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Note

Characterization of modified bovine haemoglobin by molecular filtration and electrophoresis in gradient gels

H. FOŘTOVÁ*, J. SUTTNAR, M. KRAMLOVÁ and T. I. PŘISTOUPIL
Institute of Haematology and Blood Transfusion, Prague (Czechoslovakia)
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Stroma-free haemoglobin (SFH) and its chemically modified derivatives are of interest as oxygen-carrying perfusion fluids[1–4]. Modification of SFH with pyridoxal-5′-phosphate and glutaraldehyde leads to a very polydisperse system, containing various types of modified and unmodified haemoglobin molecules and molecular aggregates with a relative molecular mass $M_r = 10^4$–10^6. In previous investigations we studied modified, mostly human, haemoglobin by several analytical fractionation techniques[5]. This paper deals with the possibility of using other methods, *viz.*, ultrafiltration, fast protein liquid chromatography (FPLC) on Superose™ 12 and vertical slab electrophoresis on gradient gels, for the characterization of native and modified bovine SFH.

EXPERIMENTAL

SFH was prepared from fresh bovine erythrocytes in the usual manner[6]. The modification of bovine SFH with pyridoxal-5′-phosphate (Fluka, Buchs, Switzerland) and glutaraldehyde (Serva, Heidelberg, F.R.G.) was performed in the presence of lysine (Lachema, Brno, Czechoslovakia) using modified versions of published methods[2,3]. Fractionation of modified bovine SFH by ultrafiltration was performed on XM 50 and XM 100 A membranes (Amicon, Lexington, MA, U.S.A.) as recommended by the producer[7]. FPLC on Superose 12 has been described elsewhere[8]. The exponential gradient of polyacrylamide gel was 10–22.5% for SDS-PAGE (polyacrylamide gel electrophoresis in sodium dodecyl sulphate) and 8–20% for PAGE without SDS. The size of the gels was 140 × 180 × 1 mm. The solutions for the preparation of the gel and the buffers for electrophoresis were prepared according to LKB Application Note 320 (ref. 9). A polyacrylamide gel of concentration 3% in buffer of pH 6.8 was used as the stacking gel.

Electrophoresis was carried out at 8°C in a discontinuous buffer system of pH 8.8 according to Laemmli[10], using 30 mA per plate for 6 h. The samples were incubated for 12 h at 37°C in buffer of pH 6.8 in the presence of 8 *M* urea and 2% SDS prior to SDS-PAGE. PAGE without the addition of denaturing agents was performed under the same conditions.

The densitometric evaluation of the electrophoretograms was carried out on a Clini Scan apparatus (Helena Labs., Beaumont, TX, U.S.A.). A low-molecular-weight calibration kit (Pharmacia, Uppsala, Sweden) was used for calibration in SDS-PAGE.

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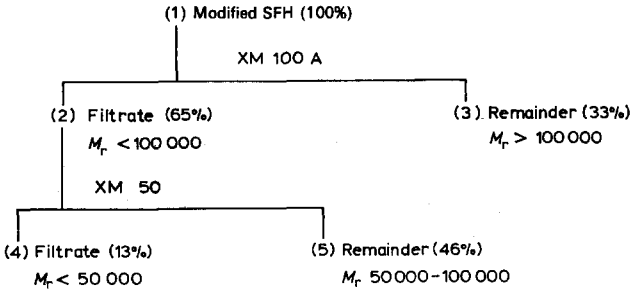

Fig. 1. Scheme of the fractionation of modified bovine SFH by ultrafiltration on Amicon XM 50 and XM 100 A membranes. Numbers in parentheses show the percentages of the individual fractions.

RESULTS AND DISCUSSION

The rough fractionation of haemoglobin samples by ultrafiltration on the Amicon membranes is shown schematically in Fig. 1.

Fig. 2 shows the results of FPLC on Superose 12 achieved with native and modified bovine SFH and also with fractions prepared by ultrafiltration. The native SFH formed a single symmetrical peak (apparent M_r = 48 000 according to the

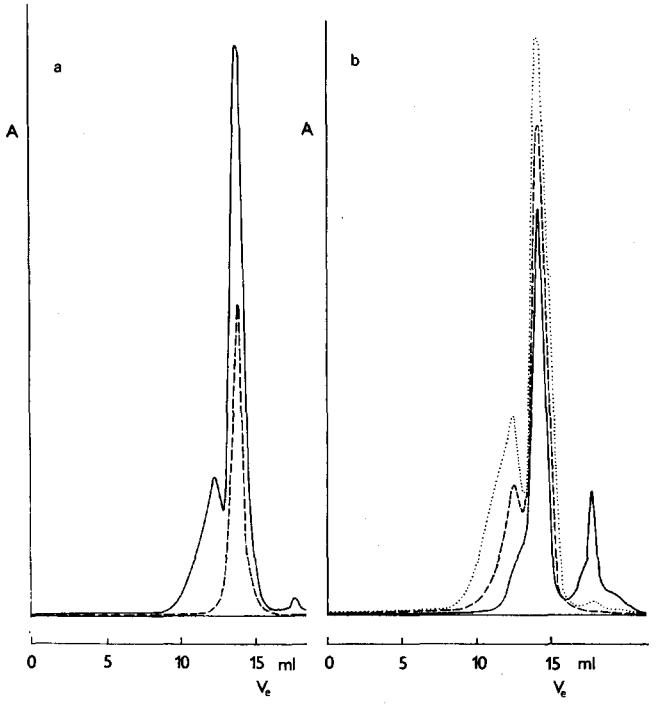

Fig. 2. Gel chromatography of haemoglobin samples on Superose 12. Buffer, 0.05 *M* sodium phosphate (pH 7.0), 0.15 *M* NaCl, flow-rate 0.9 ml/min. (a) Full line, modified SFH (see fraction 1 in Fig. 1); dashed line, native SFH. (b) Full line, fraction 4; dashed line, fraction 5; dotted line, fraction 3 (see Fig. 1).

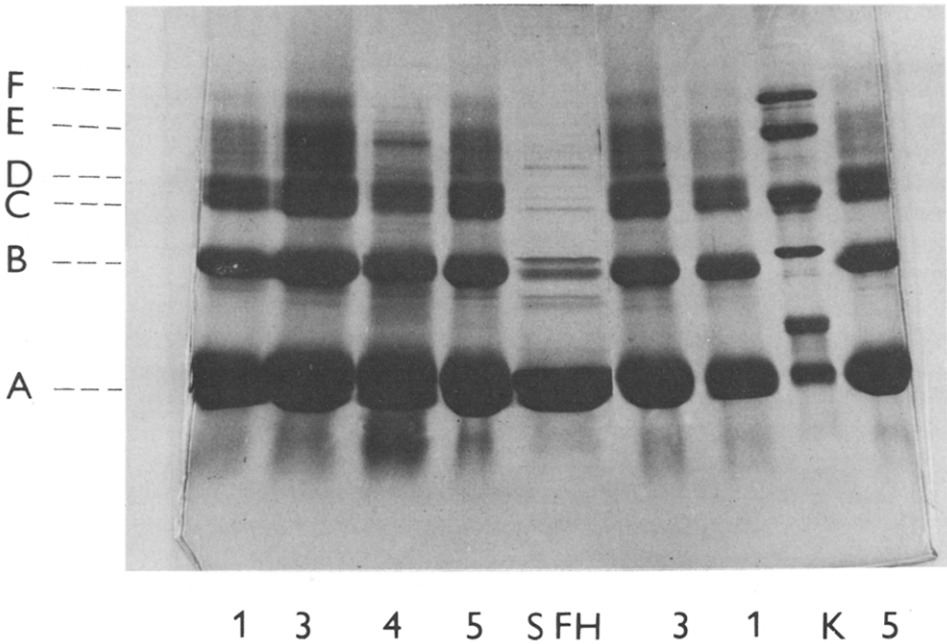

Fig. 3. Electrophoresis of haemoglobin samples on the polyacrylamide gradient gel with sodium dodecylsulphate (SDS-PAGE). For numbering of samples, see Fig. 1. SFH, unmodified bovine haemoglobin; K, calibration kit. The letters A–F correspond to the zones of the electrophoretic pattern of the modified bovine SFH (see Fig. 4).

calibration graph published in ref. 8). After modification with pyridoxal-5′-phosphate and glutaraldehyde, two peaks appeared. The peak of the molecular aggregates corresponding to a mean M_r of about 150 000 formed 30% and the position of the main peak, forming about 70% of the total mount of proteins, was identical with that of native SFH.

Similar results were obtained with the fractions from ultrafiltration (see Fig. 1). Fractions 5 and especially 3 showed a marked peak, corresponding to aggregates. In fraction 4 the peak of the aggregates was missing, and in addition to the main peak at the position of native SFH a small peak of lower relative molecular mass was found.

FPLC on Superose 12 led to analogous results to chromatography on Sepharose 6B, but better reproducibility and faster separations were obtained comparison with classical chromatography. Similar results were achieved in our previous work[8] on the analysis of human SFH.

PAGE on the gradient gels in the absence of denaturing agents confirmed the high polydispersity of modified SFH. Whereas the native SFH was characterized by a single sharp zone during electrophoresis, the modified SFH, under the same conditions, led to prolonged diffuse strips with the highest intensity at the position of native SFH.

Fig. 3 shows SDS-PAGE on the gradient gel and Table I summarizes the densitometric evaluation of the above electrophoresis. Native bovine SFH is char-

TABLE I

QUANTITATIVE EVALUATION OF FIG. 3

For numbering of the fractions, see Fig. 1.

Sample	*Proportion (%)*				
	Low M_r	*Monomer*	*Dimer*	*Trimer*	*Higher aggregates*
Native SFH		93	7		
(1) Modified SFH		60	23	12	5
(3) Fraction with M_r > 100 000		50	24	15	11
(4) Fraction with M_r < 50 000	11	55	24	8	2
(5) Fraction with M_r 50 000–100 000		54	26	14	6

acterized by one intensively stained zone corresponding to the subunits α and β (M_r = 16 000). The modified bovine SFH is characterized by several intense zones with M_r values corresponding approximately to the monomer, dimer, trimer and higher aggregates of haemoglobin subunits. Similar results were achieved with the fractions from ultrafiltration (fractions 3, 4 and 5). The ratio of the individual zones is shown in Table I. The calibration graph for the determination of M_r is shown in Fig. 4 and is related to sample K in Fig. 3.

Electrophoresis in the gradient gels has a significantly higher distinguishing ability than electrophoresis in gels having a uniform pore size. We always prepared the gels in concentration intervals most suitable for the given purpose. In one experiment it was possible to work on two gel plates, which permitted a parallel analysis of twenty samples under the same experimental conditions.

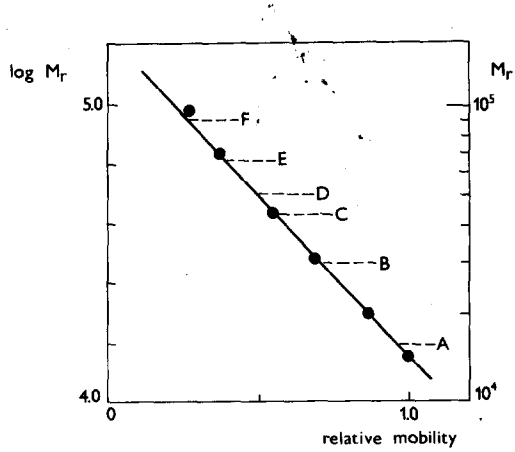

Fig. 4. Calibration graph for the determination of relative molecular mass (M_r) by SDS-PAGE in gradient gel. Relative mobility is related to the fastest zone of the calibration kit (α-lactalbumin, trypsin inhibitor, carbonic anhydrase, ovalbumin, albumin, phosphorylase B; full circles). The letters A–F correspond to the zones of the modified SFH (see Fig. 3). Their M_r values read from the calibration graph were as follows: A = 15 900, B = 29 600, C = 43 000, D = 50 100, E = 64 500 and F = 89 000.

The modified bovine SFH prepared in our laboratory generally had a lower degree of aggregation than analogous preparations of this type[2,3]. Our preparation contained about 30% of aggregates, according to FPLC on Superose 12 (Fig. 2) and ultrafiltration (Fig. 1). After treatment with denaturing agents, the presence of about 40% of aggregates was found with SDS-PAGE (Fig. 3). The remainder probably consisted of both unreacted haemoglobin molecules and their subunits and of modified molecules intramolecularly linked by glutaraldehyde to different degrees of aggregation. These molecules were assumed to be partly disintegratable to subunits by denaturing agents[8].

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