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A method of high resolution immunoelectrophoresis for the alcohol dehydrogenase isozymes

The technique of immunoelectrophoresis[1] has proven to be a powerful tool in immunological investigations but it has had only limited application in genetic studies where variant forms of enzymes are analyzed. The disadvantages of the technique for such studies are that (1) resolution is not sharp, (2) relatively high concentrations of antigen and antibody are required, and (3) a fairly homogenous antibody preparation must be employed in analyses with crude tissue extracts.

In the course of studies on the genetic control of alcohol dehydrogenase (ADH) in maize, a strong need developed for a method of analyzing allelic isozymes at the protein as well as the activity level. ADH concentration in crude extracts is too low for direct visual observation of gel electrophoresis bands stained non-specifically with protein dyes, and the polypeptides specified by some alleles are enzymatically inactive. The immunoelectrophoretic method described in this paper overcomes the disadvantages listed above. It is an extension of the technique of two-dimensional cross-electrophoresis of antigen and antibody developed by NAKAMURA[2]. A marker enzyme is used to indicate the retardation of migration of anti-ADH antibodies as they cross the ADH antigen bands. The method makes use of the fact that ADH retains its activity when complexed with antibody, but the method can be modified for use with other enzyme systems where this is not the case.

Experimental

When electrophoresis is performed at a pH where an enzyme and some of its antibodies migrate to opposite poles, precipitation should occur at the line where the two meet when they are applied as separate samples in parallel slits in the supporting medium. If the enzyme has the property of retaining activity even when complexed with antibody, the precipitation line can be detected by staining the gel for enzyme activity. With proper adjustment of antigen and antibody concentrations, all of the enzyme will be precipitated in a sharp straight line. If, however, a homo-

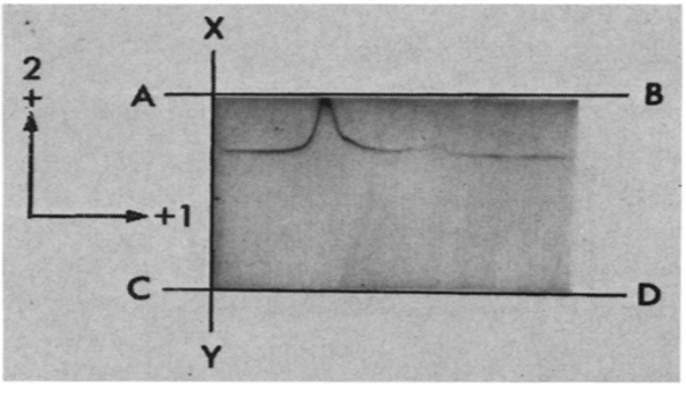

Fig. 1. Starch gel immunoelectrophoresis profile of ADH^{S106} mutant inactive enzyme. Inactive enzyme extract applied in slit X–Y for electrophoresis in the first direction. For the run in the second direction rabbit anti-ADH antiserum was applied in slit A–B and $ADH^{C(1)}$ marker enzyme in slit C–D. Gels stained for ADH activity with nitro blue tetrazolium. See text.

logous antigen was previously subjected to electrophoresis in the same gel in the other direction (90° shift), the precipitation line between marker enzyme and antiserum will not be straight but will show a peak at the position of the perpendicular antigen band (see Figs. 1 and 2). The peak results from retardation of migration of ADH antibodies by the antigen band since the marker enzyme has to migrate for a greater distance before it comes in contact with and is precipitated by the antibodies. The peaks indicate the positions of cross-reacting antigen bands and the number of peaks formed will depend on the number of cross-reacting antigens in the test sample. Peak height, *i.e.*, the degree to which migration of the antibody front is retarded, is correlated with the concentration of antigen in the perpendicular band.

The resolution of this method is very high as it does not depend on diffusion of antigen and antibody. The presence of other antigens or antibodies does not cause

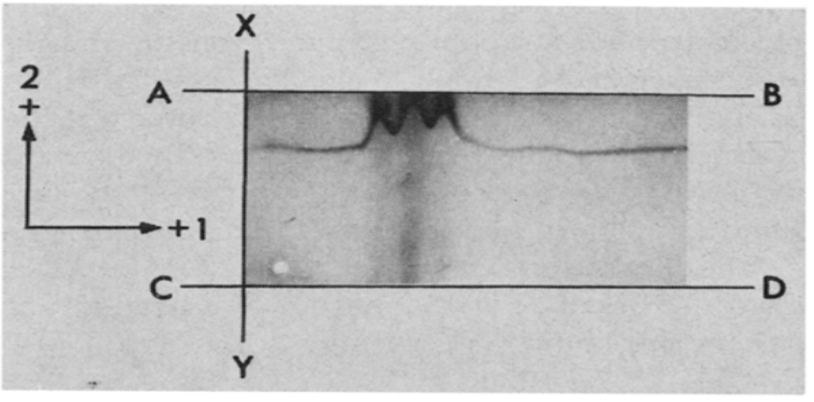

Fig. 2. Immunoelectrophoresis profile of an ADH extract from an Adh^F/Adh^S heterozygote showing the SS, SF, and FF isozyme peaks. Same as Fig. 1 except that the Adh^F/Adh^S extract was applied in slit X–Y for the first direction electrophoresis.

interference since the precipitation band is distinguished on the basis of specific enzymatic activity. Finally, only very low concentrations of antibody and antigen are required with this method. As an example of the sensitivity of the method, it is possible to detect the presence of one-thousandth of the ADH present in the embryo of a single corn seed.

The method was developed to determine the exact electrophoretic mobilities of mutant inactive ADH isozymes of maize, and to compare, at the protein level, the relative concentrations of allelic ADH isozymes in various tissues of heterozygous plants.

ADH retains its activity when precipitated with antibody. Anti-ADH antibodies migrate to both the anode and cathode in starch gel electrophoresis in a sodium borate buffer at pH 8.5. This was determined by a method of two-dimensional electrophoresis[3]. After electrophoresis of the antibody preparation in one direction, the gel was rotated 90° and an ADH enzyme preparation was electrophoresed in the second direction such that the enzyme migrated through the perpendicular bands of antibody. The gel was then stained for ADH activity with nitro blue tetrazolium[4] and the positions of the anti-ADH antibodies determined by the regions of retardation of enzyme migration. These regions were seen to occur on either side of the line of origin for the antibodies (Fig. 3).

Basically, the method consists of making a slit in a square gel slab and inserting a strip of filter paper impregnated with the test antigen sample. Electrophoresis is performed in a direction perpendicular to the axis of the slit. Upon termination of the run in the first direction, the filter paper strip is removed and two parallel slits are made perpendicular to and intersecting the first slit. If the direction of migration of the test antigen is known, the parallel slits do not have to extend on either side of the original slit. A strip of filter paper impregnated with a dilute marker enzyme solution is inserted in one of the parallel slits. If the enzyme migrates anodally the strip is inserted in the cathodal slit and where migration is to the cathode the enzyme is inserted in the anodal slit. A second strip of filter paper impregnated with low titer antiserum is placed in the other parallel slit. Electrophoresis is performed in the

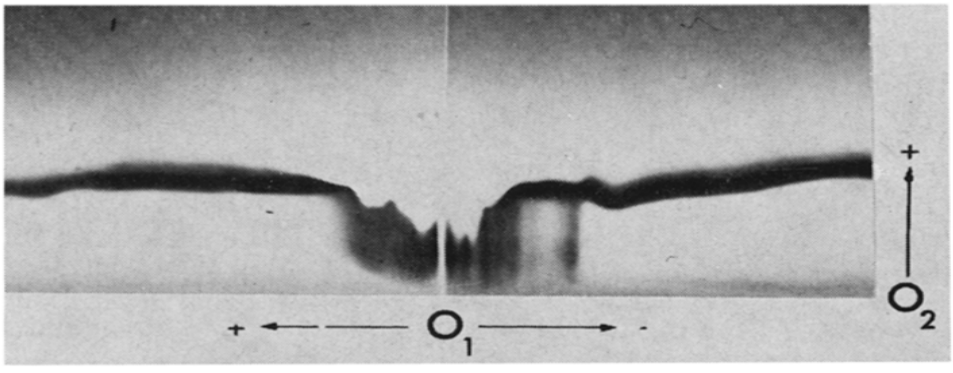

Fig. 3. Two-dimensional electrophoresis zymogram showing heterogeneity of anti-ADH antibodies and enzymatic activity of the enzyme–antibody complex. Anti-ADH antiserum was run in the first direction from origin O_1 and $ADH^{C(t)}$ marker enzyme and then run in the second direction from origin O_2.

second dimension (90° rotation of the gel) perpendicular to the axis of the two parallel slits. The distance between the parallel slits depends on the rate of migration of the enzyme and should be slightly shorter than the distance over which the enzyme and should be slightly shorter than the distance over which the enzyme migrates during the course of the electrophoretic run. The gels are sliced and stained for enzymatic activity in the usual manner.

In typical maize ADH experiments with starch gel electrophoresis performed at room temperature the durations of the runs are 2.5 and 2 h, respectively, for the first and second dimensions. When the electrophoreses are performed in the cold, the times are increased since the migration rates are decreased. The filter paper strips are approximately 5 cm long with 2.5-cm span between the parallel slits for the second dimension run when $ADH^{C(t)}$ (ref. 4) is used as the marker enzyme.

Discussion

Figs. 1 and 2 demonstrate immunoelectrophoretic runs with inactive ADH and multiple ADH isozymes produced in heterozygotes, respectively. It should be pointed out that although the height of the peak is correlated with the concentration of antigen in the band, it is also influenced somewhat by the migration rate of the antigen. For a given antigen concentration the height of the peak will increase as the migration rate increases. Since the antigen migrates toward the antibody front

in the second dimension run, an increase in the rate of migration will result in bringing an increased amount of antigen in contact with the antibodies.

As was mentioned earlier, this immunoelectrophoretic method can easily be modified for use with enzyme systems where the enzyme–antibody complexes are inactive. The modification simply requires a shortening of the duration of electrophoresis in the second dimension such that electrophoresis is terminated at the time when the marker enzyme comes in contact with the antibody front. The gel is then stained for marker enzyme activity. Regions of enzyme activity indicate positions of antigen bands. Since the marker enzyme is inactivated by its antibodies, enzyme activity will be seen only in the positions where the marker enzyme did not complex with its antibodies, *i.e.* the positions where the migration of the antibody was retarded by the antigen bands.

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Department of Botany,
Indiana University,
Bloomington, Ind. (U.S.A.)

DREW SCHWARTZ

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