Phenotyping Adenosine Deaminase Using Ultra-Thin Isoelectric Focussing

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INTRODUCTION

Adenosine deaminase (ADA: E.C. 3.5.44.4) catalyzes the conversion of the purine Adenosine to Inosine. Human red cell ADA was first found to be polymorphic by Spencer (Spencer et. al. 1968). Subsequent studies confirmed the existence of two autosomal codominant alleles: ADA^1 and ADA^2, with the ADA^1 allele being much more common than ADA^2 in all ethnic groups. The distribution of allelic frequencies is such that the ADA polymorphism falls into the category of forensic blood grouping systems which are usually not particularly useful but in a certain number of cases provide a strong indicator of identity in criminal and paternity cases.

Most forensic laboratories phenotype ADA using a modification of Spencer's original starch gel method developed by Culliford (Culliford 1978). To date there appears to be no other report of isoelectric focussing as a technique for ADA phenotyping in the literature.

Of all the focussing techniques, ultrathin isoelectric focussing (UTIEF) has proved to be particularly useful when applied to some of the standard polymorphic enzyme systems (Divall 1981, 1983, 1984) and polymorphic serum protein systems (Edwards 1986; Budoule 1987). All methods are fast, reliable and straightforward, with the added advantage of large numbers of samples being tested per plate. More importantly UTIEF offers greatly enhanced sensitivity in the detection of blood group activity in dried bloodstains (Divall 1981, 1983, 1985; Edwards 1986; Budoule 1987).

This paper presents the optimized conditions for IEF on ultrathin gels of the common ADA phenotypes.

MATERIALS AND METHODS

Glass plates measuring 22 x 15 cm were used and a gel thickness of 0.15 mm was achieved by applying PVC insulating tape along the sides of the base plate.

The following solution produces a standard gel composition (5% T, 3% C) for this type of IEF and is sufficient for 10 gels:

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Distilled water	40 ml
Acrylogel (BDH Ltd)	1.2 g
"Grade 1" Acrylamide (BDH Ltd)	0.8 g
Sucrose	5 g
Riboflavin 10 mg% sol.	0.4 ml
Ampholine pH 4-6 (LKB)	2 ml

Gels were poured using the flap technique, with top plates treated with a coat of Sigmacote (Sigma). Gels were polymerized using long wave U.V. for 3 hours and subsequently stored at 4°C before use.

Lysates were prepared by adding one drop of 3 x washed packed red blood cells to one drop of .05M dithiothreitol at least one hour prior to application on the gel.

Samples were absorbed onto 4 x 3 mm pieces of filter paper (Whatman No.1) and applied down the long axis of the plate 2 cm from the cathode edge. Anode and Cathode consist of 1 cm Wide Whatman No. 17 filter paper strips soaked in 1M orthophosphoric acid and 1M sodium hydroxide respectively. Gels were focussed for 1 hour at 2000 volts (current and power not limiting) with a coolant temperature of 10°C.

ADA enzyme activity was visualised using an agar based reaction mixture applied to the middle third of the gel.

The following reactants are dissolved in 10 ml of 0.025M phosphate buffer pH 7.5 :

Adenosine (Sigma)	20 mg
Xanthine oxidase (Sigma Grade 1)	0.08 units
Nucleoside phosphorylase (Sigma)	0.8 units
Meldola Blue (50 mg%; Boehringer)	0.2 ml
MTT Tetrazolium (Sigma)	3 mg

This was added to 10 grams of 2% agar and incubated for 30 - 40 minutes at 37°C.

RESULTS

i. Isozyme Patterns

Figure 1 shows a developed plate with the standard 3 phenotypes (the ADA 2 lysate was 2 months old and so weaker). A diagrammatic representation of the band patterns observed is given in figure 2.

With the starch gel electrophoresis technique, distinguishing between ADA 2 and ADA 2.1 is not always straightforward, as the relative strength of the bands can be difficult to assess. With the IEF technique known ADA 2 lysates always gave a very strong first band compared to the faster anodal bands, while ADA 2.1 lysates invariably gave a pattern of banding where the second band was slightly stronger.

In all lysates examined a clear area in an otherwise pale purple background occurs between the 2 band and the most cathodal 1 band. This achromatic region is caused by the enzyme Superoxide Dismutase (SOD: E.C. 1.15.1.1).

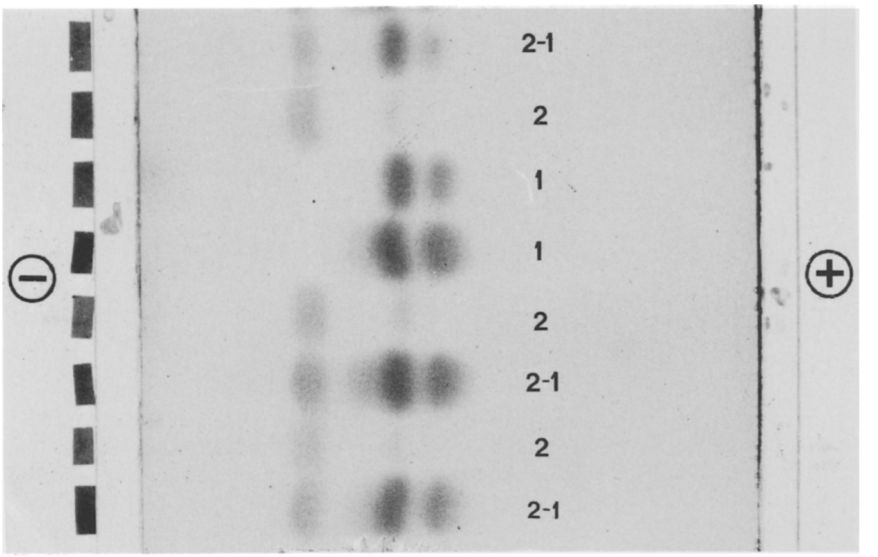

Figure 1 ADA Isozyme patterns of 3 common phenotypes.

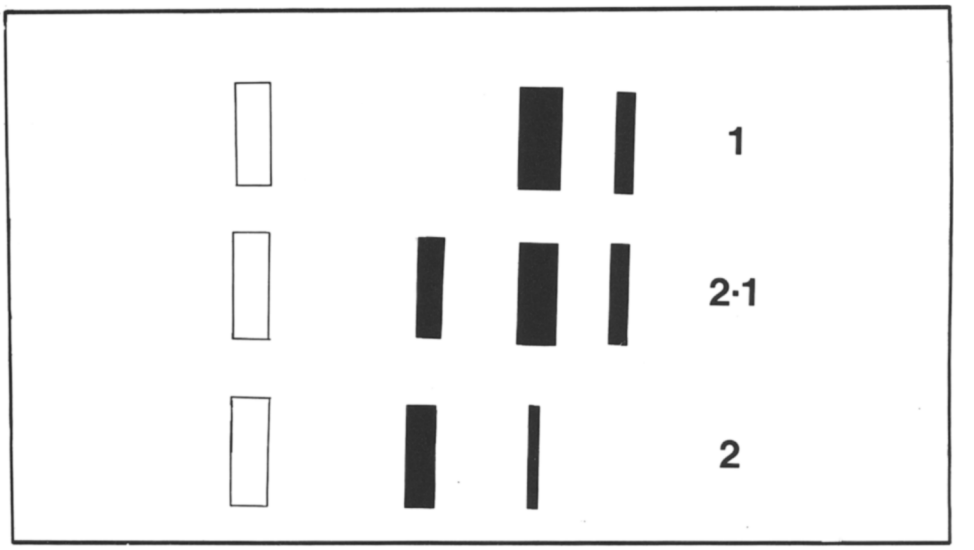

Figure 2 Diagrammatic representation of band patterns.

SOD is itself slightly polymorphic although this aspect of the enzyme was not studied in any way.

ii. Population Studies

The incidence of the three principle ADA phenotypes in three distinct ethnic groups has been studied. Population data and gene frequencies from this study are given in table 1. Gene frequencies in all population groups are in close agreement with previously published data (Steadman 1985).

Table 1. The incidence of ADA phenotypes and gene frequency estimates in three ethnic groups

Ethnic Group	Phenotype	Observed	Expected	χ^2	Gene Frequencies
Caucasian	1	1,829	1,829.943		ADA^1 = 0.9457
	2.1	212	210.124		ADA^2 = 0.0543
	2	5	5.933		
		2,046	2,046.000	0.164 = 0.6 < P < 0.7	
Asian	1	398	402.020		ADA^1 = 0.8581
	2.1	141	133.006		ADA^2 = 0.1419
	2	7	10.974		
		546	546.000	1.960 = 0.1 < P < 0.2	
Negro	1	170	170.007		ADA^1 = 0.9913
	2.1	3	2.975		ADA^2 = 0.0087
	2	0	0.017		
		173	173.000	0.001 = 0.975<P<0.95	

iii. Factors Effecting the Isozyme Patterns

While attempting to optimise the conditions for focussing ADA at this pH range, three variables had a noticeable effect on the isozyme patterns observed, these were:- duration of focussing
temperature of coolant
and position of sample application

Duration of focussing: When lysates were focussed for periods exceeding 1 hour, the isozyme patterns travelled further across the gel and became increasingly diffuse. This suggests that the ADA isozymes do not reach their respective pI's after 1 hour's duration but are still travelling to a position of equilibrium on the pH gradient where the separation between

the isozymes is less distinct. This technique therefore represents electrophoresis across a pH gradient or as it has been more specifically termed non-equilibrium focussing. (Divall 1984).

Temperature of coolant and position of sample application: When non-equilibrium focussing occurs both the temperature of the gel and the position of sample application on the gel become important considerations. Both these factors were systematically varied to find the optimum value for both. Moving the origin further from the cathode edge was found to produce incomplete separation of the ADA isozymes. The same effect was observed by decreasing the coolant temperatures below 10°C.

DISCUSSION

This study has shown that the ADA blood group system can be rapidly and reliably phenotyped using isoelectric focussing on ultra thin gels, with a technique which is ostensibly non-equilibrium focussing. Unlike equivalent work with the EsD and PGM systems, this study has not revealed any further ADA phenotypes. However, the technique does offer several advantages over the conventional electrophoretic methods: results can be obtained within 1½ hours of sample preparation and up to 35 samples can be typed on each gel. In our laboratory the IEF technique has been used to group ADA in more than 5,000 samples arriving in a variety of containers and conditions.

Of more interest in considering this technique is the observation that ADA 2 lysates are quite distinct in their isozyme patterns compared to ADA 2.1 lysates. This difference in isozyme patterns appears to remain well defined in lysates kept frozen for several months and this would imply that IEF may offer a more reliable method of phenotyping ADA in bloodstains. Results from preliminary experiments using this technique to phenotype ADA in bloodstains appear to be very encouraging and studies are now being carried out to examine its suitability for this application.

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