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COMPARATIVE SEROLOGICAL STUDIES OF MYROSINASE FROM *SINAPIS ALBA* AND *BRASSICA JUNCEA* SEEDS

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Abstract—Crude myrosinase extracts of *Sinapis alba* and *Brassica juncea* seed proteins have been investigated. Immunodiffusion results indicate that the enzyme is serologically similar in the two species but that a considerable concentration difference exists between them, that in *S. alba* being greater than that in *B. juncea*. There is also some indication that the enzyme may exist in two forms.

INTRODUCTION

IT HAS been suggested[1] that enzymes are better suited for taxonomic investigation than other proteins, and that a comparison of enzymes may allow assessment of genetic relations in and between taxa. Enzymes catalysing the same reaction have been found to differ in structure in different taxonomic groups,[2] and Stanier[3] has used bacterial cell-wall enzymes as antigenic markers to help determine taxonomic differences in species of *Pseudomonas*.

Taxonomic assessment of certain *Brassica* and *Sinapis* species has recently been made, based on electrophoretic studies of seed proteins.[4–6] Although much of this work concerned general protein analysis, some enzyme systems (β-galactosidases, β-glucosidases, and esterases) were investigated.

Serological studies following double diffusion and immunoelectrophoresis have the advantage of allowing a means of direct comparison of the proteins of different species. Several tests have been developed[7] for identification of specific enzymes in precipitin bands; e.g. Grabar and Daussant have successfully identified, and then compared, amylase in wheat and barley extracts.[8]

In a recent paper[9] a method was reported for identification of myrosinase, an enzyme restricted to the Cruciferae and certain allied families, and it was indicated that there might be some taxonomic distinction between *Brassica* and *Sinapis* based on the enzyme found in

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[2] W. S. ALLISON and N. O. KAPLAN, in *Taxonomic Biochemistry and Serology* (edited by CHARLES A. LEONE), p. 401, Ronald Press, New York (1962).
[3] R. Y. STANIER, in *Chemotaxonomy and Serotaxonomy* (edited by J. G. HAWKES), p. 201, Academic Press, London (1968).
[4] J. G. VAUGHAN, A. WAITE, D. BOULTER and S. WAITERS, *J. Exptl Botany* **17**, 332 (1966).
[5] J. G. VAUGHAN and A. WAITE, *J. Exptl Botany* **18**, 100 (1967).
[6] J. G. VAUGHAN and A. WAITE, *J. Exptl Botany* **18**, 269 (1967).
[7] P. GRABAR and P. BURTIN, in *Immunoelectrophoretic Analysis*, Elsevier, Amsterdam (1964).
[8] P. GRABAR and J. DAUSSANT, *Cereal Chem.* **42**, 531 (1964).
[9] J. G. VAUGHAN, E. GORDON and D. ROBINSON, *Phytochem.* **7**, 1345 (1968).

both genera. Total protein patterns following acrylamide gel electrophoresis indicate that certain differences exist between *B. juncea* and *S. alba*, as well as considerable similarities.[10] In the present study an attempt has been made to determine the relationship, based on serological criteria, between myrosinase in the two species.

RESULTS AND DISCUSSION

Double Diffusion

Complete seed protein extracts of *Sinapis alba* (Ac) and *Brassica juncea* (Jc) were tested reciprocally against antisera to *S. alba* (AsA) and *B. juncea* (AsJ). In all cases the precipitin lines formed were so many and so close together that it was impossible to detect accurately which band contained myrosinase activity [Plate 1(i)]. To obviate this difficulty extracts from *S. alba* (Am) and *B. juncea* (Jm) containing mainly myrosinase were tested against the antisera. Usually two precipitin bands developed in the reaction of Am against the homologous antiserum AsA [Plate 1(ii)], and the heterologous antiserum AsJ. The same was found in the Jm reaction against the two antisera, but the precipitin bands were weaker.

To determine whether one or both of these two precipitin bands contained myrosinase, the two bands were separated using a sharp scalpel and tested individually for presence of the enzyme. In each of the four reactions (Am/AsA; Am/AsJ; Jm/AsA; Jm/AsJ) the outer band nearer the antigen well stained most strongly, the colour developing immediately. The inner band nearer the antiserum well also stained blue but the colour took longer to develop and was much fainter. In reactions where more than two precipitin bands developed, the bands showing myrosinase activity were easily detected by separating the precipitin bands and testing individually for the enzyme. On no occasion did more than two blue bands appear. As the method for extracting myrosinase from starch-gel in no way ensures exclusion of other proteins (with very similar electrophoretic mobilities to that of myrosinase) from the myrosinase extract, precipitin bands other than those exhibiting myrosinase activity may develop after immunodiffusion.

In order to determine whether the myrosinase from the two species was identical, the precipitin bands formed to Am and Jm were compared by placing these extracts alternately around the central antiserum well. The two precipitin bands formed to *S. alba* joined up with those of *B. juncea* [Fig. 1(i)]. However, spur formation sometimes occurred, the spur being formed to the *S. alba* antigen. This was found in reactions against both AsA and AsJ suggesting that the spur is due to a greater concentration[11] of the enzyme in Am, rather than to any slight serological difference between Am and Jm. After testing for myrosinase, Am and Jm gave a stronger blue reaction to AsA than to AsJ.

By arranging myrosinase and complete extracts alternately around the antiserum well, it was possible to detect which of the eight or nine precipitin bands forming to the complete extract contained the enzyme since the two bands formed to the myrosinase extract joined up with two others in the complete extract [Fig. 1(ii), Plate 1(iii)].

Immunoelectrophoresis

Results obtained from double diffusion suggest that the enzyme is serologically similar in the two species investigated. Immunoelectrophoretic analysis (IEA) was performed in order to discover if there were any electrophoretic differences in the enzyme from the two

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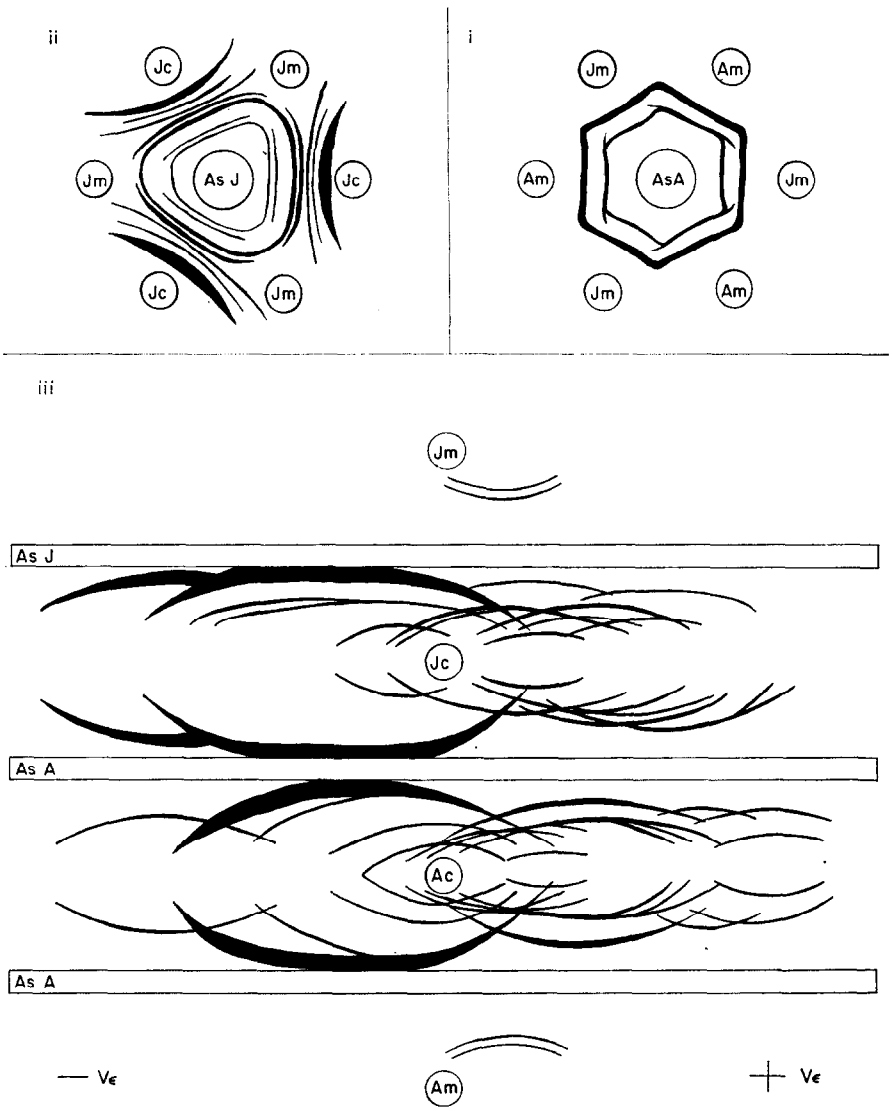

FIG. 1. (i) D.D. ANALYSIS OF *S. alba* AND *B. juncea* MYROSINASE EXTRACTS AGAINST *S. alba* ANTISERUM. (ii) D.D. ANALYSIS OF *B. juncea* COMPLETE PROTEIN AND MYROSINASE EXTRACTS AGAINST *B. juncea* ANTISERUM. (iii) IEA OF *S. alba* AND *B. juncea* COMPLETE PROTEIN AND MYROSINASE EXTRACTS AGAINST *S. alba* AND *B. juncea* ANTISERA.

Ac, *S. alba* complete protein extract; Am, *S. alba* myrosinase extract; Jc, *B. juncea* complete protein extract; Jm, *B. juncea* myrosinase extract; AsA, antiserum to *S. alba:* AsJ, antiserum to *B. juncea.*

species, and whether the two bands showing enzyme activity in the double diffusion (D.D.) plates showed any difference in electrophoretic mobility.

Complete seed protein extracts of *S. alba* and *B. juncea*, and extracts from both containing mainly myrosinase were compared. After immunodiffusion two precipitin bands were generally formed, very close together and at the same distance from antigen well, and with the same centre of curvature for both Jm and Am against homologous and heterologous

antisera [Fig. 1(iii)]. After application of the test for myrosinase, blue arcs similar in size and at the same distance from the antigen wells appeared for each of the four extracts against homologous and heterologous antisera. The colour reaction of both myrosinase extracts was markedly stronger against AsA than against AsJ. Both of the bands formed to Am and Jm were found to contain myrosinase.

The results of double diffusion, the joining up of precipitin bands of the two species, suggests that myrosinase is serologically similar in the two taxa. Judging from the differences in intensity of the blue coloration following the enzyme test, the concentration of myrosinase seems stronger in *S. alba* than in *B. juncea* seeds. This difference will be reflected in the concentration of antibodies to the enzyme in the antisera, and may help to explain the fact that Jm gives a stronger reaction to the heterologous *S. alba* antiserum than to the homologous *B. juncea* antiserum.

Conclusions drawn from IEA results support those of double diffusion. While they again show the immunological similarity of the enzyme from the two species they also show that the enzyme has similar electrophoretic mobilities.

Further evidence that the enzyme is similar in the two species is afforded by the results of specific absorption. This is a useful technique whereby addition of cross-reacting antigen to an antiserum removes corresponding cross-reacting antibodies. Thus when *B. juncea* antiserum is absorbed with *S. alba* antigen all proteins common to the two species will be removed. If myrosinase has similar antigenic determinants in the two species, it will therefore be eliminated. Extracts of Ac, Jc, Am, and Jm were tested against *B. juncea* antiserum absorbed with *S. alba* protein. Only in the Jc reaction did any precipitin bands develop and in none of the cases was any myrosinase activity detected. The result of this absorption experiment confirms the serological identity of myrosinase from the two species.

There has recently been renewed interest[12–14] in the controversy as to whether myrosinase is a one-enzyme system or a two-enzyme system containing thioglucosidase and sulphatase. Tsuruo *et al.*[14] have obtained two peaks, which show myrosinase activity, following ion-exchange chromatography, and have concluded that these peaks result from two different forms of the same enzyme. In the present paper, following IEA and D.D., two precipitin bands are found of different intensity, exhibiting myrosinase activity. This suggests that the enzyme might exist in two forms which are serologically different (or have different antigenic determinants) but which have the same molecular size and the same net charge. If this is so it would support the conclusion drawn by Tsuruo and his co-workers.

However, the fact has not been overlooked that the appearance of two precipitin bands does not necessarily indicate that more than one determinant is present on an antigen molecule, or that more than one antigen has been detected.[11]

Serology, using immunodiffusion techniques, allows a direct comparison of homologous proteins and the present work suggests complete homology between the myrosinase enzymes of *S. alba* and *B. juncea*. In a previous publication,[9] comparison of the myrosinase fractions of the seed proteins of *Brassica* and *Sinapis* species after electrophoresis in starch-gel indicated a possible difference which might support the generic distinction. One difficulty with a taxonomic comparison using this starch-gel technique is the correct assessment of the R_p values where the resulting protein bands have a fairly extensive spread. It is felt, however,

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[13] P. CALDERON, C. S. PEDERSON and L. R. MATTICK, *Agri. Food Chem.* **14**, 665 (1966)
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that immunodiffusion techniques provide a more direct assessment of protein homology and therefore the present work indicates complete homology between the myrosinase of *S. alba* and *B. juncea* although there is an obvious difference in enzyme concentration.

EXPERIMENTAL

Materials

Seeds of Bixley White Mustard (*Sinapis alba* L.) and Trowse Mustard (*Brassica juncea* Coss.) were used in this investigation.

Methods

Methods of protein extraction from seed, preparation of antisera and starch-gel electrophoresis were the same as those described in a previous paper.[9]

Double diffusion (D.D.) and immunoelectrophoresis (IEA) were carried out in starch–agarose gel.[15] A mixture of 2·5 per cent w/v starch hydrolysed (Smithies) and 0·75 per cent w/v agarose (Koch-Light Laboratories) was boiled for about 7 min in phosphate–borate buffer, pH 7·5, ionic strength 0·05 (H_3BO_3, 25·99 g/l.; NaOH, 2·4 g/l.; KH_2PO_4, 2·04 g/l.). This mixture was poured onto 15·5 × 10 cm glass plates (15·5 ml/plate) to give a gel 1 mm thick. After 20 min the gels were sufficiently set for the antigen and antiserum wells to be cut using a cork borer. This starch–agarose medium was found to give better resolution and definition than that previously described.[4]

For immunoelectrophoresis four wells, 4 mm in dia. and 2·1 cm apart, were cut across the centre of the width of the gel, with a smaller well at one side for the bromophenol blue marker. Antigen was introduced into the wells and electrophoresis carried out for 2½ hr at 15 mA/plate constant current (6 V/cm/plate), at room temperature, using ice-cold phosphate borate buffer as the tank electrolyte, and absorbent surgical lint wicks. After electrophoresis, antiserum troughs (12·5 cm long, 3 mm wide, and at a distance of 9 mm from the antigen wells) were cut parallel to the direction of current.

For double diffusion, six antigen wells 4 mm in dia. and 1 cm apart from each other were cut in a circle around a central antiserum well (0·7 mm in dia.), at a distance of 8 mm from it. (Six of these templates were cut on each plate.)

Antisera prepared to *B. juncea* and *S. alba* seed proteins were concentrated to one-fifth of their original volume in polyethylene glycol 4000 P. Absorption of serum was carried out as described in an earlier paper.[4] In this case *B. juncea* antiserum was absorbed with *S. alba* protein extract.

Immunodiffusion was carried out in a $CHCl_3$ saturated atmosphere at 37° for 24–48 hr. The plates were then washed in 0·9 per cent ice-cold saline for 24 hr, changing the wash at least once. After this the plates were tested for the presence of myrosinase.[9]

Although the presence of myrosinase in *B. juncea* is well documented,[16] considerable difficulty was experienced in detecting the enzyme in this species following IEA and D.D. It has already been suggested[9] that *B. juncea* probably contains a relatively lower concentration of myrosinase than *S. alba*. In order to obtain a more concentrated extract of *B. juncea* myrosinase, starch gel electrophoresis of the seed proteins was carried out in the first place. Following this electrophoresis the gel was sliced horizontally and a strip cut from it (parallel to the direction of current). This strip was tested for myrosinase. The region in the remaining starch gel, which corresponded to the area staining positively for myrosinase (3·3–3·8 cm from origin) in the test strip, was cut out, macerated and mixed with 10 ml buffered saline, pH 7 (1/15 M KH_2PO_4; 1/15 M Na_2HPO_4; 0·9 per cent NaCl). This mixture was left overnight at 4°, centrifuged at 4500 rev/min and the resulting supernatant concentrated to 0·5–1·0 ml in polyethylene glycol. This concentrated solution was used as antigen in IEA and D.D. analyses. The same technique was used for extracting myrosinase (2·6–4·0 cm from origin) from *S. alba* seed proteins. The use of crude myrosinase as the antigen had two advantages compared with the complete seed protein extract. Firstly, the enzyme was obtained in a more concentrated form and, secondly, the number of precipitin bands in the D.D. and IEA plates was reduced. This allowed an easier comparison between bands giving the myrosinase reaction.

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