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Structural Analysis of Human Hemoglobin Variants by Molecular Secondary Ion Mass Spectrometry

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ABSTRACT. Chemically purified abnormal peptides, α62-76 of Hb Ube-2, βT-3 of Hb G-Coushatta, αT-7 of Hb J-Norfolk and βT-1 of Hb Himeji, were subjected to molecular secondary ion mass spectrometry (SIMS). These peptides were identified by the mass number of their protonated molecular ion $(M+H)^+$ and their amino acid sequence was confirmed by a series of the mass number of fragmented ions released by a stepwise removal from their C-terminus.

Amino acid substitution of α68 Asn→Asp in Hb Ube-2, β22 Gln→Ala in Hb G-Coushatta and α57 Gly→Asp in Hb J-Norfolk was successfully determined and a questionable Hb variant, tentatively called Hb Himeji (β140 Ala→Asp) was proven to have a significant amount of the additive of the glycated N-terminus of the βT-1 peptide.

Key words : secondary ion mass spectrometry -SIMS- — Hb Ube-2 — Hb G-Coushatta — Hb J-Norfolk — Hb Himeji

In amino acid sequence analyses of protein and peptides, the stepwise Edman degradation technique has been widely used as the basic procedure and instrumentation has developed according to the principle of this procedure.[1] However, there are several difficulties for determination of PTH-amino acid by Edman's method; e.g., 1) the N-terminal amino acid of the peptide must be in the form of a free α-amino acid residue and 2) PTH-derivatives of Ser, Thr and Trp that appear in the processing cycle can hardly be detected because the thiazolinone derivatives of those compounds decompose and diminish during acid treatment. Recently, mass spectrometry, particularly, molecular secondary ion mass spectrometry (SIMS) has been employed to overcome these difficulties.[2,3] This paper describes the results of a SIMS operation for analyses of the amino acid sequence of abnormal peptides.

MATERIALS AND METHODS

Preparation of materials. Peptides to be exposed to SIMS analyses were prepared as follows:

1) abnormal α62-76 peptide of Hb Ube-2[4]: The abnormal αT-9 of Hb Ube-2 was treated with cyanogen bromide to split it at the carboxy end of α76 methionyl residue.[5] The α62-76 peptide thus obtained was subjected to high performance liquid chromatography (HPLC, reverse phase C_{18} column, 0.01 M TEA acetate, pH 6.0/CH_3CN) to obtain it in pure form.[6]

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2) abnormal peptide of Hb G-Coushatta (βT-3)[7] and Hb J-Norfolk (αT-7)[8]: The tryptic digests of the β chain of Hb G-Coushatta and the α chain of Hb J-Norfolk were satisfactorily subjected to reverse phase HPLC to obtain the aberrant peptide in pure form.
3) N-glycated βT-1 peptide of Hb Himeji[6]: The abnormal βT-1 peptide of Hb Himeji was collected by HPLC treatment of the tryptic digest of the abnormal β chain of Hb Himeji.
4) secondary ion mass spectrometry (SIMS) using an M-80B Mass Spectrometer (Hitachi): six nanomol of the testing specimen was dissolved in 6 μl of dil-HCl and 4 μl of it was mixed with one drop of glycerol, which was then placed on the probe tip. The probe was introduced into the ion source of the apparatus. The sample was then bombarded with a Xe^+ ion beam with an energy of 8 keV. The mass signals were directly recorded by the JMA-200 data analysis system.

RESULTS

The mass spectrum of the α62-76 peptide of Hb Ube-2 (α68 Asn→Asp) analyzed by SIMS is shown in Fig. 1. A protonated molecular ion $(M+H)^+$ was seen at m/z 1512, so the molecular weight of the peptide was thought to be 1511. This mass number was consistent with the amino acid compositions of the expected abnormal α62-76 peptide. Fragment ions appearing at m/z 1365, 1250, 1135, 1036, 899, 729, 658, 543, 442, 329 and 258 were interpreted as the product of bond cleavage occurring at the carboxy end of each amino acid of the peptide. Substitution of Asp for Asn was confirmed by the

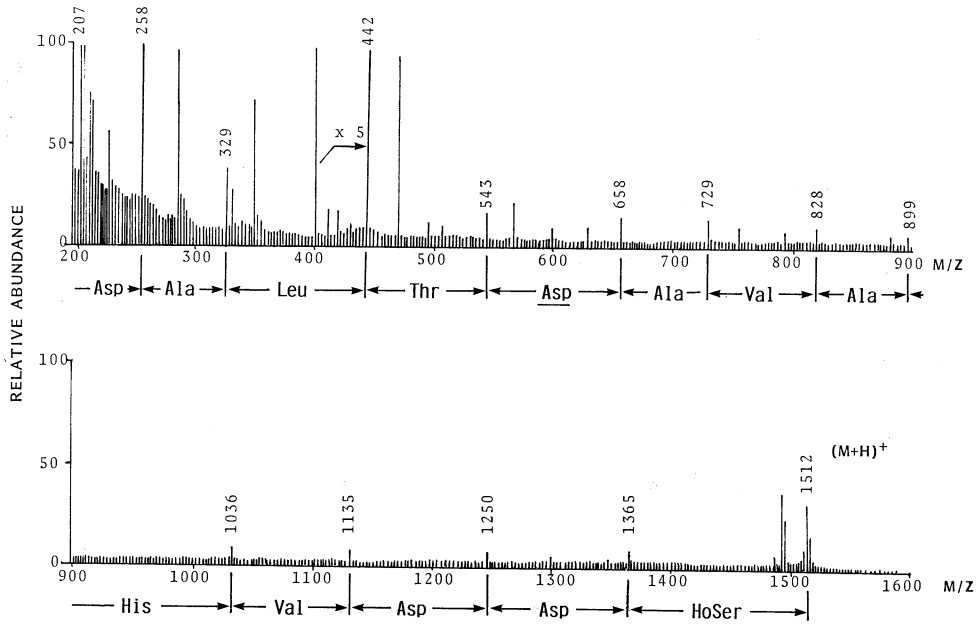


Fig. 1. Mass spectrum of the α62-76 peptide of Hb Ube-2. The fragment ion at m/z 658 indicates the substitution of Asp for Asn.

appearance of a fragment ion at m/z 658 rather than at m/z 659 after consideration the amino acid composition of the peptide. The abnormal βT-3 of Hb G-Coushatta (β22 Glu→Ala) demonstrated an abundant protonated molecular ion $(M+H)^+$ at m/z 1256 as shown in Fig. 2. This indicated that the molecular weight of the abnormal βT-3 peptide was 1255 and that one of two Glu residues at positions β22 and β26 seen in normal βT-3 was replaced by an Ala residue. The ion signal at m/z 471 was consistent with the fragment peptide Val^{18}-Asn-Val-Asp-Ala^{22}, arising by the amino acid substitution Glu→Ala at position β22.

The mass spectrum of the abnormal αT-7 peptide of Hb J-Norfolk (α57 Gly→Asp) is shown in Fig. 3. Appearance of the protonated molecular ion $(M+H)^+$ at m/z 456 signifies that one of two Gly residues normally seen at positions α57 and α59 has been replaced by an Asp residue. The fragment ion at m/z 325, 268 and 131 are thought to have been produced by splitting at the sites of a, b and c shown in Fig. 3. These results indicate that the replacement of Gly→Asp occurred at position α57. The amio acid composition of the abnormal βT-1 of Hb Himeji (β140 Ala→Asp) was identical to that of the normal βT-1 in spite of its abnormal elution pattern in HPLC. Stepwise Edman degradation studies were carried out on this abnormal βT-1, but PTH-amino acid was not demonstrable as expected in the first step product. Therefore, we believed that the abnormal βT-1 was chemically modified by a non-amino acid additive to the N-terminus of βT-1. Consequently, both the abnormal βT-1 and the normal βT-1 were subjected to mass spectrometry (Fig. 4). The protonated molecular ion $(M+H)^+$ of the normal βT-1 was observed at m/z 952, while that of the abnormal βT-1 was seen at m/z 1114. The difference of 162 corresponded to the molecular weight of glucose minus water, indicating that a glucose molecule was joined with βT-1 by a chemical

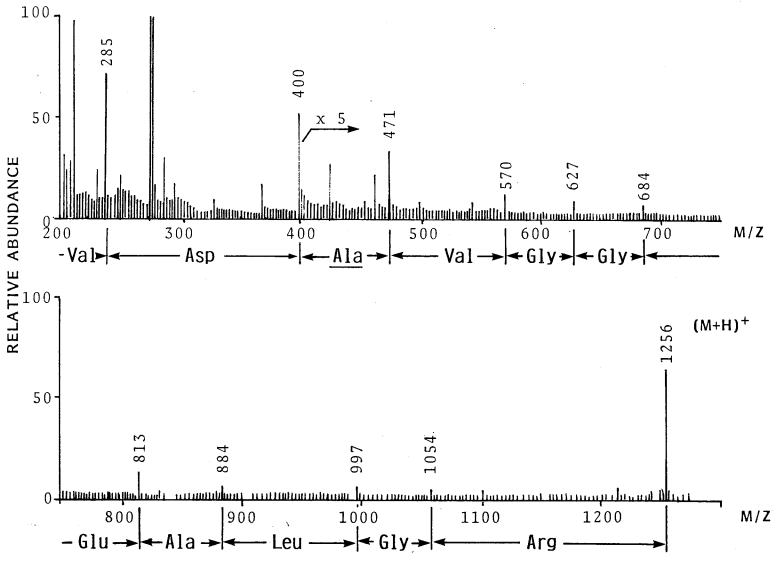


Fig. 2. Mass spectrum of the βT-3 peptide of Hb G-Coushatta. The fragment ion at m/z 471 indicates the substitution Glu→Ala at position β22.

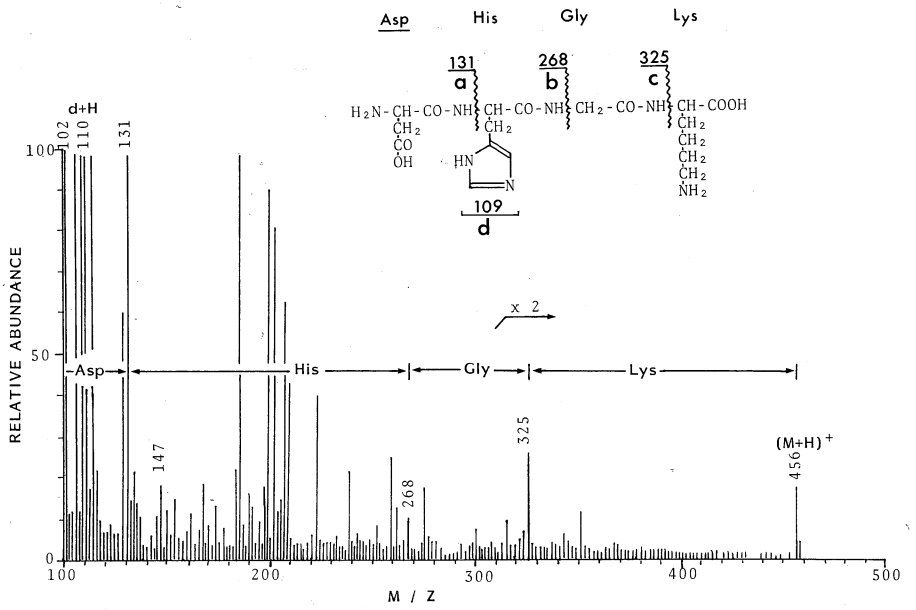


Fig. 3. Mass spectrum of the αT-7 peptide of Hb J-Norfolk. The fragment ion at m/z 131 indicates the replacement of Gly by Asp.

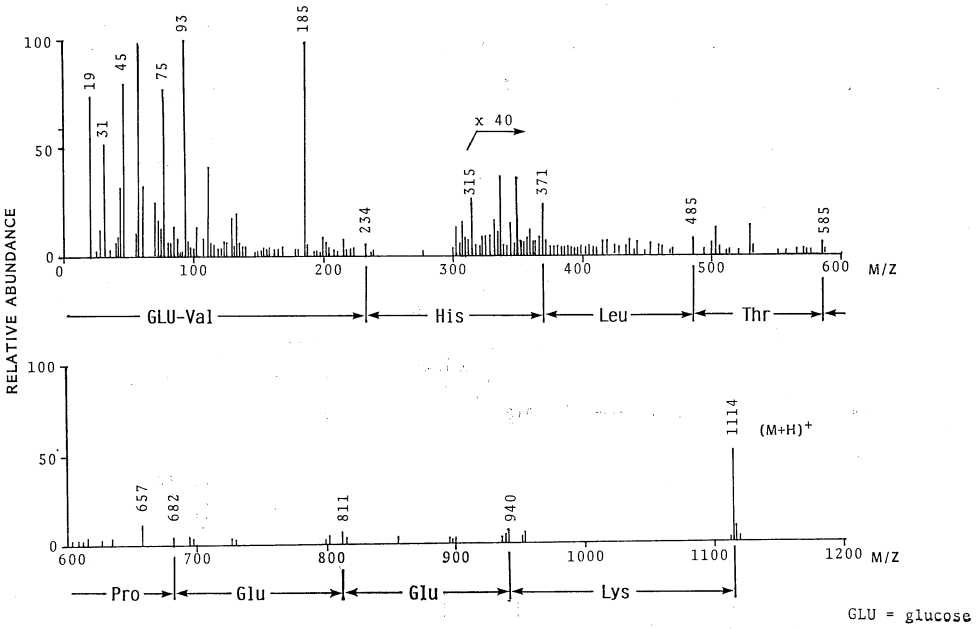


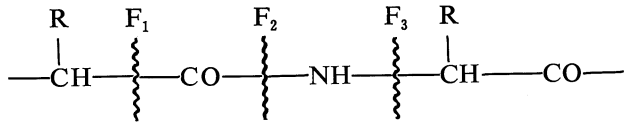
Fig. 4. Mass spectrum of the βT-1 peptide of Hb Himeji. The protonated molecular ion $(M+H)^+$ at m/z 1114 and the fragment ion at m/z 234 indicate the combination of glucose and N-terminal valine residue.

dehydration reaction. Thus, the appearance of the fragment ion at m/z 234 could be explained by the combined mass of the N-terminal Val plus a hexose. This finding led to the conclusion that the abnormal β chain consisted of a glycated βT-1 in the N-terminal residue together with the amino acid substitution of Asp for Ala at position β140.

DISCUSSION

Molecular secondary ion mass spectrometry is very effective in the sequential analysis of peptides because it discloses the protonated molecular ion $(M+H)^+$ even when the peptide is nonvolatile and mass of fragment ion provides information necessary for sequencing. It is worth noting that the mass number differences of one on the spectra could be identified with confidence at the 1500 mass level, which corresponds to the peptide of more than ten amino acids. The Edman degradation technique cannot be used in sequencing studies of peptides when their N-terminal amino acid has been modified by a non-amino acid substance, as seen in the N-glycated βT-1 of Hb Himeji, but the sequence of these peptides can be verified by SIMS because this procedure depends only on mass and not on chemical structure.

There were three common sites (F_1, F_2 and F_3) for cleavage of peptide:



One of these sites becomes preferential due to the differences in the neighbouring amino acids of the peptide, the preferential site for α62-76 of Hb Ube-2, βT-3 of Hb G-Coushatta and βT-1 of Hb Himeji was F_1, while that of Hb J-Norfolk was F_3. Peptide sequencing can be done easily and speedily with the SIMS method and the amino acid sequencing of peptides can be reliably determined with 6 nmol or less of a specimen.

Acknowledgment

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