

Detection of Abnormal Haemoglobin by Capillary Electrophoresis and Structural Identification

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Haemoglobin obtained from a male adult Ghanian with retinopathy, which was probably caused by haemoglobinopathy was analysed by capillary electrophoresis (CE) for clinical diagnosis. Two major peaks, which were in the ratio of nearly one, were detected. The elution times of these peaks (HbXI and HbXII) were faster than that of normal haemoglobin (HbA). The existence of two different abnormal types of haemoglobin was clear in the patient blood. The following sequence analysis revealed that the first peak (HbXI) was HbC and the second (HbXII) was HbS on the electropherogram, and that the patient was a heterozygote of HbS and HbC (HbSC disease). One of the diagnostic processes in a haemoglobin disease was shown by the combined use of CE, HPLC and a protein sequencer.

INTRODUCTION

Capillary electrophoresis (CE) is a powerful and widely utilized technique for analysis of biologically important molecules (Jorgenson 1984; Jorgenson and Lukacs, 1981^{a,b}, 1983; Tsuda *et al.*, 1983; Gassmann *et al.*, 1985; Lauer and McManigill, 1986^{a,b}; Cohen *et al.*, 1987; Gordon *et al.*, 1988; McCormick 1988; Lüdi *et al.*, 1988; Ewing *et al.*, 1989; Kennedy *et al.*, 1989; Grossman *et al.*, 1989; Gross and Yeung, 1990; Zhu *et al.*, 1990; Chen *et al.*, 1991). Furthermore, CE analysis has become feasible for the direct detection of biological microenvironments, specifically at the single cell level (Ewing *et al.*, 1989; Kennedy *et al.*, 1989), and has been used to separate small variations in the primary and/or secondary structure of peptides and proteins (McCormick, 1988; Lüdi *et al.*, 1988).

Since the discovery of haemoglobin S (HbS) abnormal haemoglobin has been studied intensively for medical and genetic purposes. The most popular methods for analysis of abnormal haemoglobin are gel electrophoresis and high performance liquid chromatography (HPLC) on ion exchangers. Electrophoretic analysis has made possible the discovery of numerous haemoglobin variants. The recent developments of the CE procedure offer a new approach for the rapid analysis of a micro amount of abnormal haemoglobin for clinical diagnosis (Chen *et al.*, 1991).

In this study, we employed the CE method prior to the popular and conventional methods to analyse and confirm the existence of haemoglobin variants in the blood of a patient who was a suspected case of haemoglobinopathy because of the presence of leptocytes and characteristic retinopathy.

EXPERIMENTAL

Materials. Haemoglobin solutions were prepared from the blood of both a male adult Ghanian with retinopathy in a suspected case of haemoglobinopathy and a normal adult Japanese. L-(Tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK)-treated trypsin was purchased from Sigma (St. Louis, MO, USA). Acetonitrile (HPLC grade) was obtained from Nacalai Tesque (Kyoto, Japan) and trifluoroacetic acid (TFA; sequence grade) was from Wako Pure Chemical Industries (Osaka, Japan). Biofine IEC-CM (7.5 mm i.d. × 75 mm) for a cation exchange column was from Japan Spectroscopic Co. (Hachioji, Japan) and Develosil 300 ODS-7 (4.6 mm i.d. × 250 mm) for a reversed phase column was from Nomura Kagaku (Seto, Japan).

Capillary electrophoresis. CE was performed on the Model 270A capillary electrophoretic system from Applied Biosystems (Foster City, MI, USA). An uncoated fused-silica capillary (50 µm i.d. × 122 cm length) was used. Before electrophoresis, the capillary was washed with 1 N NaOH, rinsed with distilled water, washed again with 1 N HCl, rinsed with distilled water, and equilibrated with 0.1 M sodium borate buffer (pH 9.98). Three nanolitres of haemoglobin solution (3 mg/mL in water) was introduced into the capillary by vacuum injection for 1 s. Electrophoresis ran at 30 KV constant voltage for 50 min at 30°C using 0.1 M sodium borate buffer (pH 9.98), and was monitored simultaneously at 200 nm.

Sequence analysis. The sequence analysis of globin chains was performed by the methods described previously (Ishioka *et al.*, 1986). The purified globin chains from abnormal haemoglobin were reduced and carboxymethylated and then digested with TPCK-trypsin in 0.1 M ammonium bicarbonate solution at 37°C for 24 h. Tryptic peptides were separated by reversed phase HPLC using conditions described in detail previously (Ishioka *et al.*, 1990). Elution for tryptic peptides from a chain was for 40 min with a linear gradient of 0–42% acetonitrile.

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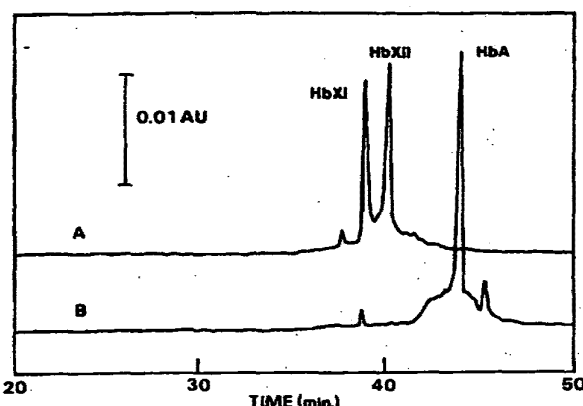


Figure 1. Capillary electropherograms of haemoglobin from a patient with retinopathy (A) and a normal volunteer (B). The electrophoretic run is described in the Experimental Section. HbXI and HbXII in (A) showed two types of abnormal haemoglobin, and HbA in (B) was a normal type of haemoglobin.

trile and for β chain was for 60 min with a linear gradient of 0–25% acetonitrile. The peptides were identified by their amino acid composition and the variant peptide of abnormal haemoglobin was identified by its amino acid sequence. Amino acid sequence determination was performed with an Applied Biosystems Model 471A protein sequencer.

RESULTS

Analysis of the patient's haemoglobin

The capillary electropherogram of the patient's haemoglobin is shown in Fig. 1. Two major peaks were detected under alkaline conditions (HbXI and HbXII in Fig. 1A). On the other hand, HbA was detected as one major peak, and the elution time of HbA was later than those of HbXI and HbXII (HbA in Fig. 1B). The amounts of HbXI and HbXII were almost the same (the ratio of HbXI was 47.4% and that of HbXII was 52.6%). The theoretical plate number was an average of 210,000. This abnormal haemoglobin presumably consists of two different types, and, under alkaline conditions, each net charge of these haemoglobin variants has less negative than the net charge of HbA.

Peptide mapping and sequence analysis of globin chains in abnormal haemoglobin

Two components of abnormal haemoglobin were separated by cation exchange HPLC. As shown in Fig. 2, two major peaks were obtained in the ratio of nearly one, which corresponded to the peaks of the capillary electropherogram except that HbXII was eluted faster than HbXI. The globin chains of HbXI and HbXII were also separated by cation exchange chromatography after being denatured in 8M urea (not shown), subjected to peptide mapping and to sequence analysis. The HPLC patterns of peptide mapping of the normal β chain are shown in Fig. 3. About 12 major peaks are obtained on each chromatogram, in which all the expected peptides were included. A peptide β T1 disappeared from the chromatogram of β I and β II (β I from HbXI and β II from HbXII in Fig. 3), and one new

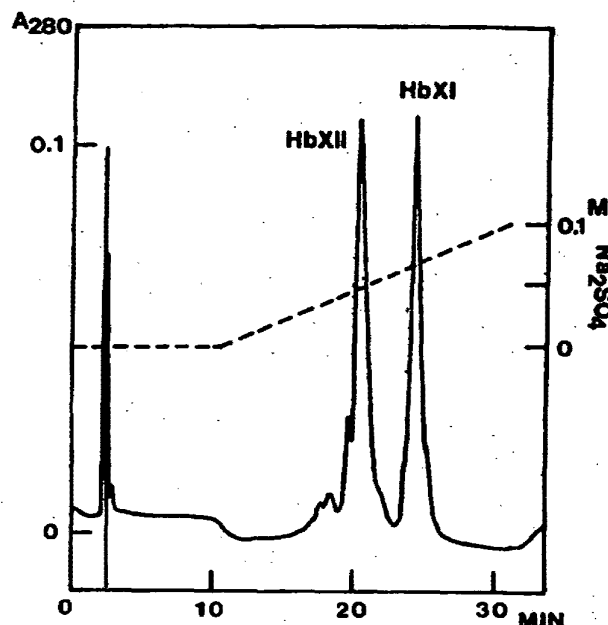


Figure 2. Separation of two components of abnormal haemoglobin on a cation exchange column high performance liquid chromatograph. Two types (HbXI and HbXII) of abnormal haemoglobin were separated at room temperature by a linear gradient of 0.1 M Na_2SO_4 (0–100%) in 0.02 M K_2HPO_4 (pH 6.5) at a flow rate of 1.0 mL/min for 20 min.

peak, β Ix and β Iix, appeared instead of β T1, respectively. The β Ix and β Iix seem to derive from the N-terminal portion, because the β T1 is the N-terminal peptide of the β chain. The maps obtained from each α chain also compare closely to one another. However, there were no significant differences on the chromatograms (not shown). It became apparent that the variants existed only in the β chains and not in the α chains. Amino acid composition and sequence analysis verified that Glu-6 was replaced with Lys-6 in the peptide β Ix,

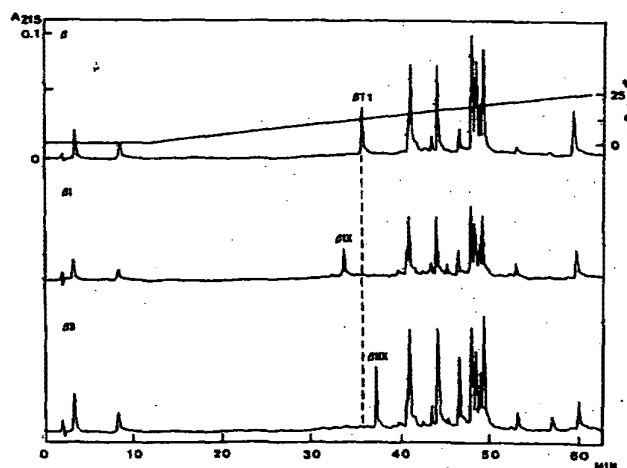


Figure 3. Peptide mappings of β globin chains. The tryptic digests of β globin chains were applied to a reversed phase column and eluted as described in the Experimental Section. β , β I, and β II in the figure showed the maps from HbA, HbXI and HbXII, respectively. A β T1 showed the N-terminal peptide of the normal β chain. β Ix and β Iix showed new peptide peaks in β I and β II, respectively, instead of β T1.

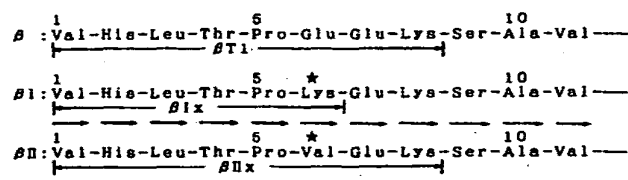


Figure 4. Amino acid sequence analysis of the tryptic peptides with the substitution obtained from abnormal haemoglobin. Amino acid residues identified by automated Edman degradation are indicated by small arrows and (*) shows the substitution sites.

as for HbXI (this [B6(A3)Glu→Lys] substitution type is HbC), and that Glu-6 was also replaced with Val-6 in the peptide β IIx, as for HbXII (this [B6(A3)Glu→Val] is HbS). These results were also supported by direct sequence determination of the N-terminal portion of the β I and β II chains (Fig. 4). The above results led to the conclusion that this patient was a heterozygote for haemoglobin S and haemoglobin C (HbSC disease).

DISCUSSION

Separation of proteins by CE in uncoated fused silica capillaries is limited by adsorption and variable rates of

electroosmotic flow. Operation at pH values above 8 to minimize these problems is useful (Lauer and McManigill, 1986^{a,b}; Lüdi *et al.*, 1988; Zhu *et al.*, 1990; Chen *et al.*, 1991), although some basic proteins can still adsorb, and the resolution may be reduced by inducing a strong electroosmotic flow. In the present study, pH 9.98 was employed and this pH value was above the isoelectric point of haemoglobin. Under these alkaline conditions, an optimal separation of haemoglobin variants was achieved, although it took a relatively long time for analysis. The analysis time might be reduced by using a short capillary, and a good example for separation of haemoglobin variants was presented by Chen *et al.* (1991). The potential, clinical and diagnostic utility of CE has been demonstrated (Jorgenson and Lukacs 1981^b, 1983; Chen *et al.*, 1991) and the effectiveness of analyses for serum proteins and haemoglobin variants was also established by comparison with conventional agarose gel-based electrophoresis (Chen *et al.*, 1991).

In this report, we have demonstrated that CE analysis is useful for clinical diagnosis of haemoglobinopathy of an actual patient with retinopathy, and we have also shown one of the diagnostic processes for the abnormality of unknown clinical samples by the combined use of CE, HPLC and a protein sequencer.

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