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Structure of human serum albumin at a foam surface

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2 ABSTRACT

3 Proteins can be adsorbed on the air-water interface (AWI), and the structural changes 4 in proteins at the AWI are closely related with the foaming properties of foods and 5 beverages. However, how these structural changes in proteins at the AWI occur is not 6 well understood. We have developed a method for the structural assessment of proteins 7 in the foam state using hydrogen/deuterium exchange mass spectrometry. Adsorption 8 sites and structural changes in human serum albumin (HSA) were identified in situ at 9 peptide-level resolution. The N-terminus and the loop (E492-T506), which contains hydrophobic amino acids, were identified as adsorption sites. Both the structural 10 11 flexibility and hydrophobicity were considered to be critical factors for the adsorption of 12 HSA at the AWI. Structural changes in HSA were observed after more than one minute 13 of foaming and were spread widely throughout the structure. These structural changes at 14 the foam AWI were reversible.

15

16 Keywords: air-water interface, hydrogen/deuterium exchange mass spectrometry, foam,
17 protein structure

20 Introduction

The air-water interface (AWI) is a hydrophobic interface.¹ Proteins are composed of 21 both hydrophilic and hydrophobic amino acids and are therefore amphiphilic. Thus, 22 23 proteins can be adsorbed to the AWI. Structural changes in proteins at the AWI, which is also referred to as surface denaturation, can occur after adsorption.² Because the 24 25 hydrophobic interaction between proteins and the AWI is a main driving force of the 26 adsorption, hydrophobic residues in the proteins, which were buried in the interior of molecule, become exposed.³ Understanding the structural changes of proteins at the AWI 27 is important for many reasons.⁴⁻⁶ For example, therapeutic proteins can be adsorbed on 28 29 the AWI during the manufacture and storage of biopharmaceuticals, which can result in changes in the structures of such proteins.⁴ These structural changes can lead to the 30 31 formation of protein aggregates, which could elicit an immune response; thus, creating a potential risk for immunogenicity.⁷ In addition, understanding the interfacial structural 32 33 instability of viruses that can be transmitted by airborne droplets is important for developing strategies to prevent the spread of viral infections.¹ 34

35 Protein adsorption at the AWI is utilized in the food industry, including in producing36 meringues, which are made from egg-white proteins that have excellent foaming

37	properties. ⁸ In addition, foam is a key quality attribute of both sparkling wine and beer,
38	and proteins play an important role in the formation of the foam of these beverages.9
39	Therefore, many studies have investigated improving the foaming ability, as well as the
40	foaming stability of foods. Previous studies have demonstrated that the surface
41	hydrophobicity of proteins was correlated with the foam properties. ¹⁰ In addition, having
42	the molecular flexibility to expose previously buried hydrophobic amino acids to the AWI
43	has also been indicated to be an important factor for foam formation. ³ The addition of
44	molecules, such as polyphenols ^{11,12} and saccharides, ¹³ and chemical modification, such
45	as oxidation, ^{14,15} has been shown to change the protein structure and/or structural
46	flexibility in solution leading to improvements in the foaming properties. Although these
47	studies have indicated that the protein structure in the foam state is related to the foam
48	properties, knowledge of the protein structure in the foam state remains elusive.
49	Analysis of protein structure at the AWI is more challenging than structural analysis
50	of proteins in solution because signals derived from proteins at the AWI, which are
51	typically weak because of the limited amount of protein at the AWI, need to be
52	selectively detected. For this reason, protein structure at the AWI has mainly been
53	evaluated using spectroscopic and scattering techniques, which provide spectra from
54	light scattered or reflected from the AWI. ^{4,16} However, such analytical techniques are

55 only applicable to controlled flat interfaces (e.g., planar interfaces produced by 56 Langmuir troughs), not to three-dimensional interfaces, such as the foam AWI, because the angles of incident light and the angles of the detectors for the reflected or scattered 57 light need to be specific. In addition, the structural resolution of spectroscopic and 58 59 solution scattering/reflection techniques is relatively low (secondary structure level), 60 thus an analytical method with higher structural resolution is desirable. 61 Hydrogen/deuterium exchange mass spectrometry (HDX-MS) can be used to study 62 protein structures and interactions by monitoring the isotopic exchange of amide 63 hydrogens with deuterium atoms in the protein backbone after a protein is exposed to D₂O.¹⁷ The deuterium exchange of each amide hydrogen in folded proteins can be 64 expressed by the following equation, the Linderstrøm-Lang model:¹⁸ 65 $cl(NH) \stackrel{k_{op}}{=} op(NH) \stackrel{k_{ch}}{\rightarrow} op(ND) \stackrel{k_{cl}}{=} cl(ND)$ (1) 66 67 where cl corresponds to the folded state and op corresponds to the open state, NH and ND are the protiated and deuterated amides, respectively, k_{op} and k_{cl} are the rate 68 constants of the opening and closing reactions, respectively, and k_{ch} is the chemical 69 deuteration rate constant. From this model, the observed deuteration rate constant (k_{HD}) 70

71 can be obtained:

72
$$k_{HD} = \frac{k_{op} \times k_{ch}}{k_{op} + k_{cl} + k_{ch}}$$
(2)

Under stable conditions, proteins can be refolded immediately after the unfolding.¹⁸

74 Thus, k_{cl} is much larger than k_{op} and k_{ch} , and then k_{HD} can be simplified as follows:

75
$$k_{HX} = \frac{k_{op}}{k_{cl}} \times k_{ch} = K_{op} \times k_{ch}$$
(3)

where K_{op} is the equilibrium constant of the opening reaction. Amide hydrogens often 76 77 form hydrogen bonds, which stabilize the protein structure and result in the value for 78 K_{op} being smaller, and thus the exchange of amide hydrogens in a folded protein is 79 slower than that in the unfolded state in which the hydrogen bonds featuring amide hydrogens are partially or completely disrupted.¹⁹ In addition, the accessibility of the 80 protein for the deuterium atoms influences the exchange rate. For example, when an 81 82 amide hydrogen is buried inside the protein or covered by additives or other proteins, 83 the exchange rate is slower compared with when the hydrogen is on the outside of the protein.¹⁹ In a typical bottom-up HDX-MS measurement, proteolytic digestion of a 84 85 protein that has been exposed to D₂O is conducted, followed by liquid chromatography and mass analysis. The deuterium uptake profiles of the resulting peptides provide 86 87 information on the secondary, tertiary, and higher order structure of the protein at peptide-level resolution,²⁰ which is a higher resolution compared with that observed 88 89 with spectroscopic and solution scattering/reflection techniques.

90	Previous studies have demonstrated that HDX-MS can be a valuable technique to
91	examine protein structures at interfaces. ²¹⁻²³ Xiao and Konermann have used HDX-MS
92	to investigate the structural change of myoglobin at the AWI and proposed a surface
93	denaturation model, although this group did not perform a detailed structural analysis at
94	peptide-level resolution. ²⁴ Another study has demonstrated that HDX-MS was useful for
95	evaluating protein structure at a flat AWI. ²⁵ However, the characteristics of the AWI
96	interface, such as the curvature of the interface and the bulk/interface volume ratio, are
97	different between flat and foam AWIs, and the protein structure at a flat AWI can be
98	different from that at a foam AWI. Therefore, a method that can evaluate protein
99	structure at a foam AWI is needed to reveal the molecular mechanism of protein foam
100	formation. In the present study, human serum albumin (HSA) was used as a model
101	protein because HSA can be highly purified and has adequate foaming properties. The
102	structure of HSA in solution has been well characterized, ²⁶ and the three-dimensional
103	structure of HSA is similar to that of bovine serum albumin, ²⁷ which is found in foods
104	such as milk. ²⁸ Thus, HSA can be used as a model protein for application to food
105	processing. We have developed an in situ method to evaluate the structure of HSA in
106	the foam state using HDX-MS. Structural information on HSA at the foam AWI was

107	successfully obtained at peptide-level resolution using the developed method. Analysis
108	after defoaming revealed that the structural changes at the foam AWI were reversible.
109	
110	Materials and methods
111	Materials
112	HSA and all other reagents were purchased from Fujifilm Wako (Osaka, Japan) unless
113	otherwise specified. The HSA monomer content, determined by size exclusion
114	chromatography (SEC) with UV detection at 280 nm, was 50.5%. Phosphate-buffered
115	saline (1×PBS; pH 7.4) and acetonitrile were obtained from Thermo Fisher Scientific
116	(Waltham, MA, USA). D ₂ O was purchased from Iwatani Corporation (Osaka, Japan).
117	
118	Preparation of HSA samples
119	Prior to analysis, HSA monomer was fractionated by SEC with a HiLoad 16/60
120	Superdex 200 column (Cytiva, Tokyo, Japan), and 1×PBS at pH 7.4 was used as the
121	elution buffer. The HSA monomer content was 99.1% after the purification. Fractionated
122	HSA monomer was dialyzed against 100 mM sodium phosphate buffer at pH 7 at 4°C,
123	and then concentrated by ultrafiltration using Amicon Ultra 10k (Merck, Darmstadt,
124	Germany) to 10 mg/mL for circular dichroism (CD) spectroscopy and interfacial tension

125 measurement, 5 mg/mL for HDX-MS measurements in the liquid state, and 100 mg/mL

- 126 for HDX-MS measurements in the foam state.
- 127

128 Interfacial tension measurement

129 Static surface tension was measured by the Whilhelmy method using a Sigma 700 130 instrument (Biolin Scientific, Gothenburg, Sweden) equipped with a platinum plate. The 131 plate was immersed in 10 mg/mL HSA solution to an immersion depth of 6 mm and 132 pulled up three times at a rate of 20 mm/min. The force (F) that acted on the plate was 133 measured and the surface tension (γ) was calculated from the equation:²⁹

134
$$\gamma = \frac{F}{L}$$
(4)

where L is the perimeter of the plate (39.24 mm). The average of three measurements was reported. Dynamic surface tension was measured by the pendant drop method using a Theta Flex instrument (Biolin Scientific, Gothenburg, Sweden). In the measurements, a 10- μ L pendant drop was generated at a flow rate of 2 μ L/s, and the shape of the pendant drop was monitored for 1200 s. The surface tension was simultaneously calculated from the Young-Laplace equation every 10 s using OneAttension (ver.4.0.3). ³⁰

142 Foaming of HSA

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	40

3 An electric milk frother (GOSCIEN, MFB1501B, Guangzhou, China) was employed 144 to foam the HSA solution (Fig. 1). The foam was generated quickly by mechanical 145 agitation created by rotating the frother head made of stainless steel at 15,000 rpm. The 146 mechanical agitation was continued until just before the analysis.

147

148 SEC after the foaming

149 HSA foam was generated for 0, 0.5, 1, 5, 10, 30, 60, and 90 min. At each time point, the foam was collected and weighed. The weight was divided by the density of the HSA 150 151 solution (1.01 g/mL) to calculate the volume of solution contained in the collected foam, 152 and then the foam was diluted with sodium phosphate buffer with 0.03% silicone 153 antifoam to achieve a concentration of 1 mg/mL for the samples at all time points. The 154 samples were centrifuged at $15,000 \times g$ at 4°C for 30 min. The supernatants were then 155 loaded onto a ACQUITY UPLC System (Waters, Milford, MA, USA) equipped with a TSKgel UP-SW3000 column (4.6 mm × 150 mm; Tosoh Bioscience, Tokyo, Japan). 156 Isocratic elution was performed using 133 mM phosphate buffer with 200 mM 157 158 KCl/acetonitrile (95:5) at a flow rate of 0.25 mL/min for 15 min. UV absorbance at 280 159 nm was monitored. Empower 3 Software (Waters) was used to calculate the peak areas.

160 The monomer (%) was calculated as a ratio of the monomer area to the total peak area at161 each time point. The measurements were performed in triplicate.

162

163 CD spectroscopy after the foaming

164 To evaluate the secondary structure of HSA before and after the foaming, CD spectra 165 were obtained using a J-1500 CD spectropolarimeter (Jasco, Tokyo, Japan) equipped with 166 a Peltier-type temperature controller PTC-510 and PM-539 detector. HSA solution (10 mg/mL) was foamed using the electrical milk frother for 1, 10, 30, and 90 min. The HSA 167 168 samples, before and after the foaming, were treated with silicone antifoam and diluted to 0.33 µg/mL with 100 mM phosphate buffer. CD spectra of the samples were collected at 169 170 a pathlength of 3 mm in the far UV region (190-260 nm) with a step size of 0.5 nm at 171 25°C. The spectrum of the blank (100 mM sodium phosphate buffer) was subtracted from 172 the sample spectra. The observed ellipticity (θ_{obs}) in millidegrees was converted to mean residue ellipticity (MRE) in deg cm² dmol⁻¹, which was calculated from the equation: 173 $MRE = \frac{\theta_{obs} \times \varepsilon_{205}}{10 \times A_{205} \times n}$ 174 (5)

175 where *n* is the number of amino acid residues (585 for HSA) and ε_{205} is the extinction 176 coefficient at 205 nm. The extinction coefficient of HSA was calculated as 2,199,430 M⁻ 177 ¹cm⁻¹ from the protein sequence using the Protein Parameter Calculator.³¹ A_{205} is the absorbance at 205 nm measured using a J-1500 spectropolarimeter. The α-helix content
was calculated from the signals in the spectra from 200 to 250 nm using BeStSel.³²

180

181 HDX-MS

182 HSA solution at 100 mg/mL was foamed using the electrical milk frother immediately 183 after 10-fold dilution with deuterated buffer (100 mM sodium phosphate buffer, pD 7 with isotope effect correction³³). The hydrogen/deuterium exchange reaction proceeded 184 185 with continuous foaming at 20°C for various time periods: 60; 600; 1800; and 5400 s. A 186 non-deuterated sample was also prepared by 10-fold dilution with 100 mM sodium phosphate buffer, pH 7.0. A quenching solution [100 mM NaH₂PO₄, 3 M guanidine 187 188 hydrochloride (GdnHCl), and 112.5 mM tris (2-carboxyethyl) phosphine hydrochloride] 189 with 0.025% silicone antifoam was used to defoam the samples and quench the exchange 190 reaction. A 100- μ L aliquot of sample in the foam state (approximately 12 μ L as solution), 191 which was taken from the foam at a site well above the frothing coil, and 988 µL of the 192 quenching solution were mixed manually at 4°C, and the pH of the mixed solution was 193 decreased to 2.5. The quenched samples were placed in vials, and subsequent analysis 194 was conducted automatically using the HDX manager and HDX PAL system (Waters). 195 Samples (50 µL) were injected into HDX manager, and then HSA was digested online by

196	two tandemly connected BEH pepsin columns (2.1 mm \times 30 mm; Waters) with 0.1%
197	formic acid in water at 100 μ L/min. The peptic peptides were trapped in a VanGuard
198	BEH Pre-column (2.1 mm × 5 mm; Waters). The online pepsin digestion and the trapping
199	were performed in 4 min. The samples were loaded from the pre-column to the separation
200	column (ACQUITY BEH C18 1.7 $\mu m,$ 1.0 mm \times 100 mm; Waters) and separated with a
201	9-min linear gradient of acetonitrile containing 0.1% formic acid increasing from 4% to
202	40% in water containing 0.1% formic acid. The flow rate of the mobile phase was 40
203	μ L/min. Eluted peptides were detected using a SYNAPT G2 mass spectrometer (Waters).
204	The deuteration rate after the quenching was distinctly low, ¹⁸ and thus the resulting mass
205	of each peptide could be used to reflect the HSA structure at the AWI. The measurements
206	were performed in triplicate $(n = 3)$.
207	The hydrogen/deuterium exchange rate of HSA was also evaluated in the liquid state
208	to compare the hydrogen/deuterium exchange rate with that of the foam state. The entire
209	measurement process was automatically performed by the HDX PAL system. Given the
210	liquid handling volume of the HDX PAL system, the HSA concentration and quenching
211	conditions were adjusted to inject the same amount of HSA as for the foam state
212	measurements. Sample solutions were diluted to 5 mg/mL, and 5 μL of each sample
213	solution was mixed with 45 μ L of deuterated buffer. The mixed solutions were incubated

214	at 20°C for the predetermined hydrogen/deuterium exchange time periods, which were
215	the same as for the foam state measurements. A quenching solution composed of 150 mM
216	NaH ₂ PO ₄ , 4.5 M GdnHCl, 168.75 mM tris (2-carboxyethyl) phosphine hydrochloride,
217	and 0.03% silicone antifoam was used for the liquid state measurements. In the quenching
218	step, the pH of the sample solution was decreased to 2.5 by mixing 25 μL of the sample
219	solution and 75 μ L of quenching solution at 4°C. The injection volume, online digestion
220	conditions, and LC-MS conditions were the same as for the measurements of the foam
221	state samples. A non-deuterated sample was prepared and analyzed in the same manner
222	using non-deuterated buffer.
223	Defoamed solution samples were also analyzed. HSA solution (10 mg/mL) was
224	foamed for 90 min using the electrical milk frother. Then, the sample was defoamed by
225	1:1 mixing with 0.05% antifoam (the final concentration of the antifoam was 0.025%).
226	HDX-MS measurements of the defoamed solution samples were conducted in the same
227	manner as for the liquid state measurements.
228	Peptic peptides detected in the non-deuterated sample were identified using the
229	ProteinLynx global server 3.0 (Waters), and the retention time and MS spectrum were
230	used to assign the deuterated peptic peptides. A full deuterated (FD) sample was prepared

 $\label{eq:231} \textbf{by incubating HSA in deuterated phosphate buffer containing 6 M urea (final D_2O\% was$

233 for the FD sample. The HDX results were mapped on the HSA crystal structure (PDB ID: 234 4K2C) using Pymol. 235 236 237 **Statistical analysis** 238 Data are expressed as the mean or mean \pm standard deviation (SD). HSA monomer% 239 and total peak area obtained by SEC were analyzed with Dunnett's multiple comparison 240 test using the data without foaming as a control. 241 The statistical analysis of differential HDX measurements was performed based on the principle of uncertainty propagation.³⁴ Deuteration differences at each time point (ΔD_t) 242 243 were calculated as follows: $\Delta D_t = m_a - m_b$ 244 (6) 245 where m_a and m_b correspond to the mean of replicate measurements of the centroid mass 246 of each peptide in states A and B, i.e., the foam and liquid states or the liquid and defoamed states. Pooled SD (s_p) was also calculated: 247 $S_p = \sqrt{\frac{\sum s_a^2 + \sum s_b^2}{2 \times n_p \times n_t}}$ 248 (7)

98%) for 24 h at room temperature. The back-exchange rate was calculated from the result

232

249 where S_a and S_b are the standard deviations of replicate measurements of the centroid 250 mass of each peptide in the two states, np is the number of detected peptides, and nt is the number of time points. The combined uncertainties of ΔD_t using the s_p were calculated 251 from the following equation:³⁴ 252 $u(\Delta D_t) = S_p \sqrt{\frac{2}{n}}$ (8) 253 254 where *n* is the number of replicates (n = 3). All deuteration difference results were tested for significance, using the coverage factor $k = 4.6^{35}$ according to 255 $H_0: |\Delta D_t| \le k \times u(\Delta D_t)$ 256 (9) 257 where H_0 is the null hypothesis. When H_0 was rejected, the difference in HDX was 258 considered as significant. 259 260 261 **Results and discussion** 262 Adsorption of HSA at the AWI 263 Surface tension measurements were conducted to determine the adsorption of HSA at 264 the AWI. The static surface tension values of the HSA solution and phosphate buffer measured by the Whilhelmy method were 50.6 ± 0.16 and 70.8 ± 0.05 mN/m, respectively 265

(Table S1). The lower surface tension of the HSA solution compared with that of thephosphate buffer indicated the adsorption of HSA at the AWI.

268 Dynamic surface tension measurements by the pendant drop method were performed 269 to evaluate the adsorption kinetics of HSA at the AWI. As shown in Fig. 1a, the surface 270 tension of the HSA solution decreased rapidly and reached a plateau in 600 s at 271 approximately 52 mN/m, which was slightly higher than the static surface tension 272 measured by the Whilhelmy method. A certain amount of HSA was adsorbed to the AWI 273 and formed a dense phase immediately after a clean surface was generated because there 274 was no time lag in the decrease in the surface tension³ Considering that the interface/bulk 275 volume ratio of the foam was much larger than that of the pendant drop, and that the mass transfer of protein is an important factor in adsorption kinetics,³ the adsorption of HSA 276 277 could be faster than that observed in the dynamic surface tension measurements and could have occurred in the second timescale, or faster, in the foam state. 278

Different from the foaming of the phosphate buffer, a firm foam was obtained immediately after the start of the foaming for the HSA solution (Fig. 1b), also suggesting rapid adsorption of HSA to the foam AWI. Because the following HDX-MS measurements were performed on the timescale of minutes, the hydrogen/deuterium (H/D) exchange rates could reflect the HSA structure after the adsorption to the foam

284	AWI. The area of the solid-liquid interface (interface with the glass container) was also
285	increased by foaming, and the interaction with the solid-liquid interface could have an
286	influence on the H/D exchange rates. However, as shown in Fig. 1b, the increase in the
287	solid-liquid interface area was approximately fourfold and this was much smaller than
288	the increase in the AWI area. Thus, the difference in the H/D exchange rates was likely
289	to mainly arise from the interaction with the AWI or the structural changes of HSA at the
290	AWI.

292 Aggregation of HSA by foaming

Structural changes at an interface can result in protein aggregation.³⁶ Because 293 294 knowledge of the aggregate distribution is crucial for the correct interpretation of the 295 HDX-MS results, SEC analysis was performed on the foam samples after the defoaming. 296 A main peak derived from the monomer was observed at approximately 6 min and the peaks of aggregates were eluted before the main peak (Fig. 2b). A significant decrease (p 297 298 < 0.01) in monomer% was observed after 90 min of foaming. The area% of aggregates 299 was 0.9% at 0 min and this was significantly increased to 10.1% at 90 min (Fig. 2a). No 300 significant differences in the total peak area were observed, suggesting that large 301 aggregates that could not be analyzed by SEC were not generated by foaming.

302	In a previous study, greater than 30% of the HSA monomer formed aggregates after
303	foaming by jetting the HSA solution. ³⁷ Although the cause of this difference in the
304	amount of aggregates generated by foaming in the previous study compared with the
305	present study might be because of differences in the formulation conditions, there is also
306	the possibility that foaming using a milk frother could be gentler than foaming by jetting
307	the protein solution. Foaming using a milk frother could be more suitable for the
308	evaluation of protein structure at the foam AWI with less influence of aggregation.

310 Structural changes in HSA at the foam AWI

311 The structure of HSA at the foam AWI was evaluated by a comparison of the H/D 312 exchange rate between the foam and liquid states. The back exchange rate, uptake plots for each peptide, and other information recommended by Glenn R. Masson et al.³⁸ are 313 314 summarized in the Supporting Information (Table S2). The H/D exchange rates for 68 315 peptides, which accounted for 57.4% of the amino acid sequence of HSA, were compared. 316 The deuterium uptake values for each peptide at each time point in the foam and the 317 liquid states were statistically compared according to the recommendations of David D. Weis.³⁴ Significant differences in deuterium uptake were observed for 23 peptides when 318 319 the deuterium uptake was compared between the foam and liquid states; 21 peptides

321

showed higher deuterium uptake and 2 peptides showed lower deuterium uptake in the foam state than in the liquid state (Fig. 3 and S1).

322 Peptides A2-F19 and E492-T506 showed significantly lower deuterium uptake at 1 323 min in the foam state compared with in the liquid state. Because the level of HSA 324 aggregates was only slightly increased by foaming for 1 min (Fig. 2), a possible 325 explanation for the lower deuterium uptake for these two peptides was that when the HSA 326 molecules were adsorbed to the AWI, the regions containing these two peptides faced to the air side of the foam AWI.¹⁰ Thus, the adsorption to the AWI resulted in lower 327 328 accessibility of deuterium at the adsorption site. These differences in deuterium uptake 329 had disappeared after 10 min, indicating that the adsorption of the HSA molecule at the 330 AWI at 1 min was reversible, i.e., an adsorption/desorption process was occurring. The 331 region that had been protected from H/D exchange was then exposed to the deuterium when the HSA molecule was desorbed into the bulk solution.³⁹ The results of dynamic 332 333 surface tension measurements showed that HSA was adsorbed on the AWI at 1 min; 334 however, given that structural changes result in very slow desorption or irreversible 335 adsorption,³ the interfacial structural changes were negligible or did not occur at 1 min. 336 Therefore, there were no significant differences in the H/D exchange rate that could be 337 related to structural perturbation at 1 min.

338	Peptides with significantly higher deuterium uptakes in the foam state were observed
339	after 10 min (Fig. 3a). The Linderstrøm-Lang model (Equation 1) ¹⁸ indicated that the
340	peptide regions were in the open state more frequently in the foam state than in the liquid
341	state. In other words, the structure of the HSA molecule was substantially fluctuating at
342	the foam AWI. The structural changes occurred over the entire peptide structure. When
343	fractional deuterium uptake was calculated based on the maximum theoretical mass
344	change (number of peptide bonds - number of prolines, which do not have an amide
345	hydrogen), the differences in the fractional uptake were comparable between the peptides
346	that showed significantly higher deuterium uptake (Fig. S3). This result inferred that the
347	extent of the structural changes for all of these peptides was equivalent. A previous study
348	has suggested that the structure of the region surrounding the adsorption site could be
349	particularly changed. ²⁵ The deuterium uptake of D1-N18 and D1-F19, peptide regions
350	that have considerable overlap with the adsorption site A2-F19, in the foam state was
351	significantly higher than that in the liquid state at 90 min (Fig. 3c). The peptide A2-F19
352	also showed higher deuterium uptake in the foam state at 90 min but the difference from
353	the liquid state was not statistically significant. These results indicated that the influence
354	of structural changes at the N-terminus on the deuterium uptake could be greater than the
355	shielding effect of the adsorption to the foam AWI. E492-T506, colored in magenta in

356	Fig. 3c, is another adsorption site and the peptide S480-R521, which includes E492-T506,
357	showed higher deuterium uptake in the foam state; however, there was no difference in
358	the deuterium uptake of E492-T506 at 90 min. The peptide E492-T506 forms a loop with
359	few hydrogen bonds between the main chains, and therefore deuterium exchange in E492-
360	T506 cannot be used to determine any structural changes because deuterium exchange is
361	sensitive to changes in hydrogen bonding in secondary structures, such as α -helix and β -
362	sheet, in which most of the amides form hydrogen bonds with carbonyl groups. ^{40,41} Thus,
363	the difference in the deuterium uptake is likely to be related to structural changes in the
364	region S480-R521, excluding E492-T506, (i.e., S480-L491 and/or F507-R521).
365	Aggregates of HSA were generated by the foaming and could prevent the deuterium
366	uptake of peptides located at the interface of the aggregates. Although this aggregation
367	could lead to an underestimation of the increase in deuterium uptake, and there was a
368	possibility that some structurally perturbated peptides could be overlooked, we focused
369	on the peptides that showed significantly higher deuterium exchange rates because these
370	peptides are likely to be the characteristic peptides that are structurally perturbated by
371	foaming. Furthermore, the presence of aggregates could lead to an overestimation of the
372	decrease in deuterium uptake; however, there were no peptides that showed significantly
373	lower deuterium uptake in the foam state.

374 To the best of our knowledge, this is the first report of the protein adsorption sites and 375 structural changes at a foam AWI. The method developed here will be useful to elucidate 376 the molecular mechanisms of protein foam properties from the viewpoint of the peptide 377 structure at the foam AWI. Protein foam properties can be altered by the presence of other components, such as polyphenols, in food products.^{11,12} While it is difficult to elucidate 378 379 the interaction sites and protein structures at a foam AWI in the presence of other 380 components using conventional methods, the developed method using HDX-MS can 381 provide insight into the molecular mechanisms of protein foam properties from the 382 viewpoint of the interaction and structure at the foam AWI.

383

384 Structure of HSA after defoaming

The secondary structure of HSA before and after defoaming was examined using CD spectroscopy. The CD spectrum of HSA before foaming showed a helix-specific pattern, which was consistent with previously reported spectra.⁴² The CD spectra after foaming for 1, 30, 60, and 90 min were measured immediately after defoaming. As shown in Fig. 4, there were no obvious differences in the spectra before and after defoaming. The secondary structure composition was estimated from each spectrum from 200 to 250 nm using BeStSel. Consistent with the visual comparison of the CD spectrum, the calculated

392	secondary structure composition was not changed obviously by the foaming and
393	defoaming. In the HDX-MS measurements, several peptides containing an α -helix
394	showed higher deuterium uptake in the foam state after more than 1 min of foaming.
395	Considering that the hydrogen bonding of amide hydrogens in an α -helix results in slower
396	deuterium exchange, structural perturbations in the α -helix regions would be caused by
397	the foaming. Although there is a possibility that CD cannot detect the structural changes
398	or the differences in solvent accessibility detected using HDX-MS because of the
399	differences in the measurement principles, the CD results after defoaming indicated that
400	the structural changes in HSA at the AWI are likely to be reversible.
401	To evaluate the reversibility of the structural changes at the AWI caused by foaming,
402	structural change was induced by foaming for 90 min, and the HDX-MS measurement
403	was also performed after defoaming of the foamed sample. Eight peptides showed
404	significant differences in the deuterium uptake between the defoamed and liquid samples,
405	and three out of the eight peptides also showed significant differences in the deuterium
406	uptake between the foam and the liquid states. Interestingly, the defoamed samples
407	showed slower deuterium uptake than the liquid samples, which was the opposite trend
408	to that of the foam samples (Fig. 5). This difference might be because of the aggregation
409	induced by 90 min of foaming, and the peptides that showed significantly lower

deuterium uptake in the defoamed condition could be those found at the interfaces of
aggregates. There is another possibility that another type of structural change, which was
not detected by CD spectroscopy, could occur after defoaming. However, in either case,
these results indicated that the structural changes at the foam AWI are likely to be
reversible. These results emphasized the advantage of in situ structural analysis of
proteins in the foam state.

416

417 Influence of hydrophobicity and surface charge

The AWI is hydrophobic,¹ and thus the hydrophobic moieties of a protein are generally 418 thought to adsorb to the AWI. Positively charged amino acids are also potential 419 interaction sites with the AWI via electrostatic interactions].²⁵ The hydrophobicity and 420 surface charge of the HSA surface were estimated using eF-site (Fig. 6).^{43,44} The surface 421 422 charge of the interacting peptides was mainly negative (shown as red in Fig. 6) and the 423 AWI is negatively charged, which suggested that electrostatic interactions do not 424 participate in the adsorption of HSA on the foam surface. There were some hydrophobic 425 moieties (yellow) identified in the interacting peptides. These results suggested that 426 hydrophobicity was the main factor for the interaction with the foam AWI. However, 427 considering that there are other hydrophobic surfaces in the HSA molecule, other

428	important factor(s) are likely to be involved in the adsorption to the foam surface. Because
429	the adsorption sites identified by HDX-MS were a loop region and the N-terminus, the
430	flexibility to position the hydrophobic side chain toward the AWI could be a potential
431	factor. The structural flexibility of protein molecules has been shown to be correlated
432	with the adsorption kinetics at the AWI. ³
433	The dehydration of the protein surface, which is essential for the induction of the
434	hydrophobic effect, can also occur for hydrophilic amino acids, which causes further
435	difficulty in identifying the protein interaction sites with the AWI. ⁴⁵ The analysis of
436	various other proteins using the developed method should provide information beyond
437	the hydrophobicity to identify the interaction sites with the foam AWI.
438	In summary, we established a method using HDX-MS and analyzed the structure of
439	HSA at the foam AWI. Using the developed method, we revealed the adsorption sites of
440	HSA at peptide-level resolution and demonstrated that reversible structural changes
441	occurred at the foam surface. The established method can be used to analyze the protein
442	structure in foam in situ with minimal influence of protein aggregation at the interface.
443	Peptides at the N-terminus and at a loop region, which had hydrophobic surfaces, were
444	identified as the adsorption sites at the foam AWI. Structural changes were induced at the

445	foam AWI after more than 1 min of foaming and were observed throughout the molecule.
446	The structural changes that occurred at the foam AWI are likely to be reversible.
447	The developed method can be applied to other proteins and proteins with additives that
448	affect the foam properties. Further research using the method established here should help
449	to understand protein foaming properties at a molecular level. In addition, structural
450	assessment of proteins at the AWI by orthogonal methods is desirable in the future to
451	support the assessment by the developed HDX-MS method.
452	

4	5	3
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454	Abbreviations
455	HDX, hydrogen/deuterium exchange; MS, mass spectrometry; AWI, air-water interface;
456	HSA, human serum albumin; CD, circular dichroism; MRE, mean residue ellipticity;
457	SEC, size exclusion chromatography.
458	
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469	- review & editing, K.E., T.T., and S.U.; Supervision, T.T. and S.U.

474	C		•	•	
4/1	Supp	orting	int	orm	ation

- 472 Figures S1–S3
- Tables S1 and S2

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631 Figures captions:

633	Figure 1. (a) Adsorption of HSA at the AWI evaluated by interfacial tension
634	measurement. The dashed line represents the static surface tension measured by the
635	Whilhelmy method [50.6 \pm 0.16 (HSA solution) and 70.8 \pm 0.05 mN/m (phosphate
636	buffer)] and the dots are the results of dynamic surface tension measurements. (b) HSA
637	solution (left) was foamed using a milk frother (right).
638	
639	Figure 2. SEC after foaming. (a) The time course of the total peak area of monomer and
640	aggregates are shown as gray bars and the time course of the monomer percentage is
641	also plotted. Error bars represent the SD of three measurements. The asterisk indicates
642	the statistical difference in monomer% tested by the Dunnett's test ($p < 0.01$). (b) A
643	representative chromatogram before foaming (0 min) and after foaming (90 min).
644	
645	Figure 3. HDX-MS results for foam samples. (a) Deuterium uptake plots of peptides
646	that showed significant differences between the foam and liquid samples. The
647	deuterium uptake was calculated by subtracting the centroid mass at $t = 0$ from the
648	centroid mass at each time point. The asterisk denotes a statistically significant

649	difference. (b) The peptides that showed significantly lower deuterium uptake at 1 min
650	in the foam state are colored cyan and (c) the peptides that showed significantly higher
651	deuterium uptake at 90 min are colored magenta.
652	
653	Figure 4. CD spectra of defoamed samples. The secondary structure composition
654	calculated by BeStSel is shown below the spectra.
655	
656	Figure 5. HDX-MS results for defoamed samples. (a) Deuterium uptake plots of
657	peptides that showed significant differences between the defoamed and liquid samples.
658	(b) The peptides that showed significantly lower deuterium uptake at 90 min in the
659	defoamed state are colored green.
660	
661	Figure 6. (a) Surface charge and hydrophobicity of HSA calculated using eF-site.
662	Positively and negatively charged surfaces are colored blue and red, respectively.
663	Magnified view of peptides A2-F19 (b) and E492-T506 (c).