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THE IDENTIFICATION OF MYROSINASE AFTER THE ELECTROPHORESIS OF *BRASSICA* AND *SINAPIS* SEED PROTEINS

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Abstract—A test has been developed for the identification of myrosinase after the separation of cruciferous seed proteins in starch gel and immunoelectrophoretic analysis. Some taxonomic distinction is shown between *Brassica* and *Sinapis* species.

SOME electrophoretic studies of seed proteins in relation to the taxonomy of certain *Brassica* and *Sinapis* species have recently been published.[1–3] In these papers, taxonomic assessment was based partly on total protein similarity as expressed by R_p values after electrophoresis in acrylamide gel, and serological identity after double diffusion and immunoelectrophoretic analysis. It has been suggested[4] that electrophoretic studies of proteins are of more value in taxonomy if protein identity can be established. This may be an aid to the evaluation of protein homology. In the previous *Brassica* and *Sinapis* studies, β-galactosidase, β-glucosidase and esterase systems were investigated after electrophoresis in starch gel.

Kjaer[5] has recorded the rather restricted distribution of myrosinase in angiosperm families although it is well represented in the Cruciferae. It is felt, therefore, that comparative studies of this enzyme in *Brassica* and *Sinapis* might have taxonomic value. As is well known, the products of myrosinase action on *Brassica* and *Sinapis* thioglucosides are generally isothiocyanate, glucose and sulphate. A recent paper[6] reports a new investigation of myrosinase action and also surveys past work. In the present paper an attempt has been made to identify myrosinase, after protein separation, by glucose production following incubation with sinigrin substrate. As far as is known the method employed for myrosinase identification has not previously been reported and, therefore, some details concerning its application are presented in the following section.

RESULTS AND DISCUSSION

Seed protein extracts of *Brassica oleracea*, *B. campestris*, *B. nigra*, *B. napus*, *B. juncea*, *B. carinata*, *Sinapis arvensis* and *S. alba* were separated by electrophoresis in starch gel. Prior to electrophoresis these extracts were dialysed to remove endogenous thioglucoside substrate. Following electrophoresis, the gel was sliced horizontally and the cut surface

[1] J. G. VAUGHAN, A. WAITE, D. BOULTER and S. WAITERS, *J. Exptl Botany* **17**, 332 (1966).
[2] J. G. VAUGHAN and A. WAITE *J. Exptl Botany* **18**, 100 (1967).
[3] J. G. VAUGHAN and A. WAITE, *J. Exptl Botany* **18**, 269 (1967).
[4] D. BOULTER, D. A. THURMAN and B. L. TURNER, *Taxon* **15**, 135 (1966).
[5] A. KJAER in *Chemical Plant Taxonomy* (edited by T. SWAIN), p. 453. Academic Press, New York (1963).
[6] I. TSURO, M. YOSHIDA and T. HATA, *Agr. Biol. Chem.* **31**, 18 (1967).

overlayed with filter paper soaked in 1 per cent sinigrin in 0·1 M sodium citrate buffer at pH 6·2. The system was allowed to incubate at room temperature for 20 min. After incubation, the substrate paper was replaced with paper soaked in a solution of glucose oxidase (7·5 units per ml), peroxidase (2·5 purpurogallin units per ml) and *o*-tolidine (1mg per ml) in citrate buffer at pH 6·2. This solution gives a blue colour with free glucose.[7]

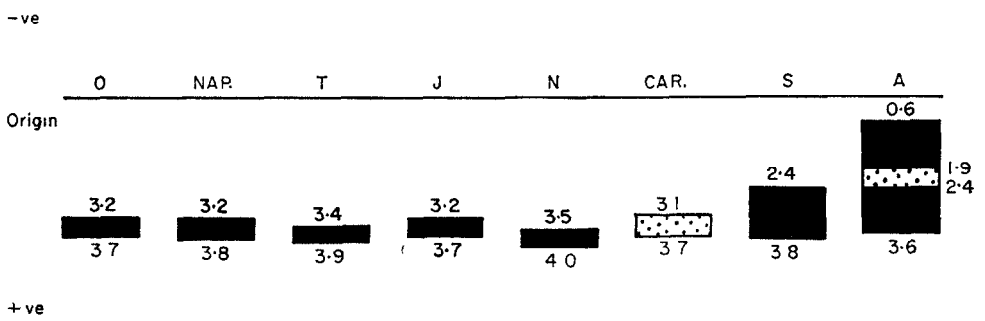

FIG. 1. STARCH GEL ELECTROPHORESIS FOR THE DETECTION OF MYROSINASE IN THE FOLLOWING TAXA: *B. oleracea* (O), *B. napus* (Nap.), *B. campestris* (T), *B. juncea* (J), *B. nigra* (N), *B. carinata* (Car.), *S. alba* (S) and *S. arvensis* (A). The average distances of the anterior and posterior margins of the glucose bands from the origin are indicated in cm.

All *Brassica* and *Sinapis* species investigated (Fig. 1) gave a positive reaction to this test and showed normally one blue band immediately or within 5 min. It is presumed that this band indicates the myrosinase fraction of the seed protein extract. All *Brassica* species showed bands virtually at the same migratory distance and of the same width (0·5–0·6 cm). Sharp distinct bands were produced for all *Brassica* species except *B. carinata* where the reaction was faint. A rather different pattern was shown by the two *Sinapis* species where, although the anterior face of the free glucose band exhibited the same migratory distance as the *Brassica* species, the longitudinal extent of the reaction was considerably greater. *S. alba* showed one very distinct band. In the case of *S. arvensis*, the reaction could be interpreted as two very distinct bands bridged by an area of faint response. This difference in myrosinase activity between *Brassica* and *Sinapis* species possibly supports the generic distinction based on morphology and other seed protein studies.[2]

Serological techniques often assist in comparative enzyme studies.[8] These allow by gel diffusion methods direct assessment of homologous enzyme systems and also, by absorption techniques, the isolation of specific protein systems. With these techniques it is, of course, necessary to have a reliable method of enzyme identification. It is known that some antigens retain their enzymatic properties after combination with the specific antibodies and can consequently be identified in precipitin arcs but relatively little work of this nature has been carried out with higher plant material.[9]

Some success has been obtained with the identification of myrosinase after immunoelectrophoretic analysis of *S. alba* seed extract against its own and *B. juncea* antisera (Fig. 2). Strips of starch gel containing the seed proteins separated by electrophoresis were embedded in agar gel, and the usual immunodiffusion procedure was followed except that "merthiolate", which inhibited the glucose identification test, was omitted from the gel medium. After the

[7] L. L. SALOMON and J. E. JOHNSON, *Analyt. Chem.* **31**, 453 (1959).
[8] P. GRABAR and P. BURTIN, *Immuno-electrophoretic Analysis.* Elsevier, Amsterdam (1964).
[9] P. GRABAR and J. DAUSSANT, *Cereal Chem.* **41**, 523 (1964).

full development of the precipitin arcs, the plate was washed in 0·9 per cent NaCl solution for 24 hr and tested for myrosinase as previously described. One blue arc appeared on the test paper but some difficulty was experienced in correlating this arc with one of the underlying antigen–antibody arcs. To obviate this difficulty, the antigen–antibody arcs in the probable region of the myrosinase activity were separated from each other using a sharp scalpel and the resulting pieces tested for free glucose as already described. With this technique it was possible to locate one precipitin arc showing myrosinase activity. No success

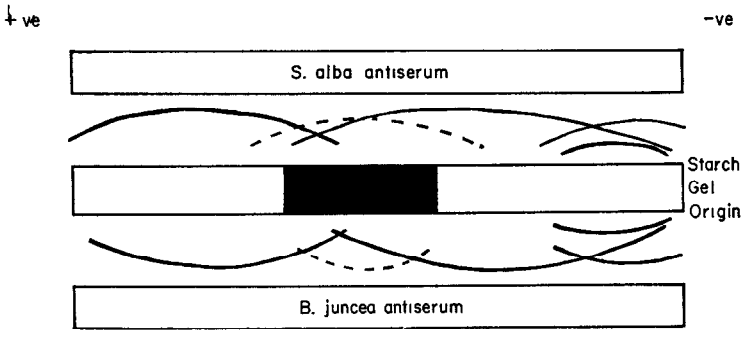

FIG. 2. IMMUNOELECTROPHORESIS ANALYSIS OF *S. alba* SEED PROTEINS, SEPARATED IN STARCH GEL, AGAINST *S. alba* AND *B. juncea* ANTISERA.
The antigen/antibody arc showing myrosinase activity is indicated by a dotted line. The myrosinase band in the starch gel is shaded.

has been obtained with myrosinase identification after the immunoelectrophoretic analysis of *B. juncea* seed extract against its own and *S. alba* antisera. This is probably the result of the relatively low concentration of myrosinase in *B. juncea* (and most likely other *Brassica* species) seed extract and consequently a retarded rate of diffusion from the starch to the agar gel during the immunodiffusion analysis. A possible means of eliminating this difficulty is to prepare highly concentrated extracts of *Brassica* myrosinase by column separation.

METHODS

Protein extracts were prepared by mixing 1 g seed powder with 1·5 ml 0·9 per cent ice cold NaCl solution and the mixture was allowed to stand for 1 hr at 4°. Natural thioglucosides were removed from the protein extracts by dialysis overnight against the saline solution. Starch gels, 15 cm × 18 cm × 6 mm, were prepared according to the method of Smithies.[10] Protein extract was introduced into the origin slit of the gel through double strips of 3 mm filter paper. Borate buffer pH 8·5 was used in the tank. Electrophoresis was carried out at 4° for 4 hr at 40 mA constant current. The test for myrosinase has already been described.

To prepare an antiserum, 1 ml of a mixture of equal parts of seed extract (10 mg protein/1 ml saline buffered at pH 7 ($\frac{1}{15}$ M KH_2PO_4, 9·08 g/l; $\frac{1}{15}$ M Na_2HPO_4. $2H_2O$, 11·88 g/l; NaCl 9 g/l.) and Freund's complete adjuvant was injected into each thigh of the rabbit at weekly intervals over a period of 3 weeks. Blood was taken from the marginal vein of the ear 4 weeks after the last injection. For immunodiffusion analysis, strips of starch gel, 9 cm × 7 mm × 3 mm, containing the separated proteins were embedded in agar gel (Oxoid Ionagar

[10] O. SMITHIES, *Biochem. J.* **61**, 629 (1955).

No. 2, 0·6 per cent w/v, veronal buffer (0·02 M barbitone/0·1 M sodium diethyl barbitone) at pH 8·6) supported on a glass plate 15 cm by 10 cm. The antiserum trough was 5 mm wide and 9 cm long and the distance between the starch strip and the trough was 1·0–1·2 cm. For the formation of precipitin arcs, incubation time was 48 hr in a chloroform saturated atmosphere.

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