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Determination of phosphoglucomutase (PGM1), acid phosphatase (ACP), and esterase D (ESD) in human bloodstains by hybrid isoelectric focusing (HIEF)

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Summary. PGM1, ESD, and ACP were determined in bloodstain extracts by isoelectric focusing (IEF) with carrier ampholytes (CA) and HIEF. HIEF yields superior results in PGM typing from bloodstain extracts, whereas for ESD and ACP typing isoelectric focusing with carrier ampholytes seems to be the method of choice.

Key words: Blood groups, PGM1, ESD, ACP – Bloodstains, PGM1, ESD, ACP determination by HIEF

Zusammenfassung. PGM, ESD, and ACP werden in Blutspuren durch IEF mit CA und durch HIEF bestimmt. Das HIEF zeigt bessere Ergebnisse bei der Typisierung von PGM in Blutspuren während sich für die Typisierung von ESD und ACP die IEF mit als bessere Methode erweist.

Schlüsselwörter: Blutgruppen, PGM1, ESD, ACP – Blutspuren, Nachweis von PGM1, ESD, ACP

Introduction

The introduction of isoelectric focusing (IEF) with carrier ampholytes (CA) caused a revolution in bloodstain analysis, not only because some enzyme and protein polymorphisms were discovered and subtyped with the method, but also because of its great power of discrimination. IEF on polyacrylamide gels has become the electrophoretic method of choice for the detection of protein and enzyme polymorphisms in bloodstains.

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The introduction of the immobilized pH gradients (IPGs) by Bjellqvist et al. in 1982 [1] appeared to hold another revolutionary promise. IPGs have a number of advantages over conventional IEF. Its power of resolution is 10 times greater, results are more easily reproduced, any pH gradient can be created, a sample of up to 10 times greater can be loaded, and finally certain IEF problems, such as salt loading and cathodic drift, are eliminated.

Nevertheless, the great potential of IPGs for the study of polymorphic enzymes in bloodstains is limited by its sensitivity, which is less than that of IEF with CA [2–4].

To overcome this disadvantage, a new method called hybrid isoelectric focusing (HIEF) was recently developed by Altland and Rossmann [5]. In HIEF, IPGs are rehydrated with the appropriate CA prior to IEF. A preliminary study of the usefulness of HIEF for the identification of PGM1 subtypes in bloodstains has recently been published by Burgess et al. [6].

In this paper we report the advantages and disadvantages of HIEF as compared to IPGs and IEF with CA for the analysis of certain enzymes in bloodstains.

Material and methods

Twenty-microliter bloodstains from people with known PGM1, ACP, and ESD phenotypes were prepared on cotton cloth and air-dried at room temperature. Two weeks later, the bloodstains were extracted in 30 µl 0.05 *M* dithiothreitol (DDT) overnight.

To test the sensitivity of the method doubling dilution series of bloodstain extracts were prepared to a final dilution of 1:64.

All experiments were carried out using LKB systems 2297 Macrodrive 5 and 2217 Ultrophor (LKB, Bromma, Sweden).

Polyacrylamide gel isoelectric focusing was carried out in 0.4 mm polyacrylamide gels at gel concentration of (T = acrylamide + bis/100) T = 4.5% and cross-linking of (C = bis/acrylamide + bis) C = 2.8%. Sucrose (Merck) was added as a stabilizing agent at a total concentration of 12% (w/v). Ampholine (LKB, Bromma, Sweden) concentration was 2% (w/v). Polymerization was carried out with 2% (v/v) riboflavin (BioRad) solution (0.3% w/v) under UV light (360 nm).

A pH range of 5–7 was used for PGM1 subtyping, and samples were applied on Whatmann 3MM filter paper (0.5 × 0.5 cm) at a distance of 2 cm from the anode. The electrode solutions were 1% ethanolamine for the cathode and 1% acetic acid for the anode. Focusing was carried out at 5 W constant power. A maximum voltage of 2000 V with unlimited current was applied. IEF was carried out for 3 h at a cooling temperature of +10°C. After focusing PGM isoenzymes were developed using the conventional agar-overlay method of Sutton and Burgess [7].

The pH range used for ESD subtyping was pH 4–6. Samples were applied on Whatman 3 MM filter paper at a distance of 1 cm from the cathode. The electrode solutions were as above. Focusing was carried out as above. Gels were stained with 4-methyl-umbelliferyl acetate according to the method of Hopkinson et al. [8].

The pH range used for ACP typing was pH 4–8, and the samples were applied using Whatman 3 MM filter paper at a distance of 3 cm from the anode electrode strip. The electrode solutions were 1% (w/v) NaOH for the cathode and 1% (v/v) acetic acid for the anode. Focusing was carried out at 5 W constant power. A maximum voltage of 1500 V with unlimited current was applied. IEF was carried out for 2 h at a cooling temperature of +10°C. Gels were stained with 4-methyl-umbelliferyl phosphate according to Swallow et al. [9].

For HIEF, IPG gels were first cast according to Bjellqvist et al. [1] on a backing of GelBond PAG film. The gel measured 260 × 100 × 0.5 mm with the pH gradient along the short

Table 1. Volume (µl) 0.2 *M* Immobiline pK

pk	Acidic dense solution			Basic light solution		
	ACP	ESD	PGM1	ACP	ESD	PGM1
3.6	351	176	338	87.5	–	91
4.6	127	375	–	61.5	431.5	–
6.2	208	431.5	408.5	65.5	431.5	353.5
7.0	66.5	–	–	172.5	–	–
8.5	173	–	–	173	–	–
9.3	–	–	–	–	101	–

pH range: ACP 5–8, ESD 4.8–5.8, PGM1 5.6–6.6

dimension. A linear pH gradient was poured using a micrograd ient mixer from a dense acidic solution containing the Immobilines (LKB, Bromma, Sweden) listed in Table 1, 1.25 ml acrylamide-Bis solution (29.1 g acrylamide and 0.9 g Bis to 100 ml with distilled water), 2.1 ml glycerol (87%), 10 µl TEMED (Bio-Rad) solution (50 µl TEMED in 0.5 ml distilled water), and 10 µl ammonium persulfate solution (50 mg ammonium persulfate in 0.5 ml distilled water) and distilled water to a total volume of 7.5 ml, basic and light solution containing the Immobilines listed in Table 1, acrylamide-Bis, TEMED and ammonium persulfate solution as above, and distilled water to a total volume of 7.5 ml.

After the solutions were poured, the mold was left for 15 min at room temperature to allow the density gradient to stabilize, after which it was warmed for 1 h at 50°C to polymerize the gel. Following polymerization, the gel was washed six times, 10 min each with distilled water and usually used within 1 day or covered with a plastic film and stored at 20°C until needed. Finally, prior to focusing, dried gels were rehydrated in their mold with a solution of 1% Ampholine pH 5–7 for PGM1, 2% Ampholine pH 4–6 for ESD, and 1% Ampholine 4–6 and 1% Ampholine 6–8 for ACP. The electrode solutions were 10 m*M* NaOH at the cathode and 10 m*M* glutamic acid at the anode. Samples were applied on Whatman no. 3 MM filter paper at a distance of 1 cm from the anode for PGM and ACP and a 1 cm from the cathode for ESD. Focusing was carried out at 7 W, 4 mA, and 3000 V for 3 h. After focusing isoenzymes were visualized as above.

Results and discussion

Although PGM1 subtypes are easily recognized using both IEF and HIEF methods, bands are sharper and straighter using HIEF (Fig. 1). This is especially true in the case of contaminated bloodstains (salts, etc.), since salts produce wavy bands in IEF with conventional CA. In addition, bands are better separated with HIEF, and a much greater quantity of samples can be loaded in HIEF gels.

HIEF gave good results down to the final extract solution of 1:32 when 10 µl was loaded on Whatman paper. These results are similar to those seen with IEF with conventional CA and superior to those observed with IPG gels without rehydration with CA (1:4). These results are equivalent to those previously published by Burgess et al. [6].

Although some of the advantages of HIEF observed for PGM (such as the sharpness and straightness of the bands, the amount of sample that can be loaded, and the lack of interference of salts and contaminants) were also seen

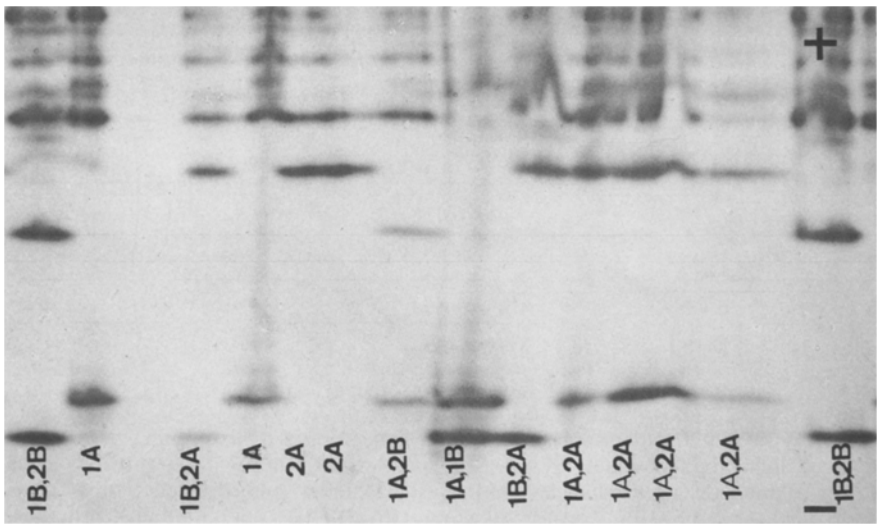

Fig. 1. PGM1 subtypes by HIEF (IPG gels pH 5.6–6.6 rehydrated with 1% Ampholine pH 5–7) in 2-week-old bloodstains

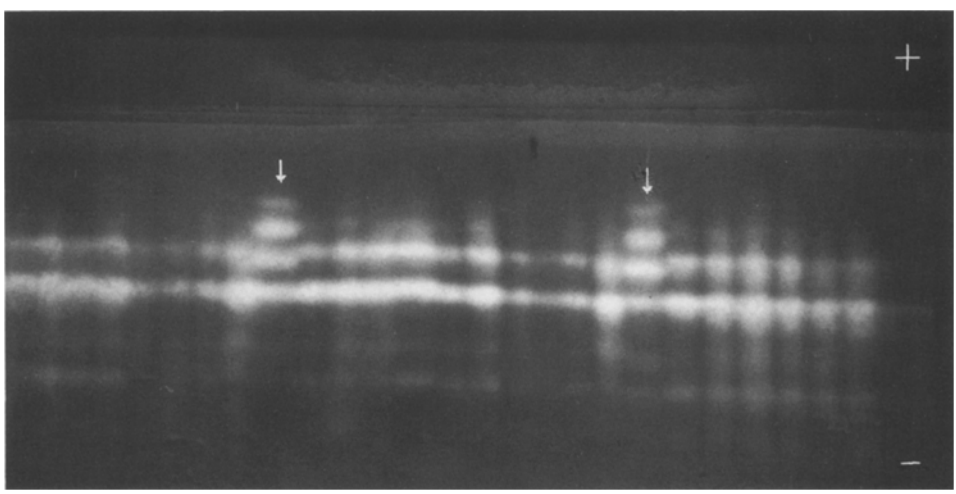

Fig. 2. ESD phenotypes by HIEF (IPG gels pH 4.8–5.8 rehydrated with 2% Ampholine pH 4–6) in 2-week-old bloodstains. ESD*5 allele (indicated by *arrows*) is clearly separated, but ESD*1 and ESD*2 alleles are not well distinguished

with ACP and ESD, HIEF has in the latter serious handicaps: ACP typing by HIEF is extremely expensive (because five different Immobilines must be used to cover a pH range of 3 or 4 units); the ACP*A allele is not perfectly separated from hemoglobin (Hb) bands; and although the ESD*5 allele (Fig. 2) can be better separated with HIEF, the alleles ESD*1 and ESD*2 are not well distin-

guished. As for ACP, ESD can be detected in 1:32 dilutions with HIEF as well as with CA.

HIEF is therefore clearly to be the method of choice for demonstrating PGM polymorphism in bloodstains, while IEF with CA is preferable for ACP and ESD.

Finally, given the clear advantage of HIEF in bloodstain analysis, its utility in the detection of other polymorphisms (proteins for example) should be investigated.

References

1. Bjellqvist B, Ek K, Righetti PG, Gianazza E, Görg A, Westermeier R, Postel W (1982) Isoelectric focusing in immobilized pH gradients: principle, methodology and some applications. J Biochem Biophys Methods 6:317–339
2. Altland K, Hackler R, Rossmann U (1986) Avoiding liquid exudation on the surface of rehydrated gels used for hybrid isoelectric focusing in carrier ampholyte supplemented immobilized pH gradients. Electrophoresis. 7:251–259
3. Altland K, Eckardstein A, Banzhoff A, Wagner M, Rossmann U, Hackler R, Becher P (1987) Hybrid isoelectric focusing: Adsorption of proteins onto immobilized pH gradient matrices and desorption by carrier ampholytes. Electrophoresis 8:52–62
4. Righetti PG, Chiari M, Gelfi C (1988) Immobilized pH gradients: Effect of salts, added carrier ampholytes and voltage gradients on protein patterns. Electrophoresis 9:65–73
5. Altland K, Rossmann U (1985) Hybrid isoelectric focusing in rehydrated immobilized pH gradients with added carrier ampholytes: Demonstration of human globins. Electrophoresis 6:314–325
6. Burgess RM, Sutton JG, Werrett DJ (1987) A comparison of the isoelectric focusing patterns of red cell phosphoglucomutase separated on an Ampholine, Ampholine/separator and immobilized pH gradient. Forensic Sci Int 35:213–218
7. Sutton IG, Burgess R (1978) Genetic evidence for four common alleles at the phosphoglucomutase-1 locus (PGM1) detectable by isoelectric focusing. Vox Sang 34:97–103
8. Hopkinson DA, Mestriner MA, Cortner J, Harris H (1973) Esterase D: A new human polymorphism. Ann Hum Genet 37:119–137
9. Swallow DM, Povey S, Harris H (1973) Activity of red cell acid phosphatase locus in other tissues. Ann Hum Genet 37:31–38

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