ISSN 0029 - 3865

CBPF-NF-044/91

A RAPID METHOD FOR OBTAINING LARGE AMOUNTS OF α AND β CHAINS FROM HUMAN HEMOGLOBIN

by

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Abstract

Human hemoglobin chains were dissociated by p-chloro-mercuribenzoate (PCMB) treatment and the resultant α and β chains were separated by batchwise techniques on DEAE and CM-cellulose resins respectively. This method, different from the classic chromatographic procedures, allows isolation of α and β chains from either diluted or concentrated solutions of hemoglobin in a short period of time.

Key-words: Hemoglobin chains; Batchwise.

Introduction

Hemoglobin and myoglobin have been widely studied and are considered as models of enzimatic action. The tetrameric structure of hemoglobin is responsible for the relevant cooperative effect of oxygenation. To develop the understanding of this effect, studies of isolated hemoglobin chains are fundamental. Additionally, separation of hemoglobin chains is important for (a) studies of the primary structure of polypeptidic chains of different hemoglobins, (b) detection of abnormal chains of mutant hemoglobins and (c) investigation on the biosynthetic ratio of the α chain to the non-α chain (Shibata, 1980).

Various procedures are known for dissociating and isolating α and β chains of human hemoglobin with different yield (Tyuma et al. 1966; Bucci & Fronticelli, 1965; Rosemeyer & Huehns, 1967). One of the problems during investigations of human hemoglobin mutants has been the difficulty of separating the α and β chains in a pure form from small quantities of starting material. This was partially resolved with the development of methods for dissociating large amounts of native α and β chains from human hemoglobin with p-chloro-mercuribenzoate (PCMB) treatment. The resultant chains are separated by chromatography on a column of CM-cellulose or DEAE cellulose by means of a pH gradient (Geraci et al. 1969). Although column chromatography techniques have proven to be the most accurate they are time consuming. The alternative of using modern techniques such as FPLC (Fast Protein Liquid Chromatography) turns out expensive and restricted to small amounts of material.

We now report on a simple and rapid method for separating any amount of hemoglobin chains by batchwise techniques on cellulose resins.

Material and Methods

Human Hemoglobin (HbA)-Blood was collected in heparin and red cells were washed three times with 0.9% saline solution. Packed cells were hemolyzed with distilled water and chloroform. The concentration of HbA solution was

determined spectrophotometrically at 540 nm after conversion to methemoglobin cyanide (ε = 11.0 x $10^3 cm^{-1}.mole^{-1}$/per heme group). A solution of oxyhemoglobin in 0.1M phosphate buffer, pH 6.2 was dissociated into its subunits by reaction with PCMB, slightly modifying Bucci and Fronticelli (1965) method. The PCMB was dissolved in a small volume of 0.1N NaOH and back titrated with 0.5N acetic acid to the first appearance of turbidity. The HbA solution was adjusted to contain 0.2M NaCℓ and then mixed thoroughly with the PCMB solution (molar ratio of PCMB to Hb 10:1). The PCMB-Hb solution was left overnight at 4^0C.

For separating PCMB α and β chains, a two batchwise selective procedure was used. To obtain α chains, one half of the PCMB-Hb solution was adjusted to pH 8.6 by centrifuge desalting through a Sephadex G-25 column equilibrated with 50mM Tris-HCℓ. The solution was then incubated for 1h at 4^0C with DEAE-cellulose resin (Whatman DE-52) equilibrated in the same buffer in a proportion of 60-120 mg protein in 2mℓ/0.5g resin. The incubation was interrupted by centrifugation at 800xg and the supernatant was again incubated with a new sample of resin (in the same conditions) for 1:30h. After this period the supernatant was collected by centrifugation as described. Under these conditions undissociated HbA and β chains remain bound to the resin.

β chains were obtained by adjusting the remaining half of the PCMB-Hb solution to pH 6.6 by centrifuge desalting through a Sephadex G-25 column equilibrated with 10mM potassium phosphate. The solution was then incubated with a CM-cellulose resin (Whatman CM-52) (60-120mg protein in 2mℓ/0.5g resin) equilibrated in the same buffer. The rest of the procedure was as detailed for α chains.

All the described chromatographic procedures are carried out in the cold. The extent of chain formation and the purity of the isolated chains were checked by 1% agarose horizontal electrophoresis in Tris-EDTA-borate acid buffer system as described by Gammack et al. (1960) for starch gel electrophoresis. For the system of Gammack et al., the following stock solution is used: Tris base 109g/liter; disodium EDTA, 5.84g/liter; boric acid, 30.9g/liter. The stock solution is diluted 1:20 for the gel and 1:7 for the electrodes. The electrophoresis lasts for 30-40min. using a voltage of 100V. The gels were stained with coomasie blue.

Results and Discussion

Figures 1 and 2 show photographs of the electrophoretic patterns obtained with typical samples of α and β chains purified by the batchwise procedure. The pattern for the chains looks homogeneous and similar to those obtained by chromatographic methods (Bucci & Fronticelli, 1965; Tyuma et al. 1966, Rosemeyer & Huehns, 1967). As expected, the β chains migrate towards the anode at a higher rate than HbA. The slowest band corresponds to α chains. In our conditions, the α chains were arrested towards the cathode, probably due to an endosmotic effect.

We modified the traditional use of starch in gel electrophoresis using agarose, making it easier to handle and giving a better resolution.

The yield obtained is approximately 47% for initial amount of α chains and 36% for β chains. These values are close to those found by Bucci & Fronticelli quoted in the review by Bucci, 1981. They obtained a yield of 50% of the initial amount of the chains using a classic CM-cellulose chromatography. The PCMB-α and -β chains were eluted by a pH continuous gradient. Bucci suggests that the observed loss is probably due to the precipitation caused by the PCMB treatment and by inespecific adsorption to cellulose resins.

In this work, we purified α and β chains from PCMB-Hb diluted solutions (and small volumes). The purified chains are mostly in the oxy-form as shown by the characteristic absorption spectra (700-300 nm) (data not shown). The possibility of handling small quantities of starting material simplifies the study of Hb rare mutants without further manipulation such as concentrating the sample. Although we used diluted solutions we can easily process by batchwise procedure large amounts of protein in the same short period of time (2:30h). In contrast, the elution time of the classic column chromatography increases with increasing protein concentration of the sample.

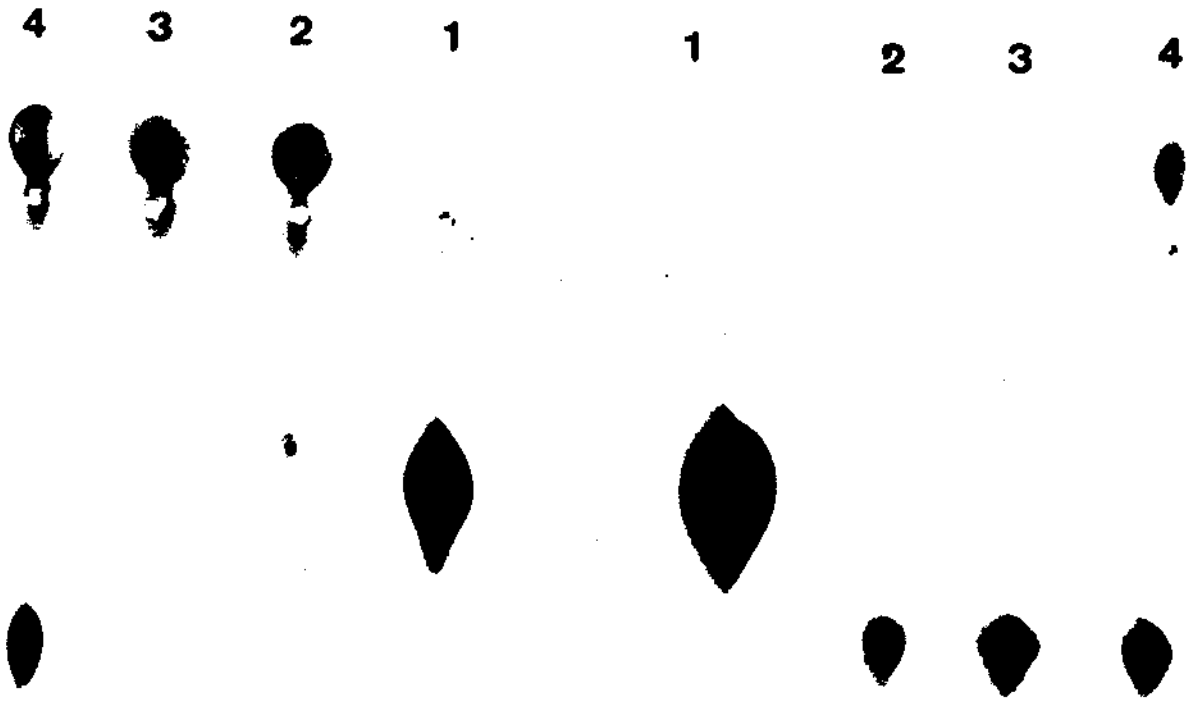

Fig. 1

Fig. 2

Figure 1 - Agarose electrophoresis of HbA subunits.

1 - HbA

2 - α-PCMB subunits (first incubation)

3 - α-PCMB subunits (second incubation)

4 - HbA + PCMB (mixture of α-PCMB and β-PCMB subunits)

Figure 2 - Agarose electrophoresis of HbA subunits.

1 - HbA

2 - β-PCMB subunits (first incubation)

3 - β-PCMB subunits (second incubation)

4 - HbA + PCMB (mixture of α-PCMB and β-PCMB subunits).

Acknowledgments

We are grateful to Dr. Eliane Wajnberg for helpful discussions and criticism of the manuscript and to the Instituto de Hematologia (Rio de Janeiro, Brasil) for kindly supplying blood samples.

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