I. INTRODUCTION

Time-of-flight mass spectrometry (TOF-SIMS) was applied to the detection of protein structural change in studies of protein-protein interactions and protein conformational changes. Though X-ray structure analysis, one of the strongest techniques for analyzing a protein structure, provides a detailed protein structure, it is not adequate to detect a partial structural change. Improved techniques are still required to study protein structure changes, although several analytical techniques, such as fluorescence resonance energy transfer (FRET) [1] and infrared spectroscopy [2], have been developed. Since TOF-SIMS provides chemical information on the uppermost surface of a protein monolayer, which cannot be obtained using other analytical methods, it is expected to contribute to an improved evaluation of the structural changes of proteins [3–8] due to protein-protein interaction or conformational changes. In addition, TOF-SIMS requires no pretreatment of samples, such as labeling with a fluorescent probe or coating with metallic thin films. In this study, the pH-dependent protein structural change was evaluated using TOF-SIMS in order to explore TOF-SIMS capabilities and limitations with regard to the investigation of protein structural change.

Staphylococcal protein A (SpA), a cell wall component of Staphylococcus aureus, binds to the Fc region of immunoglobulins. TOF-SIMS spectra of SpA-immobilized samples in a pH 7 solution, in an acidic solution, and in a pH 7 solution after having been soaked in an acidic solution, respectively, were compared to select fragment ions specific to normal SpA at pH 7 using spectral analysis techniques such as principal component analysis (PCA) and the mutual information method. A fragment ion depending on change in the B and E domains of SpA were detected with this method. These findings demonstrate that structural change of SpA depending on pH change can be evaluated with TOF-SIMS together with spectrum analysis methods for the multivariate data.

II. MATERIALS AND METHODS

A. Sample preparation

Imobilized SpA samples were prepared on a regenerable optic fluorescent immunosensor using SpA in the same manner as described in the previous study [14]. An indium tin oxide (ITO) coated glass slide (Sigma-Aldrich Co., St Louis, MO, USA) of 8 mm² was aminosilanized with aminopropyltrimethoxysilane (Tokyo Kasei, Tokyo, Japan). The aminosilanized ITO glass plates were activated by glutaraldehyde and then soaked in a 0.01M-phosphate buffered saline (phosphate buffered saline tablets containing 0.0027 M potassium chloride and 0.137 M sodium chloride, Sigma) solution at pH 7.4 containing protein A (Zymed Lab. Inc., San Francisco, CA, USA) and allowed to react in the dark for 30 hr at 277 K, and then the protein A molecules were immobilized on the ITO plates at the epsilon-aminogroups by a covalent bonding. After the ITO glass plates with the protein were washed with a sonic wave for 1 min in pH 7.4 PBS to remove adsorbed molecules, they were soaked in solutions of pH 7, 5, and 3, respectively, for an hour at 310 K. The acidic solutions at pH 5 and 3, respectively, were prepared with drops of an HCl solution while the solution was mon-
TABLE I: Positive ions related with amino acids [17-19].

| Formula   | m/z  |
|-----------|------|
| CH₂N      | 30   |
| CH₃N₂     | 43   |
| C₂H₆N     | 44   |
| CHS       | 45   |
| C₂H₆NO    | 60   |
| C₂H₅S     | 61   |
| C₂H₆N₆    | 68   |
| C₂H₅O     | 69   |
| C₂H₅NO    | 70   |
| C₂H₅N₅    | 70   |
| C₂H₆O₂    | 71   |
| C₂H₁₀N₅   | 72   |
| C₂H₆N₃    | 73   |
| C₂H₆NO    | 74   |
| C₂H₆N₂    | 81   |
| C₂H₆N₂    | 82   |
| C₂H₅O     | 83   |
| C₂H₈O₈    | 84   |
| C₂H₆N₅    | 84   |
| C₂H₅O₇    | 84   |

Positive ion spectra obtained with TOF-SIMS (TFS-2000, Physical Electronics, MI) using a Ga⁺ primary ion source, were acquired up to m/z 1000 while maintaining the primary ion dose at less than 10¹² ions/cm² to ensure static conditions. Ten time measurements were performed for each sample. All the spectra, which were composed of positive ion TOF-SIMS spectra, were calibrated to the CH₄⁺, C₂H₅⁺, and C₃H₇⁺ peaks before data analysis.

C. Spectrum Analysis

Intensities of peaks of secondary ions were normalized to the total ion count before principal component analysis (PCA). The fragment ions related to the amino acids in the proteins [17-19] used in the PCA analysis of the TOF-SIMS data are shown in Table I. Since this protein A molecule does not contain cysteine (C) or tryptophan (W), fragment ions reported to be related to these residues, CHS⁺ (m/z 45) from C, and C₉H₆N⁺ (m/z 130), C₁₀H₁₁N₂⁺ (m/z 159) and C₁₁H₁₄NO⁺ (m/z 170) from W, were omitted. In addition, C₄H₅O⁺ (m/z 69) was omitted to prevent the influence of the gallium ion. PCA was performed with the software for multivariate analysis based on Microsoft Excel, Excel Statistics (Excel Toukei) developed by Social Survey Research Information Co., Ltd. (Tokyo, Japan). Principal components were calculated based on the correlation matrix.

The values of mutual information [20-23] were calculated comparing the protein A-immobilized sample at pH 7 (SpA at pH 7) with the reference samples, the protein A-immobilized samples at pH 3 or 5 (SpA at pH 3 or 5), and those at pH 7 after being soaked in acid solutions (SpA at pH 7 after pH 3 or 5), respectively. Peaks of secondary ions at m/z = 40 to 500 were used for the calculation. The concept of mutual information and its calculation steps were described in previous papers [23-25]. The selected fragment ions were identified considering every possible fragment from the proteins. The detailed selection procedures were described in the previous papers [4, 25].

The results from TOF-SIMS spectra analysis, suggesting the specific fragment-ion-generating regions in the staphylococcal protein A (SpA), were compared to the amino acid sequence and three-dimensional structure of the B and E domains of SpA registered in the protein data bank (PDB: http://www.rcsb.org/pdb/home/home.do).

III. RESULTS AND DISCUSSION

Figure 1 shows the scores and loadings of the principal components (PC) 1, 2 and 3. According to the score plots (Fig. 1(a) and (b)), it is suggested that PCs 1 and 3 depend on the structural change of SpA which depend upon pH change. In addition, PC 2 discriminates the data of SpA at pH 7(pH 7), 5(pH 5) or 3(pH 3) from those of SpA at pH 7 after pH 3 (pH 3-7) or 5 (pH 5-7) (Fig. 1(a)), which indicates whether SpA has changed its structure. Thus, the PC scores clearly discriminate SpA at pH 7 from SpA at pH 3 or 5, and show that SpA at pH 3-7 and pH 5-7 are slightly different from SpA at pH 7, which suggests SpA at pH 3-7 or pH 5-7 does not completely regenerate the structure. However, the peaks having positive loadings of PC1 and also positive loadings of PC3, such as peaks at m/z 43, 73, 74, 81, 83 and 131 according to Fig. 1(c), are considered to be related to important structures of SpA, and their loadings of PC2 are positive, which means they are specific to SpA at pH 7 after soaked in an acidic solutions. For more detailed investigation, peaks specific to each sample were investigated based on the results of mutual information.

Fragment ions of mutual information value 1 comparing the data of SpA at pH 7 with the data of SpA at pH 3 or 5 are peaks at m/z 46, 62, 63, 81, 83, 97, 301, 393 and 394, respectively. Peaks at m/z 46, 62, 81, 301 and 393, respectively, also display mutual information value 1 comparing the data of pH 7 with the data of SpA at pH 3-7 or pH 5-7. Since these peaks are also contained in the pH 7 specific peaks in comparison to pH 3 or pH 5, they suggest parts which do not completely regenerate the structure in pH 7 after having been soaked in an acidic solution. The peaks at m/z 46 and 62 were not considered because these fragment ions were too small to determine their origin. The representative TOF-SIMS spectra of the other selected peaks are shown in Fig. 2. The peaks at m/z 81 and 83 contain multiple peaks, 80.95 and 81.07, and 82.95 and 83.09, respectively, which would affect the...
PCA results. Though the peaks at m/z 80.95 and 82.95 seem to be specific to SpA at pH 7, their formulas could not be identified as fragment ions from the protein. These ions might contain buffer salts such as sodium ions. In this study, PBS was not rinsed with pure water because the control of pH is crucial. In order to reduce the influence of PBS, every sample was soaked in a solution containing the same ingredients and the main sample was compared to the reference sample. As a result, the intensity of sodium ions in each sample is almost the same.

The peaks at m/z 301.14 and 393.04 were found to be specific to SpA at pH 7. Both peaks have many possible formulas with peak m/z 301.04 in particular having so many possible formulas that it is almost impossible to determine its original parts, and therefore the peak m/z 301.14 was selected for investigation of fragment ion generation. The possible formulas are comprised of the following: C_{13}H_{17}N_{4}O_{3} (301.130), C_{15}H_{21}S_{2}N_{4}O_{3} (301.133), C_{11}H_{19}N_{5}O_{5} (301.139), C_{11}H_{21}N_{2}O_{6} (301.140), C_{16}H_{19}N_{3}O_{3} (301.142), C_{16}H_{25}S_{2}N_{2}O_{6} (301.143), and C_{11}H_{21}S_{2}N_{6}O_{2} (301.145). The formulas of the peak were considered through all possible chemical formulas generated from proteins ranging from m/z 301.130 to 301.150. Then combinations of the amino acid residues generating each formula of the fragment ion were checked to find the appropriate formulas. As a result, C_{13}H_{21}N_{2}O_{6} (301.140) and C_{16}H_{25}N_{2}O_{3} (301.142) were considered to be the main formulas. Since C_{15}H_{17}N_{4}O_{3} (301.130) and C_{11}H_{19}N_{5}O_{5} (301.139) can be generated from almost all parts of SpA, they can be also generated from modified SpA, which means the fragment ion is not specific only to SpA at pH 7. C_{10}H_{20}S_{2}N_{2}O_{5} (301.143) contains cysteine, which is not included in SpA. There are no amino acid residues combinations generating C_{11}H_{21}S_{2}N_{6}O_{2} (301.145) in the amino acid sequence of the Ig-binding domains of SpA. Since C_{12}H_{25}S_{2}N_{4}O_{3} (301.133) is generated from only four parts of the domains, the NMP or NMI parts, its influence is very small, even if it is contained in the peak. The fragment ion specific to normal SpA at pH 7, 301.14, is considered to mainly contain the fragment ions C_{13}H_{21}N_{2}O_{6} (301.140) and C_{11}H_{19}N_{3}O_{3} (301.142). With the currently available TOF-SIMS technology, it is very difficult to identify secondary ions of high mass.

C_{12}H_{21}N_{2}O_{6} (301.140) can be generated from DNK(3-5), EQQ(9-11), LNEEQ(23-27), KDPP(36-39), and LND(52-54) in the B domain (1BDD) and EAQ(5-7), DQR(23-25), KDPP(33-36), EAQ(45-47), LND(49-51) in the E domain (PDB ID: 1EDI). C_{16}H_{25}N_{2}O_{3} (301.142) can be generated from NKFNK(4-8), AFYIEL(13-18), GFIQ(30-33), KKL(50-52) in the B domain (1BDD) and FYQV(11-14), GFIQ(27-30), KLN(48-50) in the E domain (PDB ID: 1EDI). The numbers in the brackets show the amino acid sequence order of the B and E domains of SpA, respectively, and they are shown in Figs. 3 and 4. The 3D structures of B and E domains registered in

http://www.sssj.org/ejssnt (J-Stage: http://www.jstage.jst.go.jp/browse/ejssnt/)
PDB were considered as representing the five Ig-binding domains in this study, because the domains, E, D, A, B and C have a similar amino acid sequence and structure. Though the structure obtained with X-ray analysis using the protein crystal might be slightly different from the structure of our sample protein at pH 7, the main structures, such as the helix, are retained. Therefore the structures in PDB can be employed to compare the main sample structure to the references.

Figure 3 shows the structures of the fragment ion-generating parts and indicates that the fragment ion-generating parts are gathered at one side of each domain. Considering the helix regions, the fragment ion $C_{13}H_{21}N_7O_5^+$ (301.140) is mainly generated from around the helical edges, and the fragment ion $C_{16}H_{10}N_3O_5^+$ (301.142) is mainly generated from the inside of the helix, as shown in Fig. 4, which shows the ribbon structures of the B and E domains and three helices in each domain. When the helices are unfolded because of a pH change, SpA loses its functional ability to bind IgG. Therefore the helices unfold in SpA at pH 3 or 5, and then they fold again when the solution pH changes to pH 7. Table II shows the average intensity.
of the peak at m/z 301. The average intensity of SpA at pH 3-7 or pH 5-7 is higher than those of pH 3 or pH 5, although it is lower than that at pH 7. Therefore, it is suggested that the helices of SpA at pH 3-7 or pH 5-7 are not completely folded, for example, the inside of the helices are folded, even though the edges are not completely folded. When the inside of the helices folds, the domains are able to bind IgG. In other words, the intensity of the peak at m/z 301 in the spectra of SpA at pH 7 after pH 3 or 5 decreases, because the peak in the spectra of SpA at pH 3-7 pH 5-7 contains mainly C_{16}H_{19}N_{2}O_{8}^{+} (301.142) generated from the inside of the helices due to incomplete folding, although the peak in the spectra of SpA at pH 7 contains both C_{16}H_{19}N_{2}O_{8}^{+} (301.140) generated from the edges and C_{16}H_{19}N_{2}O_{8}^{+} (301.142) generated from the inside of the helices. Thus, the results obtained by means of TOF-SIMS analysis can explain structural change in SpA which is dependent on pH change. Therefore, it is demonstrated that TOF-SIMS has the capability to evaluate detailed structural changes in a protein molecule. Currently, it is difficult to evaluate fragment ions higher than approximately m/z 400 because of the mass resolution limitation of the machines. In the near future, when TOF-SIMS machines will be improved to provide a higher mass resolution or an MS/MS application to identify high mass fragment ions, more detailed and useful information on the structural changes of proteins will be forthcoming.

### IV. CONCLUSIONS

Structural changes in the B and E domains of staphylococcal protein A (SpA) that were dependent on pH change were evaluated with TOF-SIMS. The PCA results show the difference between the SpA-immobilized samples at pH 7 and under acidic conditions. By means of the mutual information method, a peak at m/z 301, specific to the SpA-immobilized sample at pH 7 was found in comparison to TOF-SIMS spectra of SpA-immobilized samples in acidic solutions or those at pH 7 after having been soaked in acidic solutions. The peak at m/z 301 was identified by searching through every possible fragment from every part of the SpA amino acid sequence and it is considered to mainly consist of two fragments, C_{13}H_{21}N_{3}O_{6}^{+} (301.140) and C_{16}H_{19}N_{2}O_{8}^{+} (301.142). These two fragment ions are generated from a different region of each of the domains of SpA: C_{13}H_{21}N_{3}O_{6}^{+} (301.140) is generated from the helical edges and C_{16}H_{19}N_{2}O_{8}^{+} (301.142) can be generated from the inside of the helices. The results from TOF-SIMS spectra analysis show that the helices unfold in an acidic solution and then fold again at pH 7. Thus, TOF-SIMS application to protein structural change is a promising technique studying protein-protein interactions and conformational change. In addition, further improve-
ment of TOF-SIMS so as to afford higher mass resolution or an MS/MS application to identify high mass fragment ions, will be needed to accelerate the application of TOF-SIMS to the field of proteomics.

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