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Note

Electrofocusing of stroma-free haemoglobin and its derivatives in agarose isoelectric focusing gels

M. KRAMLOVÁ* and T. I. PŘISTOUPIL
Institute of Haematology and Blood Transfusion, Prague (Czechoslovakia)
and
J. KRAML
1st Department of Medical Chemistry, Charles University, Prague (Czechoslovakia)
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In a previous paper[1] we showed that isoelectric focusing of haemoglobin subfractions can be achieved in thin-layer gels of purified agarose C (Pharmacia, Uppsala, Sweden); however, the reproducibility of the results was not as satisfactory as with polyacrylamide gels[2] because of electroendosmosis. Rosén *et al.*[3] used gels of a new variant of agarose EF (LKB, Bromma, Sweden)[4] with extremely low electroendosmosis for the reproducible electrofocusing of various proteins in an Ampholine (LKB) pH gradient. Several advantages of electrofocusing in agarose EF gels were pointed out.

In this paper we describe the electrofocusing of haemoglobin and its derivatives in agarose IEF (Pharmacia)[5] gels and Pharmalyte (Pharmacia) pH gradient.

EXPERIMENTAL

Stroma-free haemoglobin (SFH) was a standard sample stored at −20°C for 1 year. Crude haemolysate of fresh human erythrocytes (either treated or untreated with carbon monoxide) was prepared by the addition of 4 volumes of distilled water to 1 volume of packed erythrocytes washed five times with sodium chloride solution (9 g/l)[2]. The reaction of oxyhaemoglobin (50 g/l) with a solution of pyridoxal-5-phosphate (Roche, Basle, Switzerland) was performed at 10°C for 30 min at a molar ratio of 1:1 and pH 7.4. The reaction of deoxyhaemoglobin (50 g/l) with glutaraldehyde was performed under a flow of nitrogen at 10°C for 60 min at a molar ratio of 1:5 and pH 7.1.

Prior to electrofocusing all haemoglobin samples were desalted on a 9 × 0.9 cm column of Sephadex G-25 Superfine in distilled water.

Isoelectric focusing in agarose IEF was performed with a Pharmacia FBE 3000 flat-bed apparatus and an ECPS 3000/150 constant-power supply following the procedure recommended by the manufacturer[5]. Pharmalyte was used to form a gradient of pH 5–8.

Procedure[5]

To prepare agarose gel, a mixture of 0.3 g of agarose IEF, 3.6 g of sorbitol and

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28 ml of distilled water was heated in a boiling-water bath until the agarose was dissolved (90°C). Then 1.9 ml of Pharmalyte (pH 5–8) were added and the mixture poured on a specially hydrophilized plastic film (Celbond) placed on a levelling table, pre-heated to 60–70°C. The gel was left to cool for 15 min on the levelling table and then transferred to a closed plastic box with moist paper, where it was stored overnight at room temperature.

Porous electrode strips were dipped in electrode solutions (anode solution, 0.05 *M* sulphuric acid; cathode solution, 1 *M* sodium hydroxide solution) and placed on a filter-paper for 1 min to remove excess of liquid. Volumes of 20 µl of desalted samples (haemoglobin concentration in the range 10–30 g/l) were applied on small pieces of filter-paper and laid on the surface of the gel. The constant-power supply was set to deliver a maximum of 15 W and 1500 V. The experiment was run for 90 min; after 45 min the run was interrupted and the sample applicators were removed. After the separation was completed the gel was immediately put into the fixing solution (5% sulphosalicylic acid + 10% trichloroacetic acid in distilled water) and left there for 30 min. Then the gel was washed twice for 15 min with the destaining solution (35% ethanol + 10% acetic acid in distilled water), dried using three pieces of filter-paper and a hair dryer, stained 10 min in the staining solution (0.2% Coomassie Blue G-250), destained for about 5 min and finally dried with a hair dryer.

RESULTS AND DISCUSSION

Fig. 1 shows that thin-layer electrofocusing in agarose IEF gel makes it possible to detect easily even slight differences between the patterns of variously treated human haemoglobin samples. There is a general similarity between the results and their reproducibility achieved in agarose IEF gel and in polyacrylamide gel[2]; however, work with agarose is easier and less hazardous[1,3,4]. Moreover, concentrated

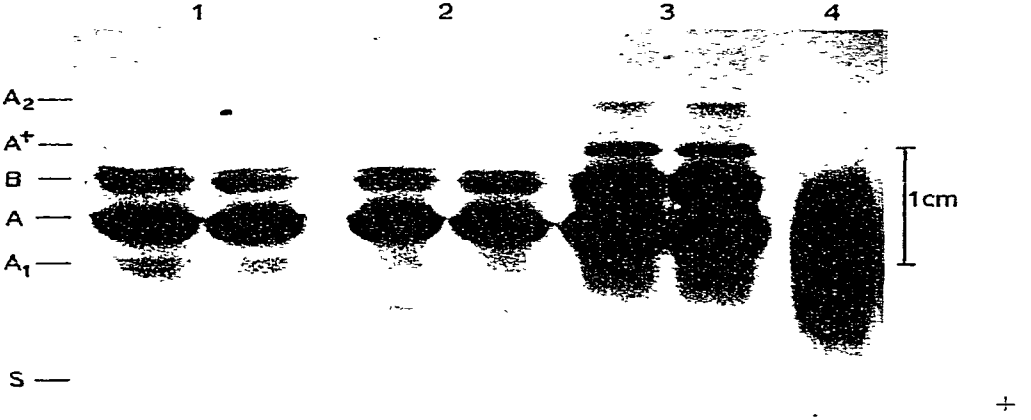

Fig. 1. Electrofocusing of native and modified human haemoglobin in agarose IEF gel. 1, Haemoglobin from fresh lysed erythrocytes; 2, as 1, treated with pyridoxal-5-phosphate; 3 = stroma-free haemoglobin (SFH) stored at −20°C for 1 year; 4 = SFH treated with glutaraldehyde. Ampholyte: Pharmalyte (pH 5–8). s = Position of the start. All samples were treated with carbon monoxide before electrofocusing; staining with Coomassie Blue G-250. Capital letters indicate the positions of main haemoglobin subfractions[1,2].

"overloaded" protein zones (haemoglobin A) seem to be more stable in agarose gels during fixation and staining than in polyacrylamide gels[2]. The pattern of native fresh haemoglobin consisted of 10–12 subfractions as usual[1,2]. After treatment with pyridoxal-5-phosphate a new distinct zone appeared in a more acidic region, due to the reaction of free amino groups of haemoglobin with the aldehydic group and to the induction of the phosphate group. In contrast to sample 1, the zones of the A_1 haemoglobin subfractions became diffuse. Samples 1 and 2 also formed about ten zones of non-haemoglobin proteins, mostly in the region of about pH 5–6.5. Stroma-free haemoglobin (sample 3) stored for 1 year at −20°C formed distinct zones of A_2, of methaemoglobin A and A_2 and of carbonanhydrase, and also some faint unidentified zones. Non-haemoglobin protein fractions in the pH region of about 5–6 were significantly less intense than in samples 1 and 2 owing to the extensive purification of SFH. Reaction of haemoglobin with glutaraldehyde caused a marked change in the pattern characterized by the disappearance of all individual zones[2].

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