species studied, with differing sensitivities towards ascorbic acid.[19] However, the differing responses of the R_m 0·25 isoenzymes for yellow mustard and turret rape to ascorbic acid suggest that these enzyme forms of comparable molecular size and charge may differ fundamentally in the ascorbic acid activation mechanism. Our findings therefore suggest that this mechanism is more complex than previously reported.

EXPERIMENTAL

Source material. Seeds of *S. alba* L. (yellow mustard), *C. abyssinica* Hochst ex R. E. Fries (crambe or Abyssinian kale), and the mustards *B. nigra* L., and *B. juncea* L. cultivars Brown, Common Oriental, and Lethbridge 22A were obtained from Northern Sales (1963) Ltd., of Winnipeg, Manitoba. The rapeseeds *B. napus* L. cv. Bronowski and *B. campestris* L. cv. Echo (minus seed coats) were obtained from the Food Research Institute, Ottawa, while *B. napus* cv. Turret was supplied by the Department of Plant Science, University of Manitoba. All seeds came from 1970 plantings, with the exception of the crambe (1969 crop), and were held at room temp. throughout the storage period.

Enzyme preparation and assay. Thioglucosidase was partially purified by three separate methods, namely EtOH,[20] acetone[5] and $(NH_4)_2SO_4$ fractionation from aq. extracts of the defatted seeds; in each case a freeze-dried powder was finally obtained. The activity was determined by the method of Schwimmer,[5] in which sinigrin disappearance was monitored at 227·5 nm (37°; citric acid–Na_2HPO_4 buffer, 3·2 mM, pH 5·7; 10 mm light path) over a period of 10–20 min in a recording spectrophotometer. Protein was determined by the method of Lowry *et al.*[21] using crystalline bovine serum albumin (British Drug Houses, fraction V) as the standard.

Disc electrophoresis on polyacrylamide gel. Sample preparation for electrophoresis was carried out according to the method of MacGibbon and Allison,[14] except that 40 mg of coarsely-ground whole seed, or 1–12 mg. of freeze-dried enzyme preparation, was used. Electrophoresis was carried out with an anionic gel system (7·5% polyacrylamide gel, running pH 9·3) described in the polyanalyst Electrophoresis Apparatus manual published by Buchler Instruments Inc., Fort Lee, New Jersey, U.S.A. The gels were modified by doubling the concentrations of riboflavin and of ammonium persulphate. Electrophoresis was at 4° at 4 mA per tube for 1·5 hr.

Development of isoenzyme patterns. The procedure employed was a slight modification of that of MacGibbon and Allison.[14] The gels were flooded in an aqueous solution of sinigrin, 2 mg/ml, $BaCl_2$, 6 mg/ml, and ascorbic acid, 3 mM. Sufficient HOAc was added to attain a final concentration in the solution of N. The white bands of $BaSO_4$, indicating thioglucosidase activity, were allowed to develop for 16 hr, after which the isoenzyme patterns were placed on permanent record by densitometric tracing.

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