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Chromatographic separation of oxy- and met-myoglobin on a DEAE-cellulose column

Since there is a strong tendency for MbO_2* to be oxidized easily to MetMb during isolation procedures, all myoglobin preparations reported earlier[1,2] were obtained in the form of MetMb. If needed, therefore, MbO_2 was prepared from MetMb by drastic reduction with sodium hydrosulphite, but it is very unstable due to autoxidation[3,4].

Recently, very stable preparations of native MbO_2 have been obtained from sources of horse heart muscle[5,6] and sperm whale skeletal muscle[7] by the introduction of chromatography on DEAE-cellulose. However, the detailed elution profile for the chromatographic procedure has not yet been shown. In this communication we present the elution profile for the separation of native MbO_2 and MetMb by DEAE-cellulose chromatography.

The myoglobin was extracted from the bovine heart muscle by SHIKAMA's[5] method with a slight modification: the myoglobin was precipitated by saturation with 60–100 % ammonium sulphate at pH 6.8 in an ice bath and dissolved in a small amount of the buffer to be used for the DEAE-cellulose chromatography, 5 m*M* Tris (Sigma, primary standard)–HCl, pH 8.5. This pH was chosen because native horse MbO_2 is most stable against autoxidation under these conditions[6]. Haemoglobin was prepared from bovine blood cells according to HEIDELBERG's modification of SUZUKI *et al.*[8]. The concentration of myoglobin and haemoglobin was determined by DRABKIN's[9] method.

Our crude sample still contained a large amount of haemoglobin. The haemoglobin can easily be separated from the myoglobin by molecular sieve chromatography which was suggested by AWAD *et al.*[10] and followed by HARDMAN *et al.*[7]. A typical elution pattern of the crude myoglobin on Sephadex G-75 (Pharmacia) is shown in Fig. 1. 10 ml of the myoglobin solution were applied to the Sephadex column (3 × 90 cm) which had been equilibrated with 5 m*M* Tris–HCl buffer, pH 8.5. The column was eluted with the same buffer at room temperature. Every 5 ml of the effluent was collected in each tube of the fraction collector. The protein level was measured by the absorbancy at 280 nm and the haeme protein level was estimated by the absorbancy at 417 nm in their Solet band region. The myoglobin and the haemoglobin were conveniently identified from the absorbancy ratio of 581 nm/576 nm at pH. 8.5. The wavelengths, 581 nm and 576 nm, are the absorbancy maxima of the α-band of MbO_2 and HbO_2, respectively. This absorbancy ratio was 1.0 for the two haeme proteins in the met-form. If the oxy-form is present in any amount, this value would be higher than 1.0 for myoglobin; on the other hand it would be lower than 1.0 for haemoglobin. In Fig. 1, the transition of the absorbancy ratio of 581 nm/576 nm from less than 1.0 to greater than 1.0 can clearly be seen. In this preparation the absorbancy ratios were 0.87 for the first peak and 1.11 for the second, indicating the presence of haemoglobin and myoglobin, respectively. These absorbancy ratios were different from one preparation to another, depending upon the amount of oxy-form in each haeme protein.

* Abbreviations: MbO_2, oxymyoglobin; MetMb, met-myoglobin; HbO_2, oxyhalmoglobin.

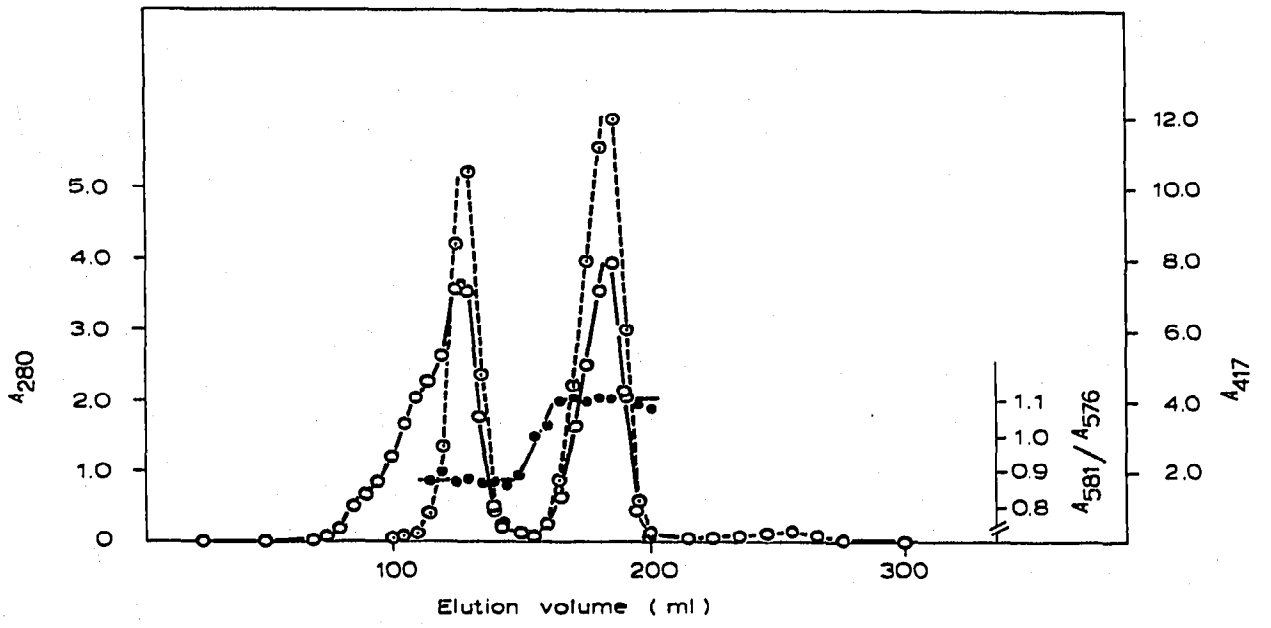

Fig. 1. Molecular sieve chromatography of a myoglobin extract on a Sephadex G-75 column. Ten ml (5.52 μmoles as haeme) of myoglobin extract were applied to a Sephadex G-75 column (3 × 90 cm). The column was eluted with 5 m*M* Tris–HCl buffer, pH 8.5, 5 ml fractions collected. The protein level was measured by the absorbancy at 280 nm (○—○). Haeme protein was estimated by the absorbancy at 417 nm in the Solet band region (⊙—⊙). Myoglobin and haemoglobin were conveniently identified by the absorbancy ratio of 581 nm/576 nm (●—●).

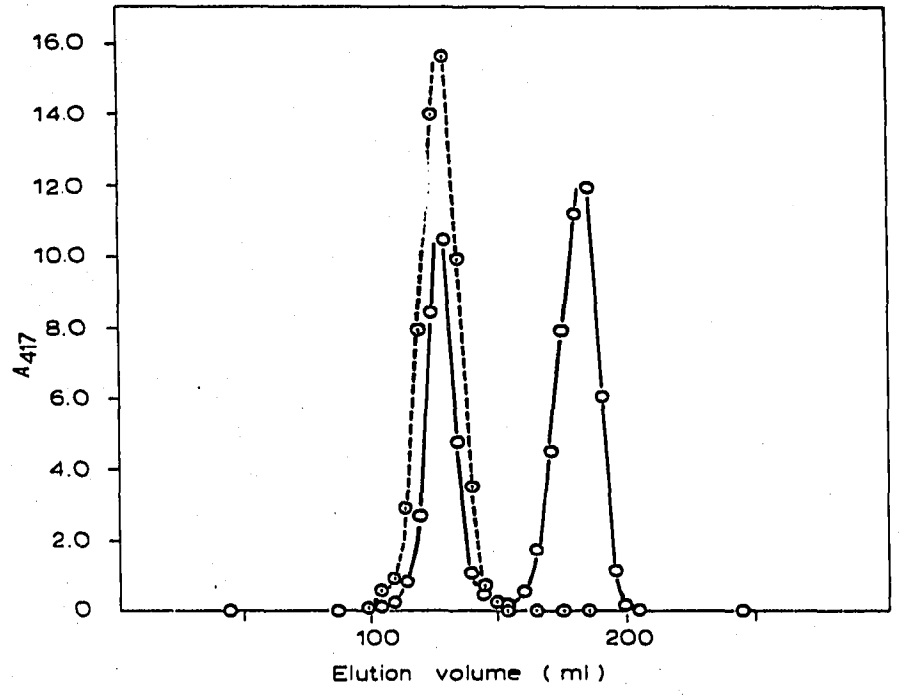

Fig. 2. Duplicate run of haemoglobin for myoglobin extract on a Sephadex G-75 column. Ten m (169 mg) of haemoglobin were applied to a Sephadex G-75 column, and the chromatography wa done under the same conditions as that for the myoglobin extract (⊙ – – – ⊙). The elutio pattern of myoglobin extract was replotted from Fig. 1 (○—○).

Fig. 2 shows a duplicate run for haemoglobin obtained from red blood cells on the same Sephadex G-75 column. The haemoglobin was eluted coincident with the first peak of the crude sample, and concurs with the identification by the absorbancy ratio of 581 nm/576 nm in Fig. 1. The gel filtration was so effective for the separation of the myoglobin from the haemoglobin that the saturation with ammonium sulphate could be decreased down to 60–100 % to minimize the loss of the myoglobin which often occurred in the case of 70–100 % saturation. Ammonium sulphate was also removed from the myoglobin solution by means of this gel filtration so that the usual dialysis procedure was not necessary for the ion-exchange chromatography following. The myoglobin at this stage was a mixture of MbO_2 and MetMb in various ratios. The separation of these two forms of the myoglobin was performed on DEAE-cellulose (Brown; exchange capacity, 0.87 mequiv./g) at room temperature. The myoglobin solution was applied to a DEAE-cellulose column (2 × 20 cm) which had been equilibrated with 5 m*M* Tris–HCl buffer, pH 8.5. The column was washed with 30 ml of the same buffer, then a linear Tris–HCl gradient was employed for the elution by mixing 500 ml of 5 m*M* Tris–HCl buffer with the same volume of 30 m*M* Tris–HCl buffer at constant pH 8.0. Every 10 ml of the effluent was collected in a tube with a flow rate of 60 ml/h. In order to identify the MbO_2 and the MetMb, the absorbancy ratio of 581 nm/593 nm was taken as a convenient indicator, though

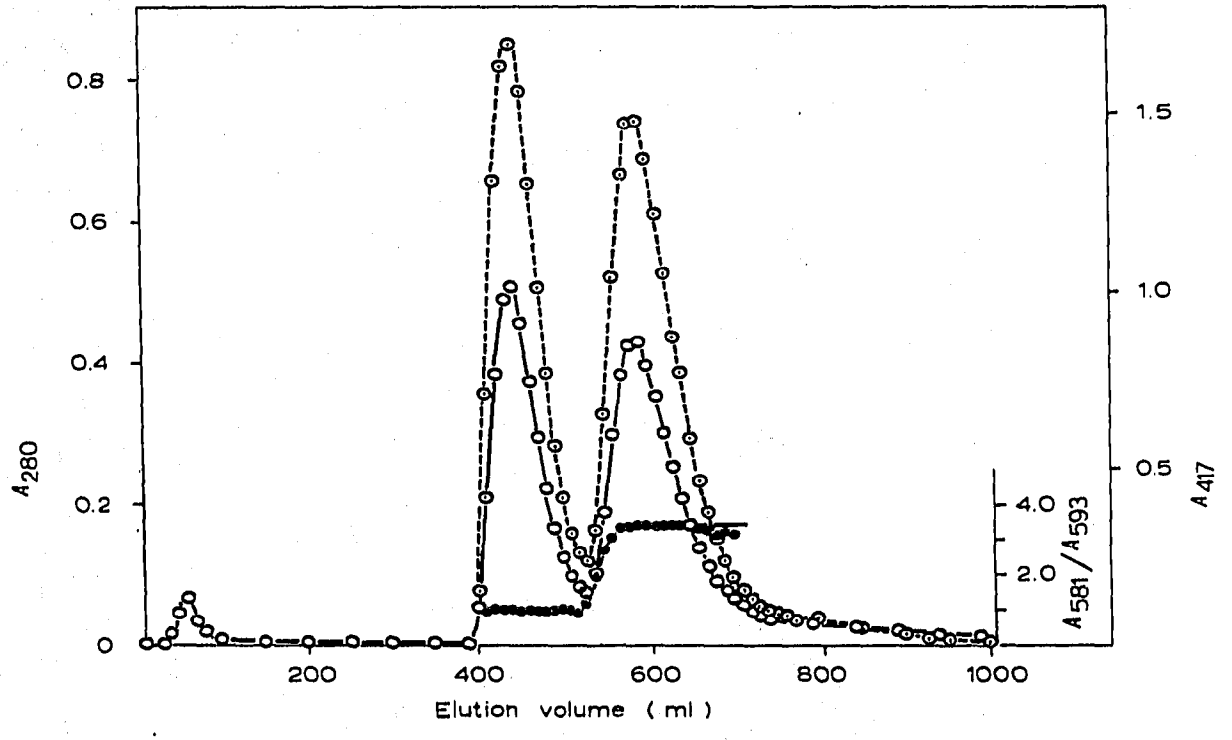

Fig. 3. DEAE-cellulose chromatography of MbO_2 and MetMb. Twenty ml (40 mg) of myoglobin were applied to a DEAE-cellulose column (2 × 20 cm) which had been equilibrated with 5 m*M* Tris–HCl buffer, pH 8.5. A linear gradient was employed for the elution by mixing 500 ml of 5 m*M* Tris–HCl buffer with the same volume of 30 m*M* Tris–HCl buffer at constant pH 8.0. Every 10 ml of the effluent was collected in a single tube at a flow rate of 60 ml/h. Protein and haeme protein were estimated by the absorbancy at 280 nm (○—○) and by the absorbancy at 417 nm (⊙—⊙), respectively. MbO_2 and MetMb were identified by the absorbancy ratio of 581 nm/593 nm (●—●).

these two forms of myoglobin are easily identified by their colours, red in the case of MbO_2 and yellowish brown for MetMb. As mentioned above, 581 nm is the α-peak of MbO_2, and 593 nm is the isobestic point for MbO_2 and MetMb at pH 8.0. This absorbancy ratio was 1.0 for MetMb. If MbO_2 exists to any extent in a fraction, this value would be higher than 1.0. The elution profile of the DEAE-cellulose chromatography for the separation of MbO_2 from MetMb is shown in Fig. 3. As clearly seen, the absorbancy ratio of 581 nm/593 nm was transmuted from 1.0 for the former peak to 3.4 for the latter, indicating the presence of MetMb and MbO_2, respectively. The recovery of the protein applied to the column was about 85%. A few faint coloured bands were left in the column. These bands seem to be the microheterogeneities of the myoglobin which have been remarked by several investigators[7,11,12]. The purity of our final product was examined by disc electrophoresis. The disc electrophoresis was carried out on a 7.5% polyacrylamide gel in 50 m*M* Tris–384 m*M* glycine buffer, pH 8.6, with a constant current of 2.5 mA per tube for 1.5 h. Fixation and staining were performed in 7.5% acetic acid containing 1% Amido Black 10B. As shown in Fig. 4, an aliquot of the MbO_2 peak in the DEAE-

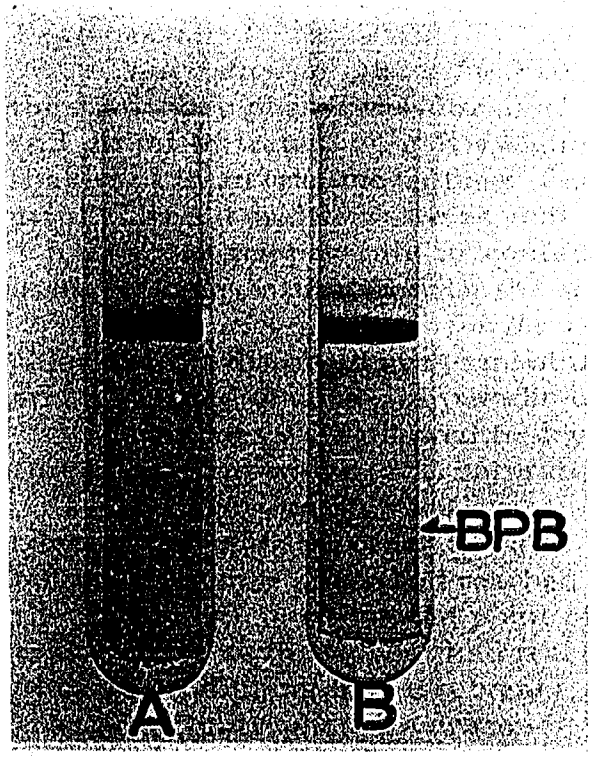

Fig. 4. Polyacrylamide disc electrophoresis of myoglobin. MbO_2(A) and MetMb(B) obtained from the fractions of the DEAE-cellulose chromatography were subjected to disc electrophoresis on 7.5% polyacrylamide in Tris–glycine buffer, pH 8.6, for 1.5 hours. Bromophenol Blue (BPB) was used as a marker.

cellulose chromatography (Fig. 3) appeared to be a single band on the polyacrylamide gel. An aliquot of the MetMb peak appeared to be one major and three minor bands on the polyacrylamide gel. The major and the third band from the top of the gel were haeme protein. The minor haeme protein seems to be a microheterogeneity of the

myoglobin. The relative mobility of MbO_2 was almost the same as that of MetMb under the conditions employed.

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