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Isoelectric Focusing in Agarose: Phosphoglucomutase (PGM Locus 1) Typing

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ABSTRACT: The subphenotypes of phosphoglucomutase (PGM) were successfully typed by isoelectric focusing with a charge-balanced agarose. The resolution was as high as that obtained with polyacrylamide, with none of the drawbacks associated with the use of polyacrylamide. Exceptionally straight iso-pH lines were obtained, making interpretation of the results more reliable. Gel preparation was simple and rapid, and the time for analysis was reduced. A way of preserving the resultant zymogram is described.

KEYWORDS: pathology and biology, genetic typing, phosphoglucomutase, isoelectric focusing

The high resolving power of isoelectric focusing (IEF) has opened up a new era in the phenotyping of polymorphic systems. The enzyme phosphoglucomutase (PGM) was the first system that was found by IEF to possess more phenotypes than were originally demonstrated by conventional electrophoresis [*1,2*]. This blood grouping system has attracted much interest because of its importance for paternity testing and forensic science work.

The phenotypes of the first locus enzyme of PGM (PGM is now known to possess ten common phenotypes rather than three) are coded by four alleles instead of two [*1-3*]. This has raised the discriminating power *DP* of the PGM system from 0.55 to 0.75 [*1,4,5*], making it almost as powerful as the Rh system (*DP* = 0.80). It is also readily applicable to the analysis of bloodstains. The high resolving power of IEF has also shown similar subphenotypes for the Gc system [*6*], among others.

Analytical IEF is often carried out with polyacrylamide gels, which have a number of disadvantages. Polymerization of polyacrylamide gels can prove troublesome: a poorly polymerized gel will never give good results. Difficulties can arise with wavy iso-pH bands, making discrimination between phenotypes difficult. Acrylamide is a known neurotoxin, and, lastly, ammonium persulfate is known to inactivate certain enzymes [*7,8*].

Agarose has none of these disadvantages. In addition, gel preparation is simple and agarose is chemically unreactive. Agarose provides ideal conditions for the detection of enzyme activity—a factor of great significance when low levels of enzyme activity, such as are encountered with bloodstains, are to be studied.

This paper reports the subtyping of the Locus 1 isoenzyme of PGM with a charge-balanced agarose specially developed for IEF. Agarose IEF is highly purified and has had residual negatively charged groups (which are an integral part of the agarose molecule)

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balanced out by the addition of stable positive charges. The problem of flooding on the gel surface—a characteristic of gels carrying unbalanced charges—has been virtually eliminated [9]. Therefore the pH gradient was highly controlled and high resolution results were obtained.

Materials and Methods

Reagents and Equipment

Agarose IEF, Pharmalyte® 4-6.5, and accessories including GelBond® were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Sorbitol, sulfuric acid, and sodium hydroxide were all of the highest available grade.

Isoelectric focusing was performed with an FBE 3000 flat bed apparatus in conjunction with an ECPS 3000/150 constant power supply (Pharmacia Fine Chemicals).

Procedure

Agarose IEF gels were cast and used according to the manufacturer's recommendations. Agarose IEF (0.3 g) and sorbitol (3.6 g) were dissolved in 27.0 mL of distilled water by boiling briefly. After the solution cooled to 75°C, 1.9 mL of Pharmalyte 4-6.5 was added, and the gel was cast upon GelBond polyester film [9].

Red cell lysates were prepared from washed and packed red cells by freezing them with an equal volume of distilled water. No anticoagulant was used because excess salt detrimentally affects both conventional electrophoresis [10] and IEF by disturbing the conductivity profile across the gel.

Bloodstains were stored at room temperature away from direct light. Bloodstain extracts were prepared by extraction with the minimum quantity of distilled water for approximately 10 min.

Because no polymerization products are present in an agarose gel, the gels were not "prerun." Paper applicators were placed on the gel surface (2 cm from the anode), and the samples were then placed on the applicators. Two or three microlitres of red cell lysates was used. For bloodstains, up to 25 μL of extract was applied to each applicator. If the sample was very dilute, more than one applicator was used for a particular sample. In this way, flooding on the gel surface was avoided. A control sample, containing a mixture of the four isoenzymes, a+, a−, b+, and b−, was applied at intervals across the gel surface. It is advisable to remove the sample applicators, particularly of bloodstains, after 45 min to prevent trailing of the sample.

The constant power supply was set to deliver a maximum of 1500 V and 15 W, current unlimited. A coolant temperature of 10°C was used, and the gel was focused for 1½ h (1800 to 2100 V·h). The distance between electrodes was 95 mm. Enzyme activity was detected with a standard overlay technique [11].

It was possible to preserve the results from the experiments using Agarose IEF on the plastic sheet (GelBond). One piece of filter paper, followed by several layers of paper towels, was placed onto the gel. Then a glass plate holding a weight of around 1 kg was placed on top. The gel was dried almost completely (it is important to remove unreacted 3-(4,5-dimethyl thiazolyl-2)-2,5 diphenyl tetrazolium bromide [MTT] and phenazine methosulfate), washed briefly with distilled water, and finally dried with a hair dryer. Gels dried in this way have been stored successfully for at least one year.

Results and Discussion

Pharmalyte 4-6.5 was selected as the correct pH interval of ampholyte for PGM subphenotyping. With this ampholyte, the PGM isoenzymes focused near the center of the gel.

The center region of an IEF gel is always least prone to disturbances and is the ideal region in which proteins of interest should be focused.

Straight iso-pH lines were obtained (Fig. 1), resulting in straight PGM isoenzyme bands. The risk of misinterpretation of the a+, a−, b+, and b− bands is therefore minimized, and so a control sample need not be included at every fourth position (the standard practice with polyacrylamide gels). The clarity of the results was attributed to the close control over electroosmosis (which causes gradient drift [9]) by the balanced charge of the Agarose IEF gel.

In Fig. 1 lysates of various ages were used, including some that had been stored deep-frozen for up to 5½ months. The four isoenzymes were easily classified. The straightness of the iso-pH lines is superior to that achieved on polyacrylamide gels [*1, 2, 12*] and is superior to agarose gels that are not charge-balanced [*12*]. Sample 10 in Fig. 1, classified as a 1+1−, shows the typical storage band that has a pI intermediate between the b− and b+ isoenzymes and is not to be confused with the c, d, e, and f bands [*13*].

Also apparent in Fig. 1 is the band of Sample 3 exhibiting PGM activity that is cathodic in position to the a− isoenzyme. The origin of this band is not known. It has been observed in both polyacrylamide and agarose gels. It is more prominent in bloodstains and old samples, which suggests it may be a breakdown product. Possibly it is an artifact, but consideration of it in the light of suggestions made by Chrambach [*14*] and others [*15*] makes this doubtful. It is important to note that this band in no way interferes with the interpretation of the results

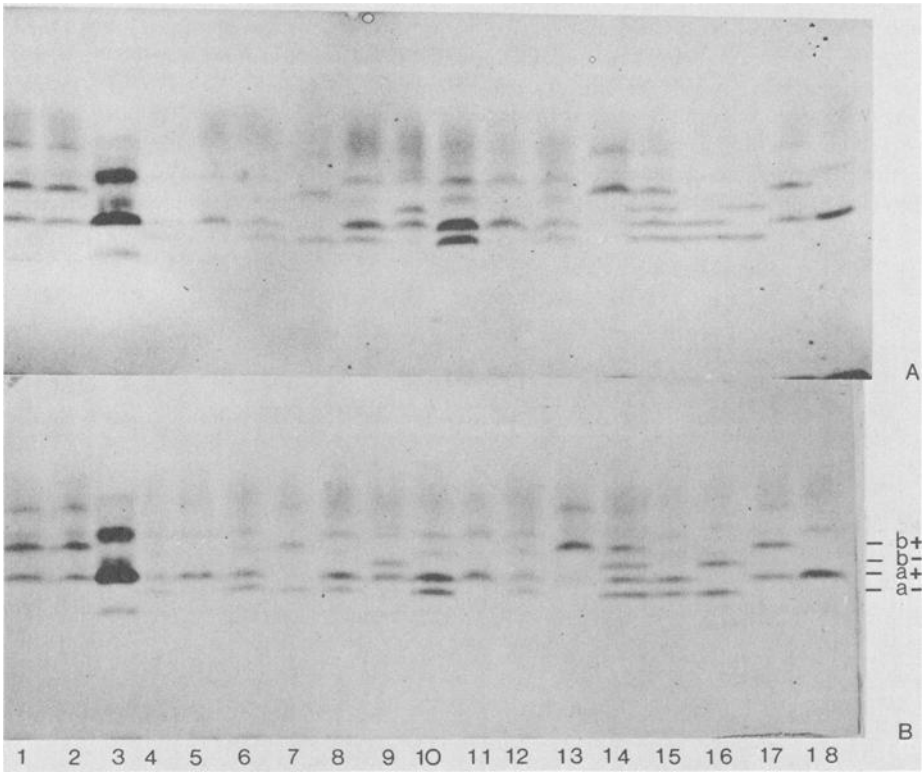

FIG. 1—*Duplicate analyses of the same samples:* (A) *a fresh zymogram and* (B) *a dried and preserved zymogram. Both analyses were done at the same time. Samples 3, 5, 11, and 18 are Type 1 + ; 1, 2, and 17, Type 1 + 2 + ; 16, Type 1 − 2 − ; 4, 6, 8, 10, 12, and 15, Type 1 + 1 − ; 13, Type 2 + ; 9, Type 1 + 2 − ; 7, Type 1 − 2 + ; and 14, control. Samples 2 and 14 through 18 were from fresh lysates. Sample 3 was a bloodstain, and the remainder were lysates stored for 5½ months at −20°C.*

as its pI point is too far removed from that of the a− isoenzyme for confusion to occur. The use of adequate control samples prevents any difficulties.

Discrimination between the four isoenzymes of importance was readily made. The results were reproducible, as can be seen by comparison of Fig. 1A with its duplicate gel (Fig. 1B). Identical results were achieved. Furthermore, Fig. 1B demonstrates that it was possible to preserve the dried zymogram. Loss in resolution was minimal. After exposure to light during photography, the background coloration increased slightly in intensity. Convenient storage of results permits evaluation of the original gel at any time. The particular gel shown was photographed after six months' storage.

Bloodstains were also analyzed by this technique, and typical results are shown in Fig. 2. Stains up to two weeks old were analyzed. The band showing PGM activity cathodic to the a− isoenzyme can be seen (and was used as a reference marker). With the exception of Sample 7, all phenotypes shown here were readily classified. Other bloodstains prepared from blood of this donor, who was Type 1+2+, were successfully typed. Distortions such as this are typical for samples containing excess salt.

No advantage was gained by the use of a lower voltage. Identical results were achieved with 600 V (maximum) for 3¼ h (1980 V·h) and with the recommended 1500 V for 1½ h (2070 V·h). There was no loss in activity through the use of a higher voltage, and the time required was reduced by half.

By using the power settings described here, satisfactory results were obtained within 1¼ to 2 h. Overfocusing must be avoided because proteins are less stable at their pI points and therefore the generation of breakdown products is encouraged. Underfocusing, in the analysis of PGM, is not a disadvantage, provided separation is adequate (that is, just over 1 h). In this instance electrophoresis in a pH gradient is achieved, rather than true isoelectric focusing, because a proper equilibrium will not have been reached.

Agarose gels are more suitable for immunotechniques than are polyacrylamide gels, and the system described here should also be successful for the sequential analysis of Gc phenotypes by immunofixation, followed by development for PGM activity on the same gel. Both Gc and PGM phenotypes have pI points that lie within the ampholyte range used here

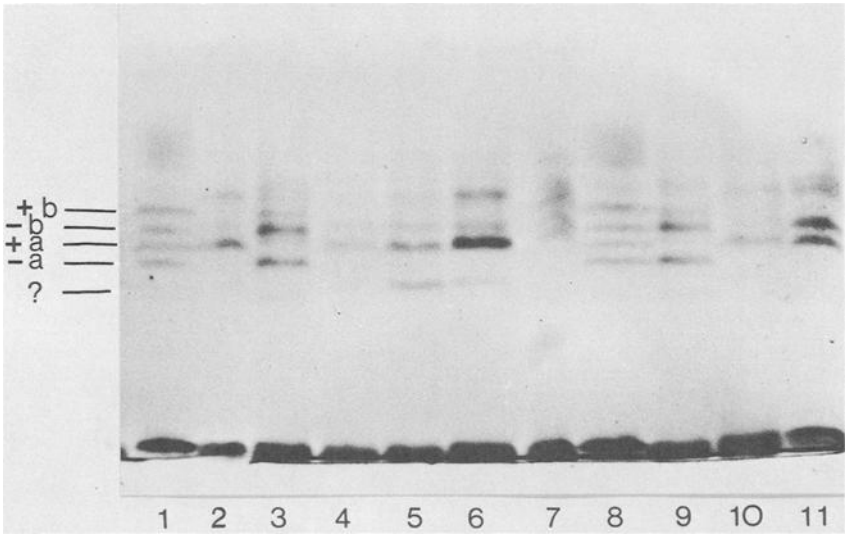

FIG. 2—*Analysis of bloodstains. Samples 2, 4, 5, 6, and 10 are Type 1 + ; 11, Type 1 + 2 − ; 3 and 9, Type 1 − 2 − ; 1 and 8, controls; and 7, inconclusive.*

(PGM isoenzymes have pI points in the range of pH 5 to 6 [*11*], while Gc components focus within the pH range 4.8 to 5.1 [*6*]). This approach not only simplifies techniques but also possibly reduces sample requirements. Consideration must be given to the use of suitable controls and sample pretreatment because PGM activity is concentrated in erythrocytes whereas Gc components are associated with serum.

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