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Note

Affinity chromatography of serum haemopexin

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In an earlier paper[1], we described a procedure for the isolation and purification of the haeme-binding serum protein haemopexin. The method involved precipitation with rivanol, ammonium sulphate and ethanol followed by chromatography of the supernatant on DEAE-cellulose. Although haemopexin obtained by this method was reasonably pure, complexity and a low yield were the main disadvantages, as in earlier procedures[2–6]. As the apohaemopexin molecule forms a strong complex with one haeme molecule[2,7,8], it seemed appropriate to utilize this property for the isolation of haemopexin from serum. As early as 1966 Heide[9] described the isolation of haemopexin on a Sephadex G-100 column with bound haeme, but no experimental details were given. In an attempt to reproduce his method, we tried several procedures for the attachment of haeme to various matrices, *e.g.*, *p*-aminobenzylcellulose, Sepharose 4B, Sephadex G-100 and Bio-Gel P-200. Of these, the affinity column with haeme attached to Bio-Gel P-200, which is described here, gave best results as far as purity and yield are concerned.

MATERIALS AND METHODS

Bio-Gel P-200 was obtained from Bio-Rad Labs. (Richmond, Calif., U.S.A.) and *p*-nitrobenzoylazide[10] and haemin[11] were prepared in our laboratory. All other reagents were products of Lachema (Czechoslovakia).

Absorption spectra were measured on a Unicam SP 700 recording spectrophotometer and circular dichroic spectra on a Jasco ORD-UV/5 instrument with a circular dichroic attachment. Sedimentation coefficients were determined on a PHYWE Model 1957 ultracentrifuge at 48,000 r.p.m.

Preparation of Bio-Gel P-200 with bound haeme

Haeme was attached to the polyacrylamide gel by the series of reactions[12,13] shown in Fig. 1.

Bio-Gel (I) was first treated with 3 *M* hydrazine for 7 h at 50° and the hydrazide derivative (II) was obtained. From the latter, the acylazide derivative (III) was prepared by the action of 0.1 *M* sodium nitrite in 0.5 *M* hydrochloric acid for 10 min at 0°. Then 2 mmole of hexamethylenediamine per millilitre of gel were added and the pH was adjusted to 11. After reaction for 1.5 h at 0°, the aminohexamethylene derivative (IV) was obtained. The amino group of IV was acylated by treatment with 0.07 *M*

Fig. 1. Reaction scheme for the binding of haeme to a polyacrylamide matrix.

p-nitrobenzoylazide in 0.2 *M* sodium tetraborate (pH 9.3) and 40% (v/v) dimethylformamide at room temperature for 1 h. The nitro derivative (V) was reduced for 40 min at 40° with 0.1 *M* sodium dithionate in 0.5 *M* sodium hydrogen carbonate solution at pH 8.5. The amino derivative (VI) was diazotized for 7 min at 0–4° with 0.1 *M* sodium nitrite in 0.5 *M* hydrochloric acid. Haemin (0.5 mg/ml of modified gel) was bound to the diazo derivative (VII) by a coupling reaction performed in saturated borax solution at 4° with gentle shaking overnight. The gel was thoroughly washed with 0.2 *M* sodium chloride solution after each step. The extent of modification of the gel was checked by reaction with trinitrobenzenesulphonic acid.

Haeme attached non-covalently to the matrix was removed by elution with 1% albumin solution in 0.1 *M* sodium phosphate (pH 7.0). Albumin molecules linked simultaneously to the covalently bound haeme were released with 0.35 *M* glycine–hydrochloric acid buffer (pH 2.4). Finally, the affinity column was poured and equilibrated with 0.1 *M* sodium phosphate solution (pH 7.0).

Affinity chromatography of haemopexin

The albumin was removed from fresh human serum by precipitation with rivanol[1,2]. The supernatant, which contained immunoglobulins, transferin and glycoproteins, was dialyzed with 0.1 *M* phosphate (pH 7.0). A volume of 150 ml of the supernatant was applied to the affinity column (50 × 2.5 cm) at a flow-rate of 150 ml/h and the unbound proteins were eluted with 350 ml of 0.1 *M* phosphate buffer (pH 7.0).

Haemopexin which had been bound to the gel matrix was released with 0.35 *M* glycine–hydrochloric acid (pH 2.4). After all of the material had been eluted (in a volume of *ca.* 100 ml), it was neutralized with 1 *M* sodium hydroxide solution, dialyzed with 0.05 *M* sodium phosphate (pH 7.0) and concentrated in an Amicon ultrafiltration cell. The column was re-equilibrated with 0.1 *M* sodium phosphate (pH 7.0) and was then ready for a further affinity chromatography cycle. The eluate obtained in the washing step was re-used as it still contained substantial amounts of haemopexin.

RESULTS

An elution diagram of affinity chromatography performed with the rivanol supernatant of human serum on Bio-Gel P-200 with covalently bound haeme is shown in Fig. 2. After the unbound proteins had been eluted with neutral buffer, the dissociation of haemopexin from the gel matrix was achieved with acid buffer, which was applied at the point indicated by the arrow. Under these conditions, the haemopexin molecule apparently unfolds and the haeme–protein interactions are disrupted[14]. Although haemopexin is eluted in three peaks, no differences in the purity of the three fractions were found on acrylamide gel electrophoresis and immunoelectrophoresis. Fig. 3a shows the acrylamide gel electrophoresis and Fig. 3b the immunoelectrophoresis of the material eluted from the column in the acid cycle. It appears that the eluate contains haemopexin with albumin and immunoglobulins as contaminants; the relative amounts of the contaminating proteins never exceeded 5%.

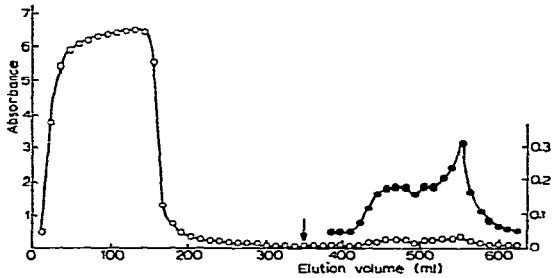

Fig. 2. Isolation of haemopexin from the rivanol supernatant of human serum on a Bio-Gel P-200 column with covalently bound haeme. Bed volume, 100 ml; eluent, 0.05 *M* phosphate buffer (pH 7.0). Glycine–hydrochloric acid buffer (pH 2.4) was applied at the point shown by the arrow. The absorbances of *ca.* 10-ml fractions were measured at 280 nm. The left-hand scale relates to the open circles, the right-hand scale to the closed circles.

Haemopexin produced by affinity chromatography binds haeme in a 1:1 molar ratio. The absorption and circular dichroic spectra in the UV and visible regions of the haeme–haemopexin complex showed no differences in comparison with those obtained for haemopexin prepared by conventional isolation methods[15,16]. Sedimentation analysis carried out with a 0.5% solution of haemopexin in 0.05 *M* sodium phosphate (pH 7.0) showed a single symmetrical peak. The sedimentation coefficient of the haemopexin preparation was $s_{20,w} = 3.42 \cdot 10^{-13}$ s.

The amount of haemopexin obtained in a single experiment performed with 150 ml of rivanol supernatant (75 ml of serum) was 5 mg. When the eluate containing

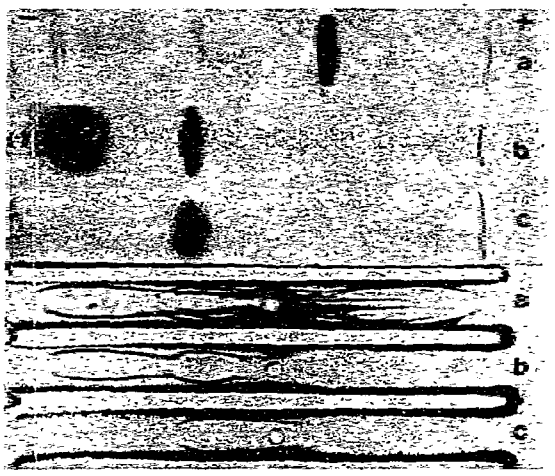

Fig. 3. Disc electrophoresis at pH 8.4 and immunoelectrophoresis at pH 8.6 of (a) human serum, (b) the rivanol supernatant of human serum and (c) haemopexin isolated by affinity chromatography on Bio-Gel P-200 with covalently bound haeme. The immunodetection was performed with horse antiserum to human serum.

unbound proteins was re-applied to the column in the next experiment, a further 2–3 mg of haemopexin were obtained. The yield, calculated on the basis of a haemopexin physiological concentration of 80 mg per 100 ml of serum, was about 20%.

DISCUSSION

Affinity chromatography on a gel matrix with haeme attached to Bio-Gel P-200 proved to be a simple and rapid method for the isolation of haemopexin from human serum. The method produces haemopexin of a sufficient purity and in a favourable yield in comparison with the conventional isolation procedures. As the acidic conditions used for the dissociation of haemopexin from the matrix might cause denaturation of the molecule, it was of crucial importance to verify whether haemopexin prepared by affinity chromatography was in the native form. We found that haemopexin that was eluted from the column in the acid cycle and immediately neutralized showed optical, chiroptical and haeme-binding properties characteristic of the native protein. This finding is in accordance with the data of Hrkal *et al.*[14], who showed that even after the action of an acidic medium on the haeme–haemopexin complex for 40 min the Soret absorption maximum reappeared fully on neutralization of the solution, which indicated that the conformation of the haeme-binding centre had been restored.

Although haemopexin produced by the above method should be completely in the apo-form, it was about 20% "haeme saturated" (*i.e.*, 20% of the molecule carried the full haeme equivalent). It appears that in spite of the careful washing with albumin solution, some haeme remains non-covalently attached to the gel matrix and is dissociated simultaneously with the bound haemopexin in the acid cycle.

The presence of trace amounts of albumin in the preparation is apparently due to the affinity of this protein for haeme, which, although much lower than that of

haemopexin, is sufficient for albumin to appear in the preparation as an impurity. The immunoglobulins that occasionally occur in the preparation probably result from their interactions with the spacer groups.

The method described appears to be a simple and efficient means for the isolation of haemopexin from human serum.

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