Red blood cell protein map: a comparison between carrier-ampholyte pH gradient and immobilized pH gradient, and identification of four red blood cell enzymes

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Applied and Theoretical Electrophoresis (1992), 3, 77–82

Red blood cell protein map: a comparison between carrier-ampholyte pH gradient and immobilized pH gradient, and identification of four red blood cell enzymes

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The aim of this study was (a) to establish a red blood cell (RBC) protein map with immobilized pH gradient for the first dimension (b) to compare the pattern with previously published RBC protein map obtained with carrier-ampholyte pH gradients and (c) to localize four new enzymes on the map (i.e. 6-phosphogluconic dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, glutathione peroxidase and superoxide dismutase). This publication provides the most updated RBC polypeptide pattern with twelve proteins or enzymes localized on the map.

Keywords: Two-dimensional electrophoresis; erythrocyte; 6-phosphogluconic dehydrogenase; glyceraldehyde-3-phosphate dehydrogenase; glutathione peroxidase; superoxide dismutase.

Introduction

Two-dimensional electrophoresis has been used to separate many kinds of proteins such as proteins excreted in urine (Anderson *et al.*, 1979), tissue proteins (Anderson *et al.*, 1978), muscle proteins (Giometti *et al.*, 1979), human semen (Edwards *et al.*, 1981), leukemic cells (Anderson *et al.*, 1983), human chorionic villi (Trnka *et al.*, 1984) and white blood cells (Gemmell *et al.*, 1982). Since the original work of O'Farrel (1975), many improvements have been made to increase the separation, resolution and detection of proteins.

Two-dimensional mapping of plasma proteins is now reaching a stage of technical adequacy, but there exists only few mapping studies on erythrocyte proteins. Accordingly, information on protein components of human red blood cells is limited. This work developed the methodologies used to obtain the two-dimensional pattern of human red blood cell proteins, the purpose being to determine the best suitable method for mapping studies.

The methods used in the present study were (a) ampholyte capillary tube gels as previously described by Hochstrasser *et al.* (1989b) and (b) a modification of the one developed by Hochstrasser *et al.* (1988a&b), which consisted in the replacement of carrier-ampholyte pH gradient by immobilized pH gradient (Bjellqvist, B. & Hochstrasser, D., manuscript in preparation). Results obtained with these two techniques are shown for comparison.

Establishment of this RBC protein map will permit the study of proteins in different pathologies. The working hypothesis is that the map will become more useful as more spots are identified, and so contribute to a further understanding of the phenomena taking place in RBC's.

In this study, four human red blood cell enzymes (6-phosphogluconic dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, superoxide dismutase and glutathione peroxidase) were also localized, therefore extending the work of Edwards *et al.* (1979).

Materials and methods

Apparatus

Isoelectric focusing (IEF) was performed either with the Multiphor II and the Immobiline DryStrip Kit (Pharmacia-LKB, Uppsala, Sweden) or with the Model 175 Tube Cell (Bio-Rad, Richmond, CA) and SDS-PAGE in the Protean II Multi-Cell (Bio-Rad, Richmond, CA). The Model 395 Gradient Former (Bio-Rad, Richmond CA), a Masterflex microprocessor pump drive (Cole-Parmer, Chicago, IL), and the Protean II Multi-Gel Casting Chamber (Bio-Rad, Richmond, CA) were used. Power was supplied with a Model 3000 Xi Electrophoresis Power Supply (Bio-Rad, Richmond, CA) for SDS-PAGE, and MultiDrive XL (Pharmacia-LKB, Uppsala, Sweden) for IEF.

Protein quantification was performed with a Perkin-Elmer 'Lambda 2' spectrometer (Perkin-Elmer, Norwalk, CT).

Reagents

Reagents were obtained from the following sources: PDA, APS, TEMED, SDS-PAGE Molecular Weight Standards, and IEF Standards (Bio-Rad, Richmond, CA); SDS, Tris, urea, sodium thiosulfate, glycine, acrylamide (Fluka, Buchs, Switzerland); CHAPS, iodoacetamide, 6-phosphogluconic dehydrogenase (EC 1.1.1.44), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), superoxide dismutase (EC 1.15.1.1), glutathione peroxidase (EC 1.11.1.9) (Sigma, St. Louis, MO); ampholytes pH 4–8, ampholytes pH 3.5–10

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Received 13 August 1991; accepted 17 April 1992

(BDH, Poole, England); Immobiline strips (courtesy of the department of internal medicine, Geneva), ampholytes pH 9–11 (Pharmacia-LKB, Uppsala, Sweden); DTE (Merck, Darmstadt, Germany).

Preparation of red blood cells

Fresh blood samples from one healthy donor were collected into a tube containing EDTA (Becton Dickinson Vacutainer Systems). Blood was then immediately centrifuged at 2500 rpm at 4°C for 10 min. Plasma and buffy coats were removed with a sterile glass pipette. Red blood cells (RBC's) were washed three times with the same volume of PBS pH 7.4 (Sambrook *et al.*, 1989). RBC's were then placed into 500 µl aliquots and stored at –80°C until analysis.

Protein quantification

Protein concentration of total RBC's was determined according to the method described in the Bio-Rad Protein Assay Kit, which is based on the Bradford dye-binding procedure.

Preparation of samples for two-dimensional analysis

Different amounts of RBC's and enzyme solutions were solubilized by mixing them with different amounts of a 6 ml solution containing 0.1 g DTE, 0.4 g CHAPS, 5.4 g UREA, 0.5 ml ampholytes pH 9–11 (Pharmacia-LKB) (Table 1).

Isoelectric Focusing (IEF) and SDS Gel Electrophoresis

IEF was executed either with Immobiline strip gels according to the method of Bjellqvist & Hochstrasser (manuscript in preparation) or with ampholyte capillary tube gels as previously described by Hochstrasser (1988b). After IEF, SDS electrophoresis was performed on vertical slab gels with the Protean II Multi-Cell. Running conditions were 40 mA per gel at 6°C.

Table 1 Amount of samples for the first dimension

Samples	*Amount of sample µl*	*Buffer solution µl*	*Loading solution µl*
RBC's	10	245	30
PGDH	1	100	30
GAPDH	10	290	30
GPX	3	30	33
SOD	3	150	30
RBC +	5		
PGDH	3	245	30
RBC +	5		
GAPDH	20	245	30
RBC +	3		
GPX	9	240	30
RBC +	5		
SOD	3	245	40

Concentration of purified enzyme solutions: PGDH 1.8 mg ml–1, GAPDH 5 mg ml–1, GPX 1.5 mg ml–1, SOD 3 mg ml–1. Concentration of RBC proteins: 187 mg ml–1. (Abbreviations: PGDH = 6-phosphogluconic dehydrogenase, GAPDH = glyceraldehyde-3-phosphate dehydrogenase, GPX = glutathione peroxidase and SOD = superoxide dismutase)

Silver staining

Gels, crosslinked with PDA and polymerized in presence of thiosulfate, were stained according to the procedure of Oakley *et al.* (1980) with the modification of Hochstrasser *et al.* (1988c), i.e. fixation with glutaraldehyde, coloration with ammoniacal silver, and development with a formaldehyde and citrc acid solution. The development lasted 15 min. Gels were then fixed in 5% acetic acid.

Identification of red blood cell enzymes

A typical run consisted of co-electrophoresis of three samples: the first used RBC's alone as the reference, the second RBC's with addition of an excess of a purified enzyme, and the third was the purified enzyme alone (Table 1). Two-dimensional electrophoresis of purified enzymes and enzyme-enriched RBC's were compared to that of RBC's alone. The gels were superimposed on a transilluminator in order to determine the position of the purified enzyme on the RBC's pattern. Gel patterns were oriented conventionally, i.e., with acidic proteins to the left and with the high MW proteins to the top of the gel. Molecular weight and isoelectric scales were determined using SDS-PAGE MW and pI markers.

The enzyme solutions contained minor impurities. To precisely localize the enzymes, unconcentrated solutions were used. The spots were identified and their MW corresponded exactly to those published previously (Table 2).

Table 2 Characteristics of erythrocyte enzymes and proteins

Enzymes	*Structure*	*MW*	*References*
PGDH	dimeric	52 kDal	Pearse *et al.*, 1975
GAPDH	tetrameric	37 kDal	Watson *et al.*, 1972
GPX	dimeric	22 kDal	Takahashi *et al.*, 1987
SOD	dimeric	19 kDal	Lieman-H. *et al.*, 1981
PK	tetrameric	63 kDal	Kahn *et al.*, 1982
Catalase	tetrameric	60 kDal	Schonbaum *et al.*, 1976
GPDH	tetrameric	53 kDal	Cohen, 1975
Actin	dimeric	42 kDal	Cohen, 1983
Carb. an.	monomeric	30 kDal	Linskog *et al.*, 1971
Hemoglobin	tetrameric	17 kDal	San george *et al.*, 1986

(Abbreviations: PK = pyruvate kinase, GPDH = glucose-6-phosphate dehydrogenase, Carb. an. = carbonic anhydrase, for the others see Table 1)

Results

Both techniques allowed good separation and focalization of proteins. About 900 spots could be detected. Molecular weights of monomeric and protein subunits ranged from about 10 kDa to 200 kDa.

Two-dimensional electrophoresis using immobilized pH gradient (Figure 1) showed its superiority for mapping studies on carrier-ampholyte pH gradient (Figure 2), because patterns obtained with carrier-ampholytes had less spots and lower resolution towards acidic and basic proteins. Moreover, immobilized pH gradients had higher loading capacities without any

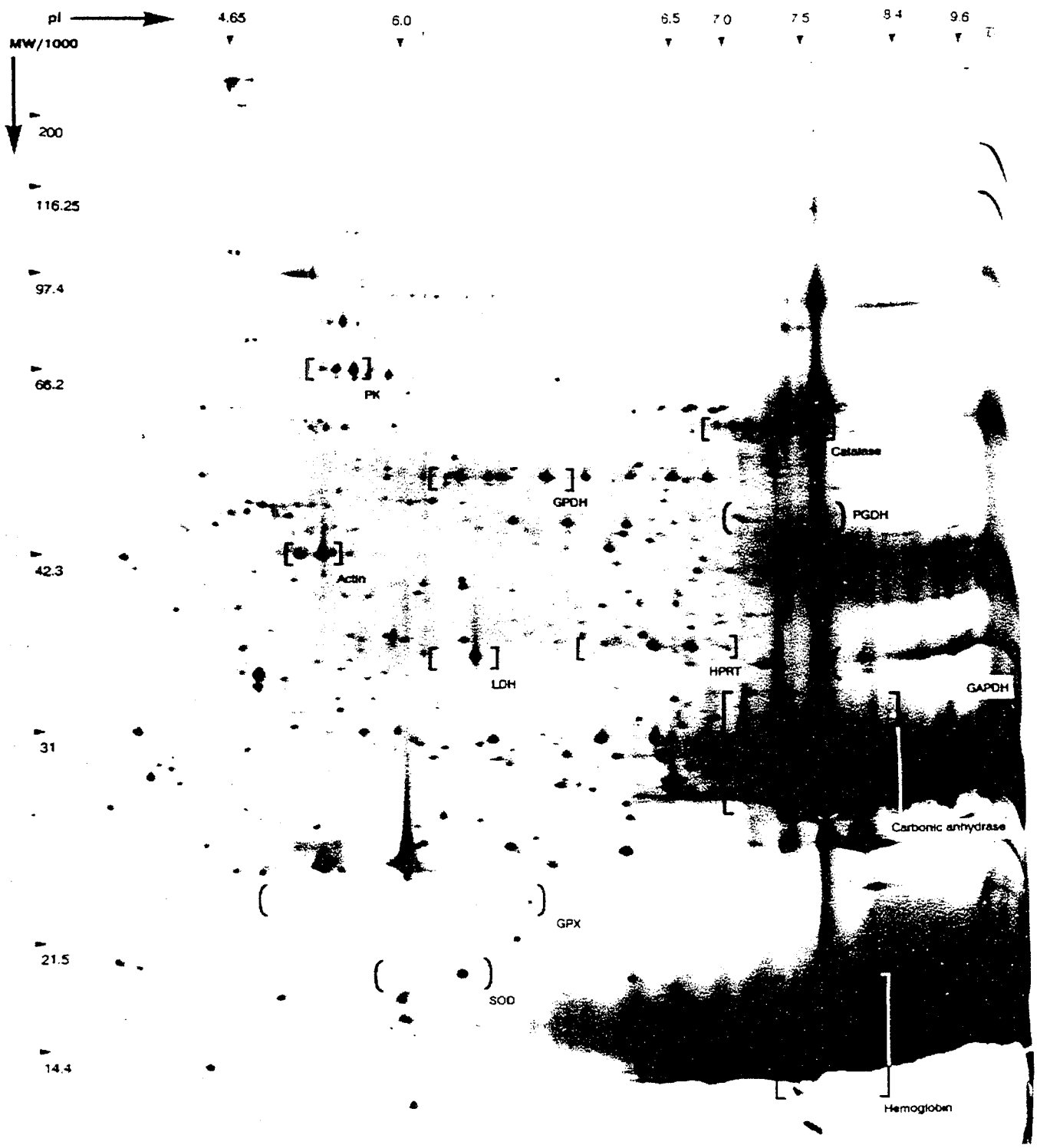

Figure 1 Two-dimensional electrophoresis of RBC proteins. First dimension with immobilized pH gradient. Identified enzyme subunits are indicated by round brackets. Enzyme subunits identified earlier by Edwards *et al.* (1979) are indicated by square brackets.

background increase, and no pH variations. Gels obtained with Immobiline strip gels could be directly superimposed on a transilluminator to determine spot positions. This procedure was not possible with 2D-gels obtained with ampholyte capillary tube gels.

The localization of enzyme subunits identified by electrophoresis of purified samples are indicated by braces in Figure 1. The enzyme subunits localized earlier by Edwards *et al.* (1979) are indicated by square brackets. Two-dimensional gels of purified enzymes and enzyme-enriched RBC's are shown in Figure 3. The sections shown in Figure 3 represent about 12.5% of the total surface of a gel. Identified proteins are between square braces.

Figure 2 Two-dimensional electrophoresis of RBC proteins. First dimension with carrier-ampholyte pH gradient.

Discussion

RBC protein patterns obtained in this study were comparable to those obtained by Edwards *et al.* (1979) and Goldman *et al.* (1985). The technique used here for the localization of proteins has been previously described (Trnka *et al.*, 1984). It is a simple technique, and avoids the utilization of antibodies.

Whole RBC protein mixtures obtained from human blood samples are quite different from commercially available enzyme solutions. Consequently, these different protein mixtures influenced differently the pH gradient obtained with carrier-ampholytes and made the RBC protein pattern comparison difficult. In Immobiline systems, the buffering groups responsible for generating the pH gradient are covalently bound to the gel matrix, and thus the pH gradient is 'pre-established'. Immobiline gradients are thus unaffected by the protein sample composition. As a consequence, Immobiline gel strips were preferentially used for our experiment instead of ampholyte tube gels.

A critical point for the localization of enzymes was the degree of purity obtained from commercially available enzyme preparations. The number of subunits and the molecular weights were the most important characteristics for enzyme localization. But, by comparison with proteins of known molecular weight (Table 2), it was easy to identify the majority of impurities. Some impurities were, on the other hand, easily identified since they were known proteins present in high concentration in erythrocytes (e.g. hemoglobin or catalase). Finally, dilution of the loading solution permitted to have only one enzyme or enzyme subunit appearing on the gel. The last

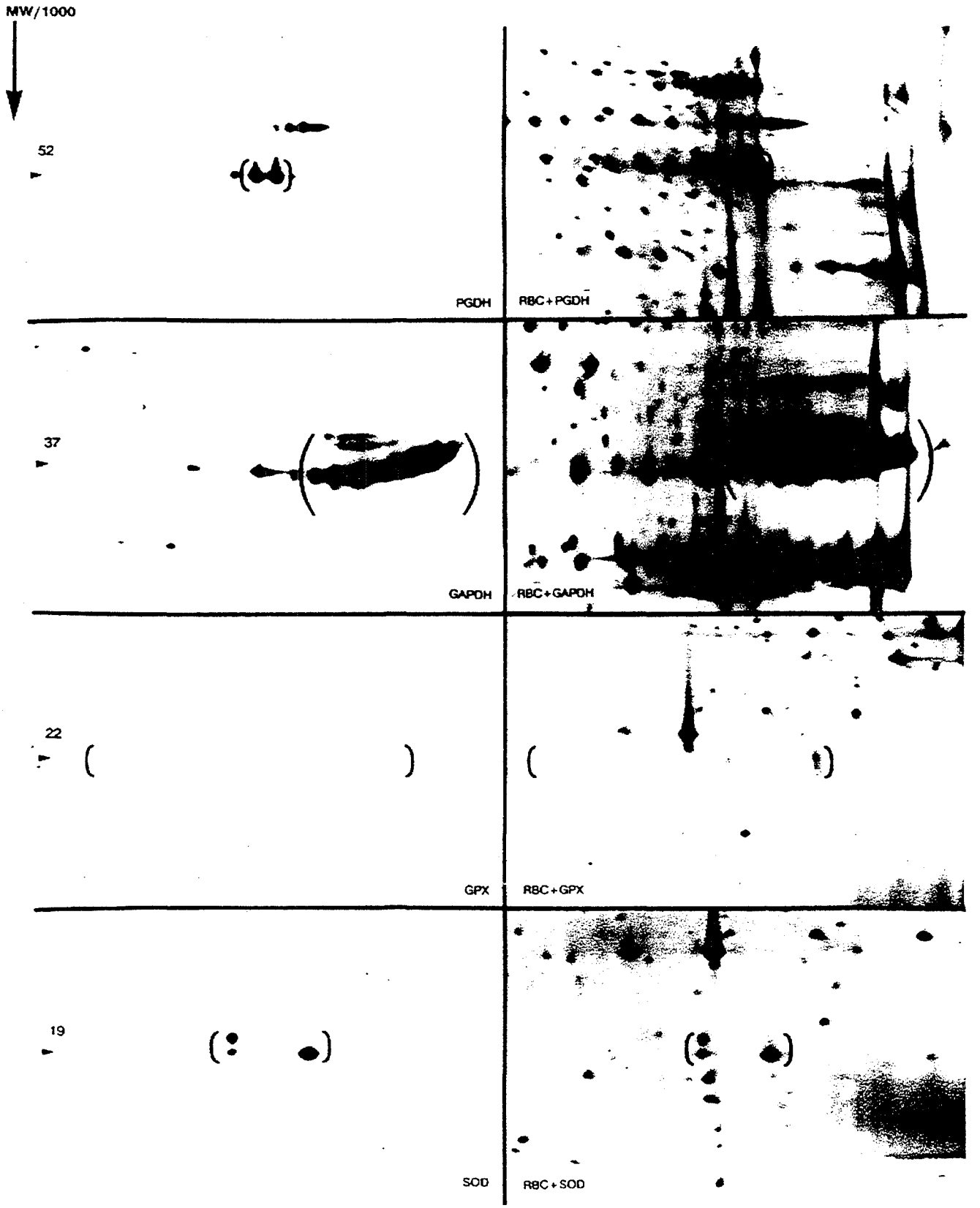

Figure 3 Two-dimensional electrophoresis of purified enzymes (left) and co-electrophoresis with RBC proteins (right). (PGDH: 6-phosphogluconic dehydrogenase, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, GPX: glutathione peroxydase, SOD: superoxide dismutase).

component appearing after several dilutions was the most concentrated, and hence the researched enzyme. As a consequence, it was by optical comparison, superimposition of gels and collection of critical information that localization of purified enzymes was achieved.

Conclusion

This work extended our current knowledge of two-dimensional red blood cell protein patterns and showed the superiority of immobilized pH gradients on carrier-ampholyte pH gradients. Mapping techniques could allow a better understanding and definition of red blood cell protein variants. Such variants will have clinical interest as they will be linked to several diseases and intracellular dysfunctions.

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