<table>
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<th>Title</th>
<th>Isolation of All Soluble Tryptic Peptides from the α Polypeptide Chain of Adult Hemoglobin from Japanese Monkey (Macaca fuscata fuscata) and their Amino Acid Compositions</th>
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<td>Author(s)</td>
<td>Terao, Yoji</td>
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Isolation of All Soluble Tryptic Peptides from the 
α Polypeptide Chain of Adult Hemoglobin from 
Japanese Monkey (Macaca fuscata fuscata) and 
their Amino Acid Compositions*

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The adult Japanese monkey (macaca fuscata fuscata) hemoglobin was 
converted into globin by removal of heme. The globin was separated into 
α and β polypeptide chains by countercurrent distribution with the solvent 
system of trichloroacetic acid containing sec-butanol, propionic acid, and 
water. The α polypeptide chain was hydrolyzed with trypsin. From the 
hydrolysate, peptides soluble at the adjustment of pH to 6.4 were isolated 
by column chromatography on Dowex 1×2 with acetate buffer containing 
organic bases such as pyridine, collidine, lutidine, and picoline, and then 
purified by descending paper chromatography with the mixture of n-butanol, 
acetic acid, and water. The amino acid compositions of the peptides thus 
isolated and purified were analyzed after HCl hydrolysis. The results were 
discussed in comparison with adult human hemoglobin and rhesus monkey 
(macaca mulatta) hemoglobin.

Concerning the amino acid compositions of the soluble tryptic peptides 
from the α polypeptide chain, it is presumed that Japanese monkey hemo-
globin is different from human hemoglobin in four amino acids of the three 
peptides, but quite the same with rhesus monkey hemoglobin which belongs 
to the same genus as the Japanese monkey does.

INTRODUCTION

Hemoglobin is a chromoprotein which exists widely in the animal 
kingdom. Though hemoglobins from various kinds of animals have a 
common function of transporting oxygen, they show their own peculi-
arities in electrophoresis, chromatography, and alkali resistance.

Studies on heme, the prosthetic group of hemoglobin, began earlier. 
The structure, which was determined by H. FISCHER in 1928, is 
already known to be common to all hemoglobins.

* This work was presented at the 41st meeting of the Japanese Biochemical Society in October, 
1968.

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With respect of globin, protein moiety, a number of reports on the structure of various hemoglobins have been made since BRAUNITZER et al. and KONIGSBERG et al. determined the primary structure of adult human hemoglobin. Reports so far published prove that though hemoglobins of various animals have nearly homologous structure, many differences among the species are found in the amino acid sequence. It is considered that these differences were caused by the accumulation of point mutations due to single base changes in genes, DNA, which control the biosynthesis of hemoglobin molecules. This also suggests that present hemoglobins of various kinds of animals have evolved from one original primitive hemoglobin. INGRAM, from this point of view, demonstrated the models of the evolution in polypeptide molecules of the α, β, γ, and δ chains of human hemoglobin by discussing the differences among their amino acid sequences. PAULING et al. figured out that 14 × 10⁶ years was required for substitution of one amino acid residue into another, on the basis of the fact that there are 17 amino acid substitutions in the amino acid sequence of the α polypeptide chain between human and horse hemoglobins. Recently, FITCH et al. pushing forward this point of view and demonstrating that mutation distance with consideration of mutable or back mutable amino acids could be calculated on the basis of variations in amino acid sequences of proteins, suggested a new type of the phylogenetic tree could be worked out. To presume the process of evolution by comparing living organisms at molecular level gives a new viewpoint to the theory of evolution which has proceeded mainly with studies on shapes and habits of living organisms.

Concerning hemoglobins of primates, interest has centered on what differences can be found between human and primate hemoglobins. ZUCKERKANDL et al. found that chimpanzee hemoglobin was quite similar to that of human hemoglobin and that gorilla hemoglobin was different from human hemoglobin at one point each in the α and β polypeptide chains. In similar ways, BUETTNER-JANUSH et al. partially compared hemoglobins of the hylobates, the papio, the perodicticus, the galogo, the lemur, and so forth with human hemoglobin and confirmed considerable differences among them.

In this laboratory, the primary structure of rhesus monkey (macaca mulatta) hemoglobin has been studied and the complete amino acid sequences were determined. According to the results, amino acid exchanges between human and rhesus monkey hemoglobins are found at four positions in the α chain and at eight positions in the β chain.

Japanese monkeys (Macaca fuscata fuscata) which live only in the Japanese Islands belong to the same genus with rhesus monkeys. It is interesting how different the hemoglobins of these two species are. The present author investigated on the amino acid compositions of the soluble tryptic peptides from the α polypeptide chain of adult Japanese
monkey hemoglobin. This paper describes in detail separation of α and β polypeptide chains, tryptic hydrolysis, isolation and purification of tryptic peptides by column and paper chromatographies, and determination of amino acid compositions.

MATERIALS AND METHODS

1) Preparation of Hemoglobin Solution

Blood drawn from the abdominal aorta of Japanese monkeys (macaca fuscata fuscata) purchased from Japan Monkey Center, Inuyama, was added 3.8% citrate sodium solution to prevent from coagulation and subjected to centrifugation at 3,000 r.p.m. for 10 min to remove plasma. To blood cells were added 2 volumes of 0.9% NaCl solution, and centrifuged at 3,000 r.p.m. for 5 min. This treatment was repeated three times. And then red cells were hemolyzed by adding an equal volume of deionized water and 1/2 volume of toluene and stirring in the cold room overnight. The hemolysate was centrifuged at 12,000 r.p.m. for 60 min. Subsequently, hemoglobin solution was obtained from the inter layer between toluene layer and stroma.

2) Removal of Heme from Hemoglobin

The solution, which was made by adding 15 ml of concentrated hydrochloric acid to 500 ml of acetone, was cooled at -20°C in the dry ice methanol. To this solution was added dropwise 50 ml of hemoglobin solution with stirring violently. After stirring the mixture for more 15 min globin which came to be precipitated was isolated by centrifugation at 5,000 r.p.m. for 10 min and washed three times with cooled hydrochloric acid-acetone mixture. The globin dissolved in the appropriate quantity of deionized water was dialyzed against deionized water and then lyophilized.

3) Separation of α and β Polypeptide Chains of Globin

Countercurrent distribution was employed for separation of α and β polypeptide chains, subunits of the globin. The solvent system used was the mixture of sec-butanol containing 0.08% of trichloroacetic acid, propionic acid, and water (8.7:1.5:11.0). In 40 ml of the lower phase of the above-described solvent, 500 mg of the globin was dissolved and to this was added 40 ml of the upper phase. The mixture was kept settled for 1/2 hour and again separated into upper and lower phases. The solution (10 ml, each) was placed in No. 3 to No. 6 tubes of the countercurrent distribution machine (20 ml capacity, 100 tubes, Shibata CDA-100). The upper phase transfer was carried out 150 times at 25°C in the constant temperature room, the machine being set for a 30-sec shifting time and a 20-min settling time. After the solution contained in each tube was added 0.5 ml of 50% ethanol to be clear, the absorbance was measured at 280 m. The solution of high-absorbance peaks
were respectively pooled, dialyzed against deionized water, and lyophilized.

4) Digestion of the $\alpha$ polypeptide Chain with Trypsin

In 50 ml of 8M urea solution was dissolved 500mg of the $\alpha$ polypeptide chain and the solution was stirred at 60°C for 45 min. After cooling, the solution was dialyzed against deionized water. According that urea was removed, the $\alpha$ polypeptide chain came to be precipitated in the dialyzing tube.

Chymotryptic activity of trypsin was removed from trypsin (Worthington Biochemical Corp., twice crystallized) by dissolving it in 1/16N HCl to a concentration of 1% and allowing to stand at 37°C for 16 hours as described by REDFIELD and ANFINSEN. The urea-denatured $\alpha$ polypeptide chain was suspended in 50 ml of deionized water. The suspension, stirred violently at 37°C, was adjusted to pH 9.0 with 0.1N NaOH, and to this was added 1 ml of trypsin solution (10 mg trypsin). Hydrolysis was carried out at 37°C for 4 hours at pH 8.0. The reaction was traced on the basis of the necessary quantity of 0.1N NaOH to maintain the hydrolysate at pH 8.0. After terminating the hydrolysis, the hydrolysate was subjected to centrifugation at 10,000 r.p.m. for 20 min to remove some insoluble material.

5) Fractionation of the Tryptic Peptides by Precipitation at pH 6.4

When the tryptic hydrolysate was adjusted to pH 6.4 with 1N acetic acid, the so-called "core", the insoluble tryptic peptide was precipitated. It was removed by centrifugation at 5,000 r.p.m. for 20 min and the remaining tryptic peptides were lyophilized.

6) Column Chromatography of the Soluble Tryptic Peptides on Dowex 1 x 2

500 ml of dry resin (Dowex 1 x 2, 200–400 mesh, Cl-form, Dow Chemical Company) was suspended in three volumes of deionized water. The suspension, after stirred, was allowed to stand for an hour and then the fine particles discarded by decantation. This treatment was repeated three times. The resin whose grain was made even in this way was suspended in two volumes of 1N NH₄OH. After allowed to stand for an hour, it was washed in a glass filter. The resin, transferred again in a beaker, was suspended with two volumes of glacial acetic acid and allowed to stand for an hour with occasional stirring. The resin, after washed with deionized water and 2 liters of the starting buffer respectively, was suspended in the starting buffer, evacuated, and poured into a column (2 x 150cm) which was warmed at 37°C. The resin in the column was equilibrated with 4 liters of the starting buffer.

The soluble tryptic peptides from 500 mg of the $\alpha$ polypeptide chain dissolved in 40 ml of deionized water was adjusted to pH 10.0 with 0.1 N NaOH. It was placed on the column. Fractions No. 1–20
were developed with the starting buffer, 1% pyridine, 1% \( r \)-collidine acetate buffer, pH 9.0 and Fractions No. 21–40 with the acetate buffer of the same composition, pH 8.5. After that, the gradient elution method was employed, that is, during the elution of Fraction No. 41–170, 0.75N acetic acid was supplied from the upper chamber into the mixing chamber containing 150 ml of 1% pyridine 1% 2.4 lutidine, \( \alpha \)-picoline acetate buffer, pH 7.5, and for the successive Fraction No. 171–240, 1.0 N acetic acid was supplied. All the organic bases (Wako Pure Chemical Industries Ltd., Osaka, Japan; special grade) used for the developers were distilled before the experiment. Development was performed at a flow rate of 200 ml/h, the column temperature being at 37°C, and the eluate was collected in 18 ml fractions. A 0.2 ml portion of each fraction, after alkaline hydrolysis, was subjected to ninhydrin reaction according to the method of Yemm and Cockings. Fractions positive to ninhydrin reaction were pooled at every peak and evaporated under reduced pressure. The residue was again dissolved in 4 ml of deionized water and lyophilized.

7) **Identification of the Peptides by Paper Chromatography**

Descending paper chromatography was carried out on a sheet of Toyo filter paper No. 50 with the upper phase of n-butanol, acetic acid, and water (4:1:5). Peptides on the chromatogram were detected by spraying 0.2% ninhydrin-n-butanol solution and heating the paper with an iron to color. As the specific amino acid color reaction, Pauly's reaction (His), \(^{17}\) \( \alpha \)-nitrosonaphtol reaction (Tyr), \(^{13}\) Sakaguchi's reaction (Arg) \(^{9}\), Ehrlich's reaction (Try) \(^{16}\) were employed.

8) **Purification of the Peptides by Paper Chromatography**

Peptides contained in each peak which was fractionated by column chromatography were purified by paper chromatography with the same system as that used for identification. Peptides on the chromatogram were located by coloring lightly with 0.02% ninhydrin-n-butanol solution. The colored spots were cut out and washed with acetone to remove ninhydrin. Elution of the peptides from the paper was carried out with 5% acetic acid, and the eluate was evaporated under reduced pressure.

9) **Amino Acid Analysis of the Peptides**

The purified peptides in 4 ml of twice-distilled constant boiling point HCl were hydrolyzed in a sealed tube at 105°C for 24 hours. The hydrolysates, after repeating evaporation to dryness under reduced pressure to remove as much HCl as possible, were subjected to Hitachi KLA-2 amino acid analyzer for their amino acid compositions.
RESULTS AND DISCUSSION

Hemolysate of adult Japanese monkeys contains hemoglobin consisting of one main component, which is almost similar in electrophoretic mobility to human hemoglobin. It is already known according to the results of the N-terminal analyses by the DNP method that as in the case of human hemoglobin, the hemoglobin molecule from Japanese monkey is composed of two Val-His polypeptide chains (α chains) and two Val-His-Leu polypeptide chains (β chains). After heme was removed by the method of ANSON and MIRSKEY, the globin was separated into its subunits, α and β polypeptide chains, by countercurrent distribution. The result is given in Fig. 1. The average recoveries of the α and β polypeptide chains by this method were 64% and 72%, respectively, and the purity was proved to be over 90% from the result of the N-terminal structural analysis by the DNP method.

The α polypeptide chain after denatured at 60°C for 45 min in 8 M urea solution, was hydrolyzed with trypsin. Hydrolysis was carried out at 37°C and pH 8.0, enzymatic concentration against the substrate being 2%. The hydrolytic process was traced by recording the uptake of 0.1N NaOH. The result is shown in Fig. 2. As shown in this figure, the alkali uptake was almost ceased two hours after trypsin was added, but hydrolysis was continued for 4 hours. Even at that time, the solution was pretty turbid. It appeared that the turbidity was caused by the indigest, which was subjected to centrifugation at pH 8.0 and the precipitate was discarded. It is generally known that among the tryptic peptides of a hemoglobin, there exists the so-called "core"
Fig. 2 The course of the tryptic digestion of the α chain of Japanese monkey hemoglobin which is insoluble at pH 6.4. The precipitation method at pH 6.4 was carried out for the first fractionation of the tryptic peptides of the α polypeptide chain. The hydrolysate brought to pH 6.4 with 1N acetic acid was centrifuged, and then from the supernatant, the so-called "soluble tryptic peptides" were obtained. The soluble tryptic peptides were isolated by column chromatography with the adsorbent, Dowex 1×2, and with developer, acetate buffer containing volatile organic bases, and then purified by paper chromatography. Fig. 3 and Fig. 4

Fig. 3 Column chromatogram of the soluble tryptic peptides from the α polypeptide chain from Japanese monkey hemoglobin
Concerning the column chromatography of peptides with this system, RUDLOFF and BRAUNITZER have described in detail and HILSE and BRAUNITZER used it for isolation and purification of the tryptic peptides from the α and β polypeptide chains of human hemoglobin, and they obtained satisfactory results. In comparing the chromatogram given in Fig. 3 with that of the soluble tryptic peptides from the α polypeptide chain of rhesus monkey hemoglobin, both are very similar to each other except that the chromatography in the present study well isolated basic peptides owing to the use of the starting buffer, pH 9.0. In order to know purity of the isolated peptides in 10 peaks fractionated in this way, paper chromatography with ninhydrin color reaction was carried out. The result is given in Fig. 4, together with the results from special amino acid color reactions. Some could be purely isolated only by the column chromatography but some fractions contained more than two ninhydrin-positive substances. They were, however, proved to be fully purified by the paper chromatography with this system. Therefore, all the peptides were purified as described above.

Those purified peptides were hydrolyzed with constant boiling point HCl at 105°C for 24 hours, and then the amino acid compositions of these peptides were analyzed. The results are shown in Table I. Analytic values were given as molar ratios without correction for losses during hydrolysis. Yields of peptides given in % were calculated rega-
Table I
Amino acid compositions of the soluble tryptic peptides from
the α polypeptide chain of Japanese monkey hemoglobin

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Yield | 31 | 43 | 38 | 25 | 36 | 55 | 38 | 17 | 49 | 30 | 64 |

According to 500 ml of the α polypeptide chain as 31 μmoles. As for tryptophan, the peptides which were positive to EHRLICH's reaction on the paper chromatogram was marked with (+) in Table I.

Tryptic peptides from the α polypeptide chain of Japanese monkey (MFα) obtained from the spots on the paper chromatogram in Fig. 4 will be discussed in comparison with the soluble tryptic peptides from the α polypeptide chain of rhesus monkey hemoglobin (MMαT) and human hemoglobin (HαT) whose amino acid sequences are already known.

Ia The amino acid composition was Lys, 1.97; His, 0.93; Gly 2.10. The yield was 23%. It has lysine one mole more than IIIa and the composition is the same as HαT7−8 or MMαT7−8. Therefore, this should be designated MFαT7−8.

IIa As a result of amino acid analysis after HCl hydrolysis, lysine was only recognized and also on the paper chromatogram, it was identified as lysine. It corresponds with HαT8 or MMαT8.

IIb It is quite similar to MMαT10 or HαT10, Leu-Arg, both on the paper chromatogram (Rf Leu 0.50) and in the amino acid analysis.

IIIa It has the same composition with αT7 (Gly-His-Gly-Lys) which
is called the basic center of the \( \alpha \) polypeptide chain.

**IIIb** It has the same composition with that of MM\( \alpha \)T2, however, it has serine one mole more and threonine one mole less than HaT2.

**IIIc** It is a methionine-containing peptide and has quite the same composition as MM\( \alpha \)T5 or HaT5. From the lightly colored spot located above **IIIc** on the paper chromatogram, a peptide which has the same composition with **IIIc** was obtained. It appears to be produced owing to the change of methionine.

**IVA** It is only one spot positive to the tryptophan reaction on the paper chromatogram. The Rf Leu value was 0.41. The amino acid composition shown in Table I is similar to that of MM\( \alpha \)T3 or HaT3.

**Va** Rf Leu 0.44. It is a dipeptide composed of tyrosine and arginine, and similar to the C-terminal peptide of MM\( \alpha \)T41 or HaT14. MAITa, in isolation of the tryptic peptides from rhesus monkey hemoglobin, reported that \( aT3 \) and \( aT14 \) could not be separated from each other.\(^{11}\) In addition, in the report of HILSe and BRAUNIZER concerning human hemoglobin,\(^{7} \) these two peptides were eluted in the same fraction by column chromatography. In the present study, these two peptides could be completely separated by column chromatography. It is probably because of the starting buffer, pH 9.0.

**VIa, VIb** They are similar in the amino acid composition to MM\( \alpha \)T1 and MM\( \alpha \)T11, respectively. They are also similar to those of human hemoglobin. It is because VIb is located just in front of the so-called “core” peptide that its yield was 17%, which is smaller than any other peptide.

**VIIa** It is a comparatively long peptide of 16 amino acid residues and is quite the same as MM\( \alpha \)T6 or HaT6 both in amino acid analysis and on the paper chromatogram.

**VIIia** The amino acid composition, though not shown in Table I, is as follows; Lys, 1.92; His, 2.84; Asp, 4.69; Thr, 0.91; Ser, 1.83; Pro, 1.10; Gly, 1.12; Ala, 6.13; Val, 3.11; Met, 0.51; Leu, 5.13. The yield was 12%. In comparison with **IXa**, this peptide has lysine one mole more than **IXa** and so it is considered to correspond to MM\( \alpha \)T8 –9 or HaT8–9. The combination of a lysine residue and \( aT9 \) at its N-terminus like this peptide was also found in the case of rhesus monkey hemoglobin.

**IXa** It is a long peptide composed of 29 amino acid residues. Its amino acid composition is quite the same with that of MM\( \alpha \)T9. The analytic value of aspartic acid seems to be a little smaller for 5 moles. It is probably because of the existence of Asp-Asp linkage. In comparison
with HaT9, this peptide has glycine and leucine one mole more and aspartic acid and alanine one mole less than HaT9.

Xa It is an acidic peptide and was completely purified by column chromatography. The yield was in 64%. This peptide has quite the same composition as MMαT4, but has glycine one mole more and alanine one mole less than HaT4.

The above-described results are summerized in Table II. As shown in this table, the α polypeptide chains of rhesus monkey hemoglobin and Japanese monkey hemoglobin have the same amino acid composition as far as their soluble tryptic peptides composed of 101 amino acid residues.

Table II
Comparisons of the Amino Acid Compositions of the Tryptic Peptides among Japanese monkey, rhesus monkey, and Human Hemoglobins

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However, between human hemoglobin and Japanese monkey hemoglobin, four differences of the amino acid sequence in three peptides are presumed.

On the other hand, an investigation on the amino acid composition of the core insoluble at pH 6.4 of the tryptic peptides is being made in this laboratory, and according to the results so far obtained, it is likely that Japanese monkey hemoglobin is quite similar to rhesus monkey hemoglobin in the amino acid composition of the insoluble tryptic peptide. It is presumed from this fact that the α polypeptide
chain of Japanese monkey hemoglobin has the primary structure similar to that of rhesus monkey hemoglobin. With respect of the $\beta$ polypeptide chain, which is also being studied in this laboratory, only one amino acid difference has been found between rhesus monkey and Japanese monkey hemoglobins. The above-mentioned data suggest that differences between Japanese monkey and rhesus monkey hemoglobins are the slightest as far as observed in the comparison of their amino acid compositions.

CONCLUSION

The soluble tryptic peptides from the $\alpha$ polypeptide chain of adult Japanese monkey \textit{(macaca fuscata fuscata)} hemoglobin were isolated and purified by column chromatography and paper chromatography, and then the amino acid compositions of these peptides were analyzed. The results were compared with human hemoglobin. Four differences in the amino acid composition were recognized in three peptides. In addition, between Japanese monkey and rhesus monkey hemoglobins, no difference was found in the amino acid composition of their $\alpha$ polypeptide chains.

ACKNOWLEDGEMENTS

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