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CROSS-LINKING OF STROMA-FREE HEMOGLOBIN MONITORED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The potential of gel-permeation high-performance liquid chromatography for the study of the reaction of stroma-free hemoglobin (SFH) with glutaraldehyde has been investigated. Using a I-125 protein column and a 50 m*M* phosphate buffer–acetonitrile solvent system, the formation of cross-linked derivatives from native SFH can easily be followed. The results suggest that this technique is a powerful alternative to electrophoresis and isoelectric focusing.

INTRODUCTION

Hemoglobin preparations free from stromal components (SFH) have been shown to meet the criteria of an ideal blood substitute[1]. However, it is known that *in vivo* this hemoprotein has a short half-life[2] and is rapidly cleared by the kidneys and through other metabolic routes[3]. In order to increase the molecular weight of the hemoglobin molecule, this protein has been coupled to activated dextrans[4,5], polymerized or cross-linked with albumin by means of polyfunctional reagents[6]. Glutaraldehyde is currently used as a cross-linking reagent in protein studies, and recently the changes in human SFH after its reaction with glutaraldehyde have been examined by means of gel chromatography, electrophoresis and isoelectric focusing[7,8].

High-performance liquid chromatography (HPLC) is now being widely applied to the separation of proteins[9–11] and offers the advantages of high resolutions, lower analysis times, easy quantitation and sensitivity.

In present study, gel-permeation HPLC on an I-125 protein column has been used for monitoring the reaction of SFH with glutaraldehyde, and the results are compared with those obtained by means of electrophoresis and isoelectric focusing.

EXPERIMENTAL

Reaction of SFH with glutaraldehyde

SFH was a standard sample produced in our laboratory from outdated human

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blood according to ref. 12. The methemoglobin content[13] was less than 1%.

The reaction of SFH (2.5%) with glutaraldehyde (0.08%) (Fluka, Buchs, Switzerland) was carried out in a phosphate buffer (50 m*M*, pH 7.5) at 4°C with stirring in a stream of nitrogen. The reaction was stopped at different times by adding tris(hydroxymethyl)aminomethane (Sigma, St. Louis, MO, U.S.A.). In another series of experiments, the reactions of SFH with glutaraldehyde in the presence of Dextran T 20 (1.66%) (Pharmacia, Uppsala, Sweden) were investigated.

Gel chromatography

Hemoglobin derivatives (0.1 ml) were chromatographed on columns (48 × 1.5 cm) of Bio-Gel A-0.5m (Bio-Rad Labs., Milan, Italy) in a phosphate buffer (50 m*M*, pH 7.5) at 4°C and in the dark. The flow-rate was 0.4 ml/min and the elution curves were recorded automatically at 280 nm. The apparatus consisted of a 2138 Uvicord S, a 2210 Channel Recorder, a 2111 Multirac and a 2132 Microperpex (LKB, Bromma, Sweden).

Cellulose acetate electrophoresis

A Model Boskamp pherostat was used. The buffer was Tris (10.2 g/l)–EDTA (0.6 g/l)–boric acid (3.2 g/l) (TEB), pH 8.5. After different times (2, 15 and 60 min), 40 µl of the reaction mixture were treated with 25 µl of 0.1 *M* Tris buffer, pH 7.9. Four samples (1 µl), including a SFH (1.5%) control, were applied simultaneously at the cathode end of a cellulose acetate strip measuring 5.7 × 14 cm (Chemagel; Chematron, Milan, Italy) and 200 V were delivered for 60 min.

The plates were then stained with Ponceau S (0.3 g in 100 ml of 3% trichloroacetic acid) for 5 min, then decolorized with 3% acetic acid until the background was white.

Gel isoelectric focusing

A Model LKB 2117 Multiphor apparatus cooled at 4°C and a LKB 2103 power supply were used. The pH range was 7–9 and the thickness of the gel was 1 mm.

The procedure for the preparation of a gel 120 × 120 × 1 mm was as follows. The stock solution of acrylamide consisted of acrylamide (30 g) (Fluka), bisacrylamide (Fluka) (1.2 g) and distilled water (100 ml). To 2.75 ml of the acrylamide stock solution were added 0.75 ml of 40% ampholines pH 7–9 (LKB), 75 µl of 40% ampholines pH 3.5–10 (LKB), 11.7 ml of distilled water and 5 µl of N,N,N′,N′-tetramethylethylenediamine (TEMED) (Sigma). The solution was degassed for 2 min and 20 µl of a freshly prepared solution of ammonium peroxodisulphate (40%) were then added.

The prerun was performed for 15 min at 4°C, 600 V and 3 W. The electrode strips (LKB) were impregnated on a glass plate with 1 *N* sodium hydroxide for the cathode and 1 *M* phosphoric acid for the anode.

Four samples (15 µl), obtained as described under Cellulose acetate electrophoresis, were applied simultaneously at the cathode end and the run took 2 h (600 V, 3 W, 4°C). The plates were stained with Fast Stain G-250[14] for 30 min and then washed with water.

Gel-permeation HPLC

A Model 6000 A solvent delivery system, Model U6K Universal injector, Model 440 UV detector and Model 730 Data Module (Waters Assoc., Milford, MA, U.S.A.) were used. A pre-packed I-125 protein column was obtained from Waters Assoc. A protein guard column (bulk material, Part No. 85290; guard column kit, Part No. 84550 from Waters Assoc.) was used. Samples were eluted isocratically using 50 m*M* phosphate buffer pH 6.9–acetonitrile (90:10 v/v).

The flow-rate was 2.0 ml/min (55 bar), and the temperature was ambient. Chromatographic peaks were monitored at 254 nm and the range setting was fixed at 0.01 a.u.f.s.

Sample solution. A 40-μl volume of the reaction mixture (2.5, 10, 15, 20, 30 and 60 min) and 25 μl of 0.1 *M* Tris buffer, pH 7.9 were diluted with 0.5 ml of the eluent. The resulting solution was mixed with a tyrosine solution in the ratio 4:1 (v/v).

Tyrosine solution. Tyrosine (10 mg), as internal standard, was dissolved in 0.5 ml of 0.1 *N* NaOH and diluted in the eluent to give a concentration of 2 mg/ml.

Standard solutions. Purified samples of aldolase, alcohol dehydrogenase (ADH), L-lactic dehydrogenase (LDH), human transferrin, ovalbumin and trypsin inhibitor (all from Sigma) served as standard for the comparison of retention times *vs.* the log of the molecular weight.

Samples of 5–10 μg in volumes of 5–10 μl were injected.

RESULTS AND DISCUSSION

The elution profiles of glutaraldehyde-treated and native SFH on Bio-Gel A-0.5m (Fig. 1) show that SFH is almost completely cross-linked in 15 min, since there is no significant peak at the elution volume of free SFH.

This method does not allow one to differentiate fractions according to their relative molecular size. Better results were obtained using cellulose acetate electrophoresis and isoelectric focusing. The electrophoretic patterns of free and cross-linked SFH (reaction times 2, 15 and 60 min, respectively) indicate that modified SFH has several fractions of higher molecular weight (Fig. 2) as a result of covalent inter- and intramolecular bonding. As shown in Fig. 3, the cross-linking of SFH was more

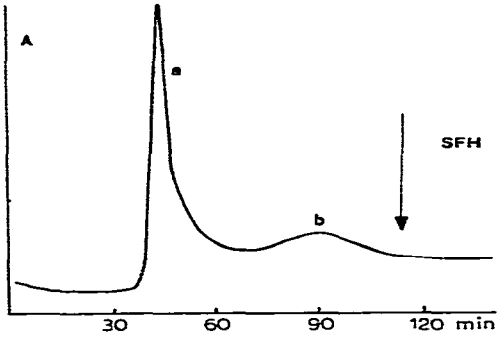

Fig. 1. Chromatography of modified SFH (reaction time = 15 min) on Bio-Gel A-0.5m. a, b = Cross-linked SFH. Column: 48 × 1.5 cm. Elution rate: 0.4 ml/min. Eluent: 50 m*M* phosphate buffer pH 7.5.

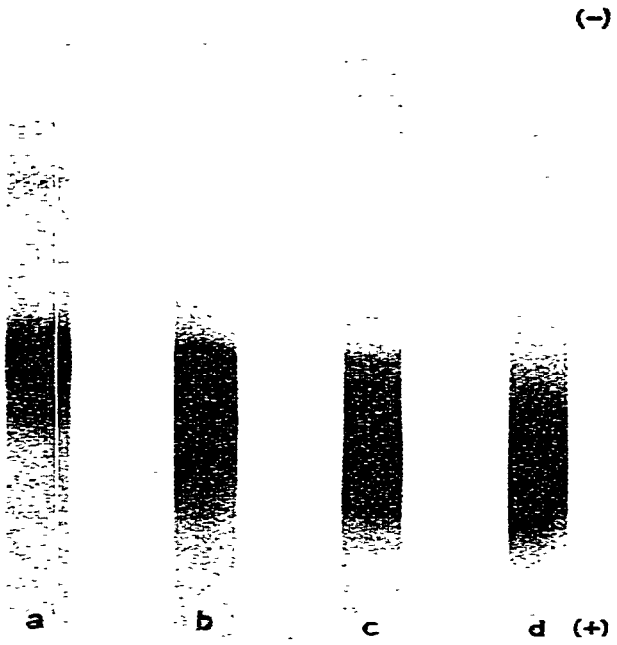

Fig. 2. Cellulose acetate electrophoresis of electrophoresis of SFH (a) and after reaction with glutaraldehyde (b, 2 min; c, 15 min; d, 60 min). Electrolyte: TEB buffer, pH 8.5. Stained with Ponceau S.

clearly followed by isoelectric focusing. However, both these techniques are time-consuming and laborious and it was preferable to detect the changes in SFH during the reaction with glutaraldehyde by HPLC. An isocratic elution mode was adopted and the separation on a I-125 protein column of SFH and products corresponding to various reaction times is shown in Fig. 4. These chromatograms indicate that SFH is converted into the main derivative (I) through intermediates of lower size. Furthermore, the peak area ratio, I/IS, is linearly dependent on the reaction time up to 60 min:

$$y = 0.043\,x + 0.22\ (r = 0.984)$$

To estimate the molecular weight (MW) of modified SFH a group of standards were chromatographed and the retention times are plotted against log MW in Fig. 5. It can be concluded that the first peak (I, 2.82 min) represents a mean MW of 230,000, while the slowest eluting peaks correspond to MW 90,000 (II, 3.12 min) and MW 22,000 (III, 3.50 min). SFH (IV) is eluted at 3.73 min and tyrosine (IS) at 5.12 min. On the I-125 column, solutes are separated by size exclusion; nevertheless, a solute–column interaction occurred and the use of 10% acetonitrile was more efficient than an increase of the ionic strength in obtaining sharp peaks and complete reproducibility. The amount injected was 6.5 μg and the detection limit was about 0.5 μg. The analysis time was less than 6 min.

Owing to its rapidity and suitability, this HPLC procedure was used to optimize the reaction conditions. First, the optimum amount of glutaraldehyde was

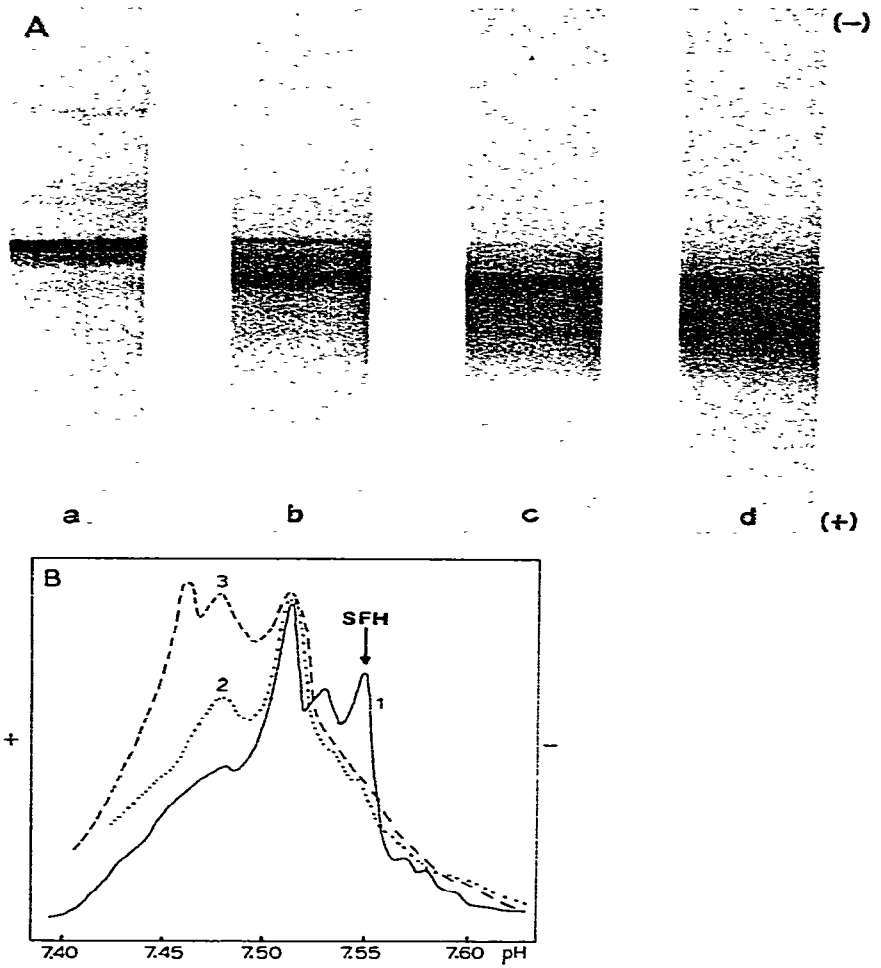

Fig. 3. A, Gel isoelectric focusing of SFH (a) and after reaction with glutaraldehyde (b, 2 min; c, 15 min; d, 60 min). Electrolyte: 1 *M* phosphoric acid (anode), 1 *M* sodium hydroxide (cathode). Ampholines pH range 3.5–10. Stained with Fast Stain G-250. B, Densitometric scan of modified SFH after gel isoelectric focusing. Curves: 1, 2 min; 2, 15 min; 3, 60 min.

investigated, keeping constant the concentration of SFH. Concentrations of glutaraldehyde higher than 0.10% yielded gels in a short time. The best results were obtained using 0.08% glutaraldehyde within 15 min at pH 7.5. Higher pH values as well as longer reaction times yielded amounts (up to 10%) of methemoglobin, while under the optimal conditions the amount of methemoglobin present was in the order of 5%.

Addition of L-ascorbic acid (5%) to the reaction mixture lowered the amount of methemoglobin (2.5%) and the resulting preparations could be stored for several days without methemoglobin increasing.

In order to avoid extensive cross-linking the reaction of SFH with glutaraldehyde was carried out in the presence of Dextran T 20. The reaction was followed by HPLC and the chromatograms were analogous to those obtained without dextran.

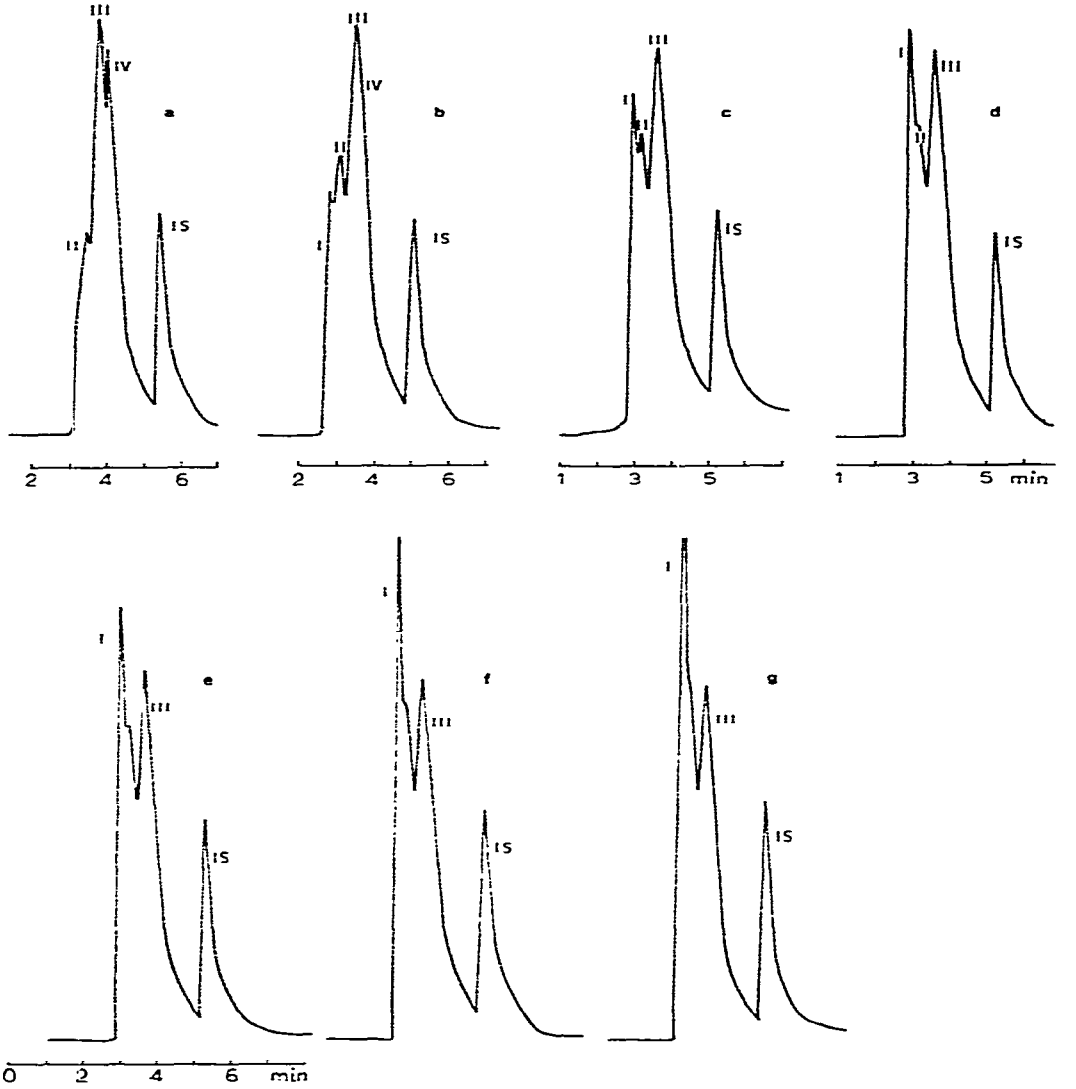

Fig. 4. Chromatograms of modified SFH on I-125 column. The reaction was stopped after 2 min (a), 5 min (b), 10 min (c), 15 min (d), 20 min (e), 30 min (f) and 60 min (g). Eluent: 50 m*M* phosphate buffer pH 6.9–acetonitrile (90:10). Flow-rate: 2.0 ml/min. Peaks: I = modified SFH, MW ≈ 230,000; II = modified SFH, MW ≈ 90,000; III = modified SFH, MW ≈ 22,000; IV = native SFH; IS = internal standard (tyrosine).

These results were confirmed by analyzing the reaction products by electrophoresis and isoelectric focusing. Quantitation of dextran[15] in the fraction corresponding to the main derivative indicated that only 10% of the total dextran was linked.

In conclusion, this HPLC method is simple, rapid and reproducible. It provides an alternative to electrophoretic and isoelectric focusing techniques, for following the reaction of SFH with glutaraldehyde.

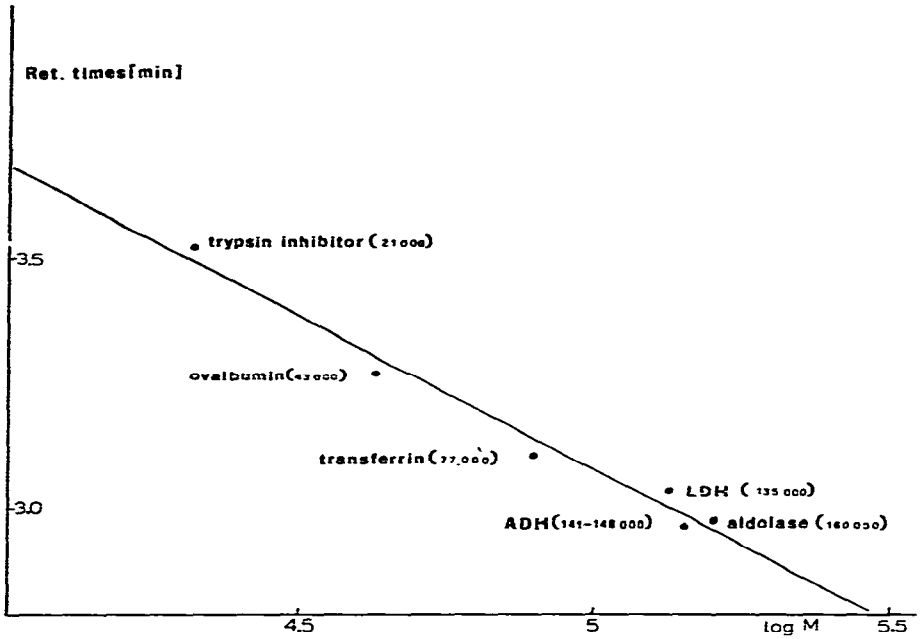

Fig. 5. Molecular weight calibration curve for I-125 column.

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