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Research Notes: United States: Superoxide dismutase (SOD) and tetrazolium oxidase (TO) zymograms observed in gradient PAGE gels and preliminary inheritance data for type 3 zymograms

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1) The use of gradient PAGE gels to study soybean isozymes.

Soybean isozymes have been studied genetically primarily with simple PAGE and starch gel electrophoresis (see Rennie et al., 1986, for a comparison and Kiang and Gorman, 1983, or Palmer et al., 1985, for reviews). Our objective was to test if the use of vertical gels with a PAGE concentration gradient would enhance isozyme resolution over simple horizontal PAGE gels. Gradient gels were tested for their ability to resolve: amylase (Am), acid phosphatase (AP), Diaphorase (Dia), glucose-6-phosphate dehydrogenase (GPD), 6-phosphogluconate dehydrogenase (PGD), phosphoglucomutase (PGM) and superoxide dismutase/tetrazolium oxidase (SOD/TO) isozymes.

We have found that the use of 3% to 12% gradients significantly improved the resolution of certain soybean isozymes over that obtained with simple 7%, horizontal PAGE gels. The vertical arrangement allows for a larger sample to be loaded into the gel, improving the resolution of weakly staining isozymes, while the concentration gradient causes proteins to "stack" as they migrate, resulting in sharper bands. The gradient also enhances molecular sieving, allowing for greater separation of conformational or size isozymes. However, vertical gradient gels in general are not as economical or as practical for germplasm screening as are thick horizontal PAGE or starch gels.

Materials and methods: We used precast gradient gels obtained from Integrated Separation Systems (44 Mechanic St., Newton, MA 02164). These gels were 16x12x1.5 cm in size, with a linear gradient running from 3% at the top of the gel to 12% at the bottom cast in Margolis buffer (0.09 M Tris, 0.08 M boric acid, 0.0026 M EDTA and 0.005% sodium azide pH 8.3). We found that using a 3% stacking gel in 0.125 M Tris-HCl (pH 6.8) buffer not only facilitated sample loading, but it also enhanced protein stacking. The samples used were the clear supernatant (at least two 20-min centrifugations at 12,000 g, with filtration through glass wool until a clear supernatant is obtained) of overnight-soaked soybean seeds, less their seed coats. The seeds were ground to a thick slurry in 1 ml/g of cold Margolis or stacking gel buffer with a mortar and pestle. Each sample was then diluted to 10 mls/g for filtration and centrifugation. After centrifugation, 5% (wt/vol) sucrose and 0.25% bromophenol blue was added to each sample from a 10x stock solution made in Margolis buffer. Only about a third of one seed is needed to have a sufficient sample, so that the remainder can be germinated. A sample, 100 µl, was loaded with a syringe into each sample well (10/gel), which are formed by placing a comb in the stacking gel before it polymerizes. The gels were electrophoresed in a 4°C cold room at 150 volts (constant voltage) for 4-8 hr (depending on the isozyme) using Hoefer "studier" gel apparatus and Margolis tank buffer. Staining was done as described by Gorman (1983).

Results: The most significant improvement in resolution was obtained for Am and SOD/TO isozymes, where several additional isozymes not observed in horizontal PAGE gels were resolved (see subsequent newsletter articles). In addition, the zymogram bands were much sharper. A fast-migrating AP band (an additional band faster migrating than the variable Ap-a, Ap-b or Ap-c allele products), not usually resolved in horizontal gels was clearly visualized in the gradient gels. The products of the Pgml gene, which stain weakly
on horizontal gels, were much more distinct on gradient gels, but their migration was faster than the Pgm2 gene's product. This is opposite from what was observed in simple gels, suggesting that the Pgml protein has a smaller configuration and weaker charge than the Pgm2 protein. This would result in the Pgml protein having a faster mobility in the gradient gel where molecular sieving plays a more important role, but slower migration in the simple gel where charge is more important. PGD zymogram patterns were similar between the gradient and simple gels for the faster migrating bands, but the slower variable bands (products of the Pgd gene) were not well-resolved in the gradient gels. This may be because of the high background staining observed or because the variable bands are caused by charge differences which the simple gel does a better job in separating. GPD zymograms were similar between gradient and simple gels (neither does a good job of resolving distinct bands). No diaphorase bands were detected on gradient gels for unknown reasons. The PGM, PGD and Dia gradient zymograms need further testing along with other enzymes we have not yet tested.

In conclusion, it appears that gradient gel systems may be useful for answering certain specific questions regarding soybean isozymes and for providing additional molecular information on the difference between isozyme variants. However, the greater cost, lower number of samples/gel, increased amount of sample preparation, and the ability to stain only for one enzyme/sample makes gradient gels impractical for most labs to use for large-scale germplasm screening.

References


There have been numerous papers dealing with amylase isozymes in soybeans (i.e., Morita and Yagi, 1979; Hildebrand and Hymowitz, 1980; Kiang, 1981; Adams et al., 1981; Mikami et al., 1982). All have reported beta-amylase as the predominant amylase type in most soybean lines and that varietal differences in beta-amylase either for electrophoretic mobility, pI, or enzyme activity were observed. Gorman and Kiang (1978), Hildebrand and Hymowitz (1980), Kiang (1981), and Griffin and Palmer (1986) reported similar results concerning the inheritance of different beta-amylase electrophoretic variants (i.e., variants were the result of five different alleles at the Spl locus). However, there has been a great deal of confusion over the number of amylase (both alpha and beta) isozymes found in soybeans and in correlating genetic electrophoretic variants with other biochemical results. A recent paper by Norby and Rinne (1985) reported 10 different varietal amylase zymograms with some varieties having as many as 18 different amylase bands. They claimed that a more sensitive staining procedure (including starch in the gel with running gels at low temperatures) allowed for detection of weak isozymes. However, the zymograms they reported have no resemblance to other reported zymograms and known genetic variants. The lines 'Chestnut' and 'Altona', which are known to have at most a trace of beta-amylase (Adams et al., 1981; Hildebrand and Hymowitz, 1980), were reported by Norby and Rinne as having numerous beta-amylase isozymes with no apparent visible intensity differences as compared with other lines tested.

We felt that there was a need for further investigation to attempt to rectify the apparent confusion and inconsistencies in the soybean amylase literature. Our objective was to test gradient PAGE gels with crude samples from lines with known genetic differences in amylase isozymes to see if additional isozymes were detected. Gradient gels allow for a larger sample volume since they are run in vertical apparatus and constantly stack proteins into sharp bands. We hoped that any weak isozymes not detectable by simple electrophoresis and conventional staining might be observed in gradient gels.

Nakamura and Futsuhara (1985) have already shed considerable light with regard to beta-amylase, as they have found that the Spl-a variant corresponds to the 7 band high pI type of Mikami et al. (1982) and Spl-b corresponds to the 7 band low pI type. Further, they found, through renatured SDS gel analysis, that only a single molecular weight species of beta-amylase exists in homozygous seed. The Spl-a protein was found to have a slow SDS migration (likely due to having a higher molecular weight) than the Spl-b protein did. Unfortunatley, they did not test Altona, Chestnut or PI 132.201, the lines homozygous for the Spl-an and sp-1 alleles. Two dimensional (isoelectric and SDS) electrophoresis of Spl-a and Spl-b variants revealed two corresponding families of isozymes with two major and two to three minor isozymes. The patterns were identical (except for placement), suggesting that isoelectric focusing is simply fragmenting the same protein into different bands for unknown reasons, or that two multigene families exist with the Spl gene acting as a regulator.

Methods: Samples were prepared and electrophoresed as described in the previous report, except that it was necessary to dilute samples with normal beta-amylase activity (all lines except Altona, Chestnut and PI 132.201) to 20 ml/g. Gels were stained by first soaking the gel for 1 hr
in 1% hydrolyzed starch in 0.2 M acetate buffer (pH 5.0). Then, the gels were rinsed with water and placed in 0.1% iodine plus 0.5% potassium iodine solution, which visualized amylase as achromatic bands in a blue background. To identify alpha-amylase from beta-amylase, varieties with known beta variants were compared and a 1% solution of soluble starch (limit dextran, which acts as a substrate only for alpha-amylase) was substituted for the hydrolyzed starch when staining gels. Sweet potato beta-amylase from Sigma was used as a control. We also tried to use a beta-amylase inhibitor obtained from Sigma, but this seemed to have no effect on any of the isozymes under the conditions tested.

Results: The gradient gels revealed a total of 10-11 amylase bands. Four strong beta-amylase bands were found. We believe these bands to be beta-amylase because of their intensity, the fact that they did not stain with limit dextran, and because all four were altered in comparing lines with known beta-amylase variants. The mobility of all four bands changed when comparing samples homozygous for the Spl-a and Spl-b alleles. The lines Chestnut and PI 132.201 (homozygous for the Spl-an allele) displayed all four bands, but at a greatly reduced intensity, while F2 seeds from the cross PI 132.201 by Altona (sp-1 allele) segregated with some seeds having zymograms similar to the PI 132.201 and others not having any of the four bands. Thus, it appears that the four bands are all products of the same gene (Spl). They may represent different conformational forms or result from the binding of the beta-amylase protein to unknown charged factors. Morita et al. (1976) suggested, after amino acid analysis, that conformational changes were responsible for the difference between two soybean beta-amylase forms separated by ion exchange chromatography. The idea that multiple beta-amylase electrophoretic forms are conformational forms of the same protein is further supported by Nakamura and Futsuhara's (1985) finding that soybean beta-amylase is a simple protein and that there is only a single molecular weight form of beta-amylase in homozygous seeds when tested in SDS gels. Nakamura and Futsuhara (1985) observed 4-5 beta amylase bands in two dimensional gels and Morita and Yagi (1918) found four different bands with isoelectric focusing.

The two strongest of the other 10-11 bands visualized on gradient gels were clearly alpha-amylase as they stained with limit dextran. The remaining weaker bands are also likely to be alpha-amylase, but they did not stain using limit dextran as a substrate. We feel that this is probably because of their lower activity and the lower degree of resolution obtained with limit dextran. No mobility differences were observed in any of the six varieties tested for any bands other than the four beta-amylase bands. The slowest migrating band (one of the two certain alpha-amylase bands) did appear to have lower activity in some samples (Chestnut, PI 132.201 and Altona). The other putative alpha-amylase bands appeared to have the same intensity in all samples. None of the alpha-amylase varietal differences reported by Norby and Rinne (1985) were observed. However, it is clear that they are correct in suggesting that there may be additional alpha-amylase isozymes not detected on simple PAGE gels.

In conclusion, we suggest that, despite the ability to resolve multiple beta-amylase bands with isoelectric focusing and gradient gels, a single structural beta-amylase gene with five known alleles is active in soybeans. There is a possibility that some of these alleles may actually represent variation in a cis regulatory gene. It appears that soybean beta-amylase allozymes differ in electrophoretic mobility, pH and molecular weight and
may each have several conformational forms. Studies starting from crude soybean meal rather than pure seed lines (i.e., Morita et al., 1975) probably isolated more than one allozyme. Alpha-amylase isozyme patterns will require additional work before any genetic interpretation is possible.

References


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3) Superoxide dismutase (SOD) and tetrazolium oxidase (TO) zymograms observed in gradient PAGE gels and preliminary inheritance data for type 3 zymograms.

We tested gradient PAGE gels for their ability to resolve isozymes with a specific SOD (EC 1.15.1.1) stain as well as with a nonspecific TO stain. In particular, we wanted to test gradient gels for use in scoring F2 seeds segregating for the cultivar-specific type 1 and type 3 TO zymogram patterns first reported by Gorman and Kiang (1977). TO electrophoretic bands are caused by any number of enzymes (particularly SOD) that prevent the spontaneous reduction of tetrazolium dyes in the electron-transfer staining systems used to detect dehydrogenases.

The type 3 TO pattern observed in simple horizontal PAGE gels had slower migrating bands 9 and 10 (out of a total of 11 bands) than did type 1 or 2 cultivars (Gorman and Kiang, 1977). Gorman and Kiang (1978) suggested a codominant mode of inheritance based on the observation of a few apparent heterozygous seeds with both slow and fast bands; however, genetic analysis was not done. Scoring of type 3 seeds proved to be difficult with this electrophoretic system. Gorman and Kiang (1978) reported that the type 1 zymogram was dominant to the type 2 (lacking bands 5 and 6). The type 1 and 2 TO zymograms were found to correspond with the int-oxidase zymogram variants reported by Larson and Beeson (1970). Griffin and Palmer (1984) reported observing the same three cultivar-specific types upon SOD staining of vertical PAGE gels (Davis system). However, their zymogram patterns were somewhat different, with type 2 lacking bands 4 and 5 and type 3 having slower migrating bands 8 and 9 (out of a total of 9 bands).

Methods: The electrophoretic methods described in the previous newsletter article were followed, except that samples were frozen until ready for use and then recentrifuged. The 0.125 M Tris-HCl (pH 6.8) stacking gel buffer was used to prepare samples. Only about one-third of each F2 seed was used per sample with the rest of the seed germinated for producing an F3. Gels were run at 150 volts for 6-7 hr (until 1.5 hr after the bromophenol blue marker dye had run out of the gel). The gels were stained for TO as described by Gorman and Kiang (1977) and for SOD by the method of Beuchamp and Fridovich (1971).

Results: A total of 14 bands (not counting the Ep locus peroxidase band which appears on some samples) was observed for homozygous seed on TO-stained gels and 11 bands on SOD-stained gels. Four weak fast-migrating bands were observed on the TO-stained gradient gels that were not observed on simple PAGE gels, but otherwise the type 1 zymograms were the same. The type 2 gradient zymogram was found to lack the fourth and fifth bands using either stain. The type 3 zymogram in TO-stained gradient gels had a slower migrating tenth band than type 1 or 2 zymograms had. The mobility of only one band was affected, rather than two as observed in the simple horizontal or Davis systems. Contrary to the finding of Griffin and Palmer (1984), we did not observe this variable band at all on SOD-stained gels. The SOD gels also lacked the two fastest migrating bands. Band 13 was a weak band and may simply have not been resolved as well with the SOD stain, but the variable band 10 and band 14 were strong bands.

A total of 45 F2 seeds from the cross 'Agate' by 'Kingston' were scored with TO-stained gradient gels. Sixteen were found to have the fast band
only, twenty had both the fast and the slow bands and nine had only the slow band. Fitting these observed results with a 1:2:1 codominant ratio yielded a chi-square of 2.73, which has a P value of 0.26. While the gradient gels proved to be a superior system for detecting type 3 zymograms and scoring segregating seeds than simple horizontal PAGE gels, resolution still was not sharp enough to conclude whether or not heterozygous zymograms included any intermediate (heterodimer) bands.

We are currently growing F2 plants so that an F3 analysis can be made, as well as making a reciprocal cross. Once this information is obtained, a gene symbol can be assigned. However, unless we are in error about type 3 variants not involving SOD isozymes or unless another specific enzyme activity is identified, the nonspecific TO designation will need to be used despite its falling into disuse in isozyme literature.

References


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