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Agarose gel electrophoresis of fern extracts to detect enzyme polymorphisms. An alternative method to starch gel electrophoresis

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Abstract

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Agarose gel electrophoresis, as a method to detect enzyme polymorphisms in ferns, is presented. Compared to starch gel electrophoresis gels can be prepared quickly and easily. The running time of 1–2.5 h is much shorter than in the starch gel method. The resolution of the bands is very good. However, agarose gels cannot be sliced like starch gels because they are only about 1.5 mm thick. The costs of the agarose gel method are about the same as those of the starch gel method.

Introduction

Following a comprehensive publication on the methodology of electrophoresis of fern extracts by Soltis et al. (1983), a considerable number of investigations of protein variation in ferns have been published. All these publications were based exclusively on starch gel electrophoresis (for example: Gastony 1986, 1988, Gastony & Gottlieb 1985, Haufler 1987, Soltis & Soltis 1987, Werth et al. 1985), and a high degree of variation of fern enzymes was demonstrated.

We propose here a different method of enzyme separation that uses horizontal agarose gels. It is already a well established method in the medical and some biological fields e.g., in human genetics (Spielmann & Kühnl 1982) or in anthropological genetics (Scheffrahn 1990).

The advantages and disadvantages of the two methods are discussed. We think that agarose gel electrophoresis provides a favourable alternative to starch gel electrophoresis because of the ease of preparing the gels, the short running time and the excellent resolution of bands.

Materials and methods

As an example, we present the detailed description of horizontal agarose gel electrophoresis of sporophytes and gametophytes of *Asplenium ruta-muraria* L. and *Athyrium filix-femina* (L.) Roth. Plants were collected in natural habitats or spores from wild plants were sown in the laboratory (north exposed window and room temperature) to obtain gametophytes. Fresh leaf material, or whole gametophytes (10–100 mg or more), were homogenised in grinding buffer (Table 1; two to three times the weight of the plant parts) in polypropylene microcentrifuge tubes (10 mm diameter) with a plastic rod approximately 3 mm in diameter. Single gametophytes about 0.5 mm in diameter were ground on a glass plate using a few drops of grinding buffer. The homogenate (except that from single gametophytes) was centrifuged for 5–10 min at 5000–6000 r.p.m., and the supernatant was collected and recentrifuged. It is important that the supernatant contains no solid material, which can cause smearing. A 0.05 M DTT (Dithiothreitol) solution (2 µl) was added to 50 µl aliquots of the supernatant, which was either used immediately for analysis or frozen at −70 °C.

The samples can be kept frozen for more than one year without loss of enzyme activity.

To prepare a 1% agarose gel, 900 mg of agarose (Seakem LE. FMC Bio Products, 5 Maple Street, Rockland, ME 04841-2994, USA) and 90 ml buffer solution were mixed and heated to boiling while stirring. The hot agarose-buffer solution is poured on a carefully cleaned and levelled glass plate (210 × 260 × 1.0 m) as a mechanical support.

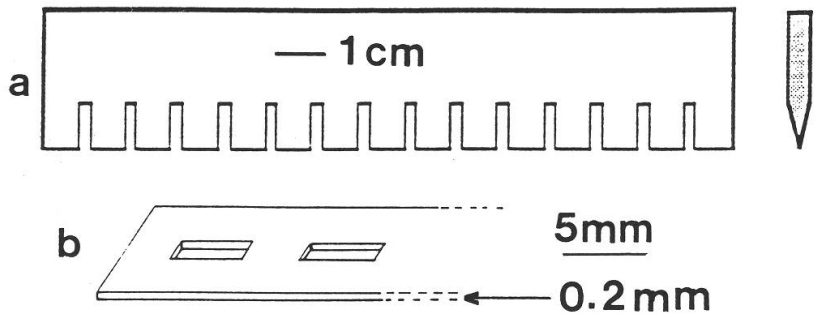


Fig. 1. Equipment for sample application. a) comb for casting slots. b) polypropylene slot template

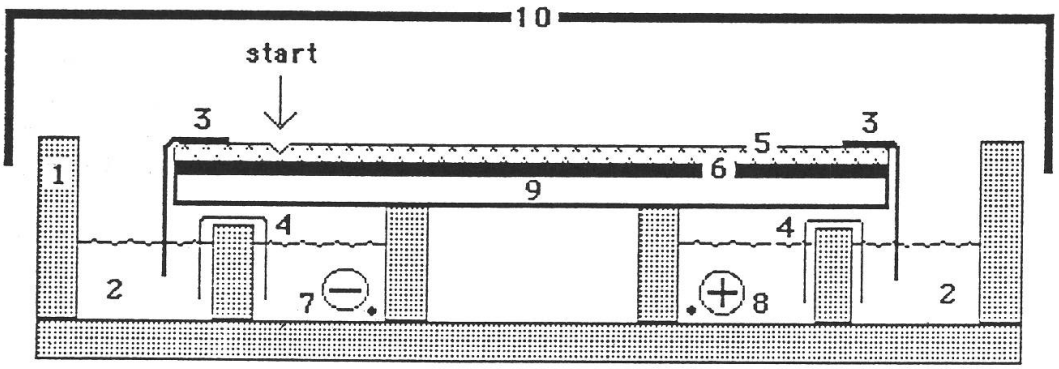


Fig. 2. Setup for horizontal agarose gel electrophoresis. 1) Electrophoretic chamber; 2) Buffer compartments; 3) Two fold Whatman 3 paper to connect buffer with gel; 4) Two fold Whatman 3 paper as electrode vessel bridge; 5) 1% agarose gel; 6) Glasplate; 7, 8) Electrodes; 9) Cooling block; 10) Cover of the chamber.

Because of surface tension the gel does not flow over the border of the glass plate (cleaned glass!). The gel is then allowed to set (solid after about 10 min; about 1.5 mm thick). After cooling in the refrigerator (4 °C) the gel is then ready for use or can be stored at 4 °C for up to 7 d. The samples are applied to slots (5 × 1 mm or 10 × 1 mm) in a template of polypropylene (1.5 × 27 cm × 0.2 mm) placed on the surface of the gel. As little as 5 µl of test solution are sufficient in most cases, so the same preparation can be used to test for several enzymes. When using single gametophytes, the homogenate is applied directly to the slots, because the small volume makes it difficult to centrifuge the samples. Instead of using a slot template (Fig. 1 b), slots for sample application can be cast in the gel by a comb (Fig. 1 a) suspended in the molten agarose when the gel is poured. The glass plate is then placed on a cooling plate kept at 5–6 °C by a cooled circulating water bath. In our experiments we used equipment produced by LKB (Sweden) or DESAGA (Heidelberg), consisting of a power supply, thermostatic circulator and electrophoresis unit. Electrophoresis can be started after the sample solution has diffused completely into the gel, i.e., after about 15 min.

The apparatus is illustrated in Fig. 2. We tested 2 different electrode buffer systems published by Soltis et al. (1983) (Table 2) with some changes. In most cases the buffer system no. 5 of Soltis et al. (1983) was used. In this case the current was stabilised at 54–60 mA during electrophoresis, which corresponded to a voltage of about 420–460 V. A running time of 1.5 to 2.5 h was adequate for most of the enzyme systems and for the different fern species. The running time can be adjusted depending on buffer systems and plant species. During the electrophoretic run, the staining-overlay is prepared following

Table 1. Buffer solutions.

Grinding buffer	
0.1 M Tris-HCl, 25 ml, contains:	
0.1% v/v 2-mercaptoethanol (2-ME)	0.025 ml
0.001 M EDTA (tetrasodium salt)	0.010 g
0.010 M potassium chloride	0.019 g
0.010 M magnesium chloride $6 \cdot H_2O$	0.050 g
4%/20% w/v PVP 40 000	1 g/5 g

Table 2. Electrode and gel buffers used.

Electrode buffer	Gel buffer
No. 5: 0.223 M Tris 0.069 M citric acid (27.00 g Tris, 13.33 g anhydrous citric acid or 14.5 g monohydrate) pH = 7.2 [1]	0.032 M Tris-citric acid (dilute 70 ml electrode buffer to 1 l) [2]
No. 1: 0.400 M citric acid Na_3 salt (117.64 g citric acid Na_3 salt dihydr.) with 1.0 M HCl to pH 7.0 [1]	0.020 M histidine-HCl (4.19 g L-histidine-HCl monohydr.) with 1.0 M NaOH to pH 7.0 [1] 0.040 M histidine-HCl pH 7.0 [2]

[1] After Soltis et al. (1983); [2] Double the concentration as in Soltis et al. (1983).

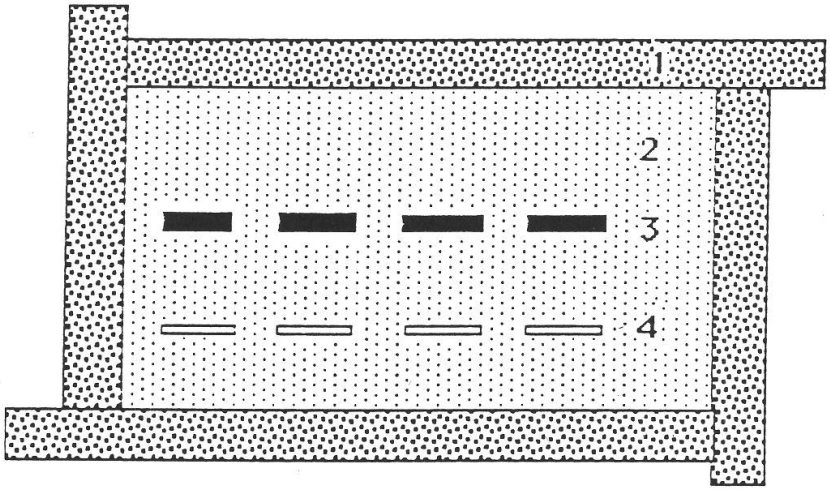
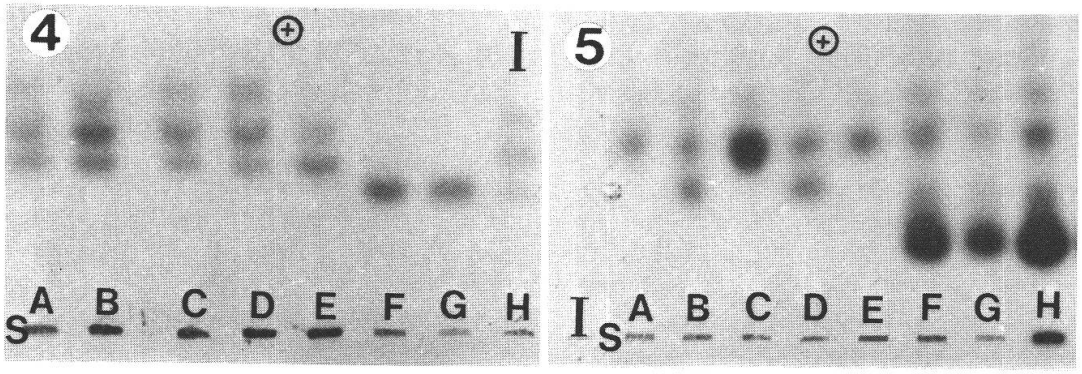


Fig. 3. Scheme of agar overlay. 1) Rubber frame; 2) Agarose gel with agar overlay on top; 3) enzymes (seen due to formazane reaction); 4) Start.



Figs. 4 and 5. Enzyme bands resulting from agarose gel electrophoresis S = start, + = anodal side, bar = 5 mm.

4. Phosphoglucoisomerase (PGI-2) A–E: *Asplenium ruta-muraria,* all plants were heterozygous for two alleles forming two homodimeric bands (fast and slow) and one more intensly stained heterodimeric intermediate band. Note E (arrow) with different pattern possibly due to tetrasomic inheritance, fast band only very weak. F–H: *Athyrium filix-femina,* two homozygous plants for the slow allele and one heterozygous (with two homodimeric bands and one more intensly stained heterodimeric band).

5. Phosphoglucomutase (PGM-2), A–E: *Athyrium filix-femina.* A, C, E, homozygous for fast allele; B, D, heterozygous (monomeric). F–H: *Asplenium ruta-muraria* showing more complicated patterns not yet understood (because of autopolyploidy and the presence of more than two loci).

the procedure described by Harris & Hopkinson (1976) or by Soltis et al. (1983). We preferred the agar overlay technique to the direct overlay because it gave sharper and more intensely stained bands. The volume of the 1% agar containing incubation buffer (after Soltis et al. 1983) and reaction mixture depends on the size of the overlay desired; in most cases it was 30 ml. The overlay is poured into a rubber frame (Fig. 3). Table 3 shows a list of the enzymes we tested. Examples of the banding patterns of two different enzymes are shown in Figs. 4, 5.

Table 3. Buffer systems and enzymes tested.

Buffer system	Enzyme
No. 1	ALD, G-3-PDH, PGI, MDH
No. 5	ALD; EST (fl), G-6-PDH, GDH, IDH, LAP, MDH, PGI, PGM, 6-PGD, SkDH

Abbreviations: ALD, Aldolase; EST (fl), Fluorescent esterase; G-6-PDH, Glucose-6-phosphate dehydrogenase; GDH, Glutamat dehydrogenase; G-3-PDH, Glyceraldehyd-3-phosphate dehydrogenase; IDH, Isocitrate dehydrogenase; LAP, Leucine aminopeptidase; MDH, Malate dehydrogenase; PGI, Phosphoglucoisomerase; PGM, Phosphoglucomutase; 6-PGD, 6-Phosphogluconate dehydrogenase; SkDH, Shikimate dehydrogenase.

Discussion

Preliminary experiments will reveal which enzymes migrate slowly and which rapidly. In further experiments two or more overlays can then be placed on well-separated regions of the gel, making it possible to test two or sometimes three enzymes on one gel (this is true also for starch gels). Both methods involve similar costs per sample, and allow the testing of about 25 samples per run. So far as we could see the agarose method led to sharper bands. One advantage of the agarose method is the short time required to make and run gels, i.e., about 15 min, and 1–2.5 h, respectively. Starch gels need a preparation time of ca. 8 h (cooling) and a running time of 8–16 h. The increased time required for handling the starch gels is compensated partly by the possibility of slicing the gel. This allows the testing of 3–5 systems on one gel. We think that agarose gel electrophoresis could in many cases represent a favourable alternative to the starch method, especially in those cases where only one enzyme has to be revealed.

Zusammenfassung

Die Agarose-Gel-Elektrophorese, eine Methode zur Analyse von Enzympolymorphismen von Farnen, wird vorgestellt. Im Vergleich zur Stärkegel-Elektrophorese ist das Herstellen der Gele sehr einfach und rasch, zudem ist die Laufzeit von 1–2,5 Stunden sehr kurz. Die Auflösung der Bänder ist sehr gut. Agarose-Gele können aber, weil sie sehr dünn sind, nicht wie Stärkegele in dünne Schichten zerlegt werden. Die Kosten für das Material sowie für die Reagenzien zum Enzymnachweis sind für beide Methoden etwa gleich.

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