Structure of human serum albumin at a foam surface

Kanta Enomoto[1], Tetsuo Torisu[1, *], Junya Mizuguchi[1], Ryosuke Tanoue[1], and Susumu Uchiyama[1,2, *]

1. Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka, 565-0871, Japan

2. Exploratory Research Center on Life and Living Systems, National Institutes of Natural Sciences, 5-1 Higashiyama, Myodaiji, Okazaki, Aichi 444-8787, Japan

*Corresponding author

Address correspondence to: Susumu Uchiyama, Ph.D., Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan, Tel. +81-6-6879-4216, Fax: +81-6-6879-7442, E-mail: suchi@bio.eng.osaka-u.ac.jp

Tetsuo Torisu, Ph.D., Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan, Tel. +81-6-6879-4216, Fax: +81-6-6879-7442, E-mail: tetsuo.torisu@bio.eng.osaka-u.ac.jp

ABSTRACT

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

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

Introduction

The air-water interface (AWI) is a hydrophobic interface.[1] Proteins are composed of both hydrophilic and hydrophobic amino acids and are therefore amphiphilic. Thus, 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 hydrophobic interaction between proteins and the AWI is a main driving force of the 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 is important for many reasons.[4-6] For example, therapeutic proteins can be adsorbed on 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 formation of protein aggregates, which could elicit an immune response; thus, creating a potential risk for immunogenicity.[7] In addition, understanding the interfacial structural instability of viruses that can be transmitted by airborne droplets is important for developing strategies to prevent the spread of viral infections.[1]

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

properties.[8] In addition, foam is a key quality attribute of both sparkling wine and beer, and proteins play an important role in the formation of the foam of these beverages.[9] Therefore, many studies have investigated improving the foaming ability, as well as the foaming stability of foods. Previous studies have demonstrated that the surface hydrophobicity of proteins was correlated with the foam properties.[10] In addition, having the molecular flexibility to expose previously buried hydrophobic amino acids to the AWI has also been indicated to be an important factor for foam formation.[3] The addition of molecules, such as polyphenols[11,12] and saccharides,[13] and chemical modification, such as oxidation,[14,15] has been shown to change the protein structure and/or structural flexibility in solution leading to improvements in the foaming properties. Although these studies have indicated that the protein structure in the foam state is related to the foam properties, knowledge of the protein structure in the foam state remains elusive.

Analysis of protein structure at the AWI is more challenging than structural analysis of proteins in solution because signals derived from proteins at the AWI, which are typically weak because of the limited amount of protein at the AWI, need to be selectively detected. For this reason, protein structure at the AWI has mainly been evaluated using spectroscopic and scattering techniques, which provide spectra from 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
57 the angles of incident light and the angles of the detectors for the reflected or scattered
58 light need to be specific. In addition, the structural resolution of spectroscopic and
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
64 D_2O.[17] The deuterium exchange of each amide hydrogen in folded proteins can be
65 expressed by the following equation, the Linderstrøm-Lang model:[18]

66 $$\mathrm{cl(NH)} \underset{k_{\mathrm{cl}}}{\overset{k_{\mathrm{op}}}{\rightleftharpoons}} \mathrm{op(NH)} \overset{k_{\mathrm{ch}}}{\rightarrow} \mathrm{op(ND)} \underset{k_{\mathrm{op}}}{\overset{k_{\mathrm{cl}}}{\rightleftharpoons}} \mathrm{cl(ND)} \qquad (1)$$

67 where cl corresponds to the folded state and op corresponds to the open state, NH and
68 ND are the protiated and deuterated amides, respectively, k_{op} and k_{cl} are the rate
69 constants of the opening and closing reactions, respectively, and k_{ch} is the chemical
70 deuteration rate constant. From this model, the observed deuteration rate constant (k_{HD})
71 can be obtained:

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

Under stable conditions, proteins can be refolded immediately after the unfolding.[18] Thus, k_{cl} is much larger than k_{op} and k_{ch}, and then k_{HD} can be simplified as follows:

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

where K_{op} is the equilibrium constant of the opening reaction. Amide hydrogens often form hydrogen bonds, which stabilize the protein structure and result in the value for K_{op} being smaller, and thus the exchange of amide hydrogens in a folded protein is 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 protein for the deuterium atoms influences the exchange rate. For example, when an amide hydrogen is buried inside the protein or covered by additives or other proteins, 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 protein that has been exposed to D_2O is conducted, followed by liquid chromatography and mass analysis. The deuterium uptake profiles of the resulting peptides provide 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 with spectroscopic and solution scattering/reflection techniques.

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

successfully obtained at peptide-level resolution using the developed method. Analysis after defoaming revealed that the structural changes at the foam AWI were reversible.

Materials and methods

Materials

HSA and all other reagents were purchased from Fujifilm Wako (Osaka, Japan) unless otherwise specified. The HSA monomer content, determined by size exclusion chromatography (SEC) with UV detection at 280 nm, was 50.5%. Phosphate-buffered saline (1×PBS; pH 7.4) and acetonitrile were obtained from Thermo Fisher Scientific (Waltham, MA, USA). D_2O was purchased from Iwatani Corporation (Osaka, Japan).

Preparation of HSA samples

Prior to analysis, HSA monomer was fractionated by SEC with a HiLoad 16/60 Superdex 200 column (Cytiva, Tokyo, Japan), and 1×PBS at pH 7.4 was used as the elution buffer. The HSA monomer content was 99.1% after the purification. Fractionated HSA monomer was dialyzed against 100 mM sodium phosphate buffer at pH 7 at 4°C, and then concentrated by ultrafiltration using Amicon Ultra 10k (Merck, Darmstadt, 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 $$\gamma = \frac{F}{L} \qquad (4)$$
135 where L is the perimeter of the plate (39.24 mm). The average of three measurements
136 was reported. Dynamic surface tension was measured by the pendant drop method using
137 a Theta Flex instrument (Biolin Scientific, Gothenburg, Sweden). In the measurements,
138 a 10-μL pendant drop was generated at a flow rate of 2 μL/s, and the shape of the pendant
139 drop was monitored for 1200 s. The surface tension was simultaneously calculated from
140 the Young-Laplace equation every 10 s using OneAttension (ver.4.0.3). [30]
141
142 **Foaming of HSA**

143 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,
150 the foam was collected and weighed. The weight was divided by the density of the HSA
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 × *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
156 TSKgel UP-SW3000 column (4.6 mm × 150 mm; Tosoh Bioscience, Tokyo, Japan).
157 Isocratic elution was performed using 133 mM phosphate buffer with 200 mM
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 at
161 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
167 mg/mL) was foamed using the electrical milk frother for 1, 10, 30, and 90 min. The HSA
168 samples, before and after the foaming, were treated with silicone antifoam and diluted to
169 0.33 μg/mL with 100 mM phosphate buffer. CD spectra of the samples were collected at
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
173 residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$, which was calculated from the equation:
174 $$MRE = \frac{\theta_{obs} \times \varepsilon_{205}}{10 \times A_{205} \times n} \qquad (5)$$
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 $M^{-1}cm^{-1}$
177 [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]

HDX-MS

HSA solution at 100 mg/mL was foamed using the electrical milk frother immediately after 10-fold dilution with deuterated buffer (100 mM sodium phosphate buffer, pD 7 with isotope effect correction[33]). The hydrogen/deuterium exchange reaction proceeded with continuous foaming at 20°C for various time periods: 60; 600; 1800; and 5400 s. A non-deuterated sample was also prepared by 10-fold dilution with 100 mM sodium phosphate buffer, pH 7.0. A quenching solution [100 mM NaH_2PO_4, 3 M guanidine hydrochloride (GdnHCl), and 112.5 mM tris (2-carboxyethyl) phosphine hydrochloride] with 0.025% silicone antifoam was used to defoam the samples and quench the exchange reaction. A 100-μL aliquot of sample in the foam state (approximately 12 μL as solution), which was taken from the foam at a site well above the frothing coil, and 988 μL of the quenching solution were mixed manually at 4°C, and the pH of the mixed solution was decreased to 2.5. The quenched samples were placed in vials, and subsequent analysis was conducted automatically using the HDX manager and HDX PAL system (Waters). 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 × 30 mm; Waters) with 0.1%
197 formic acid in water at 100 μL/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 μm, 1.0 mm × 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 μL/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 μL 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_2PO_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 μL 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
231 by incubating HSA in deuterated phosphate buffer containing 6 M urea (final D_2O% was

98%) for 24 h at room temperature. The back-exchange rate was calculated from the result for the FD sample. The HDX results were mapped on the HSA crystal structure (PDB ID: 4K2C) using Pymol.

Statistical analysis

Data are expressed as the mean or mean ± standard deviation (SD). HSA monomer% and total peak area obtained by SEC were analyzed with Dunnett's multiple comparison test using the data without foaming as a control.

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) were calculated as follows:

$$\Delta D_t = m_a - m_b \qquad (6)$$

where m_a and m_b correspond to the mean of replicate measurements of the centroid mass 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:

$$S_p = \sqrt{\frac{\sum {s_a}^2 + \sum {s_b}^2}{2 \times n_p \times n_t}} \qquad (7)$$

where S_a and S_b are the standard deviations of replicate measurements of the centroid mass of each peptide in the two states, n_p is the number of detected peptides, and n_t is the number of time points. The combined uncertainties of ΔD_t using the s_p were calculated from the following equation:[34]

$$u(\Delta D_t) = S_p\sqrt{\frac{2}{n}} \qquad (8)$$

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

$$H_0: |\Delta D_t| \leq k \times u(\Delta D_t) \qquad (9)$$

where H_0 is the null hypothesis. When H_0 was rejected, the difference in HDX was considered as significant.

Results and discussion

Adsorption of HSA at the AWI

Surface tension measurements were conducted to determine the adsorption of HSA at 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

266 (Table S1). The lower surface tension of the HSA solution compared with that of the
267 phosphate 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 tension[3] Considering that the interface/bulk
275 volume ratio of the foam was much larger than that of the pendant drop, and that the mass
276 transfer of protein is an important factor in adsorption kinetics,[3] the adsorption of HSA
277 could be faster than that observed in the dynamic surface tension measurements and could
278 have occurred in the second timescale, or faster, in the foam state.

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

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

Aggregation of HSA by foaming

Structural changes at an interface can result in protein aggregation.[36] Because knowledge of the aggregate distribution is crucial for the correct interpretation of the HDX-MS results, SEC analysis was performed on the foam samples after the defoaming. 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 ($p < 0.01$) in monomer% was observed after 90 min of foaming. The area% of aggregates was 0.9% at 0 min and this was significantly increased to 10.1% at 90 min (Fig. 2a). No significant differences in the total peak area were observed, suggesting that large 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.
309
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
313 for each peptide, and other information recommended by Glenn R. Masson et al.[38] are
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.
318 Weis.[34] Significant differences in deuterium uptake were observed for 23 peptides when
319 the deuterium uptake was compared between the foam and liquid states; 21 peptides

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

Peptides A2-F19 and E492-T506 showed significantly lower deuterium uptake at 1 min in the foam state compared with in the liquid state. Because the level of HSA aggregates was only slightly increased by foaming for 1 min (Fig. 2), a possible explanation for the lower deuterium uptake for these two peptides was that when the HSA 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 accessibility of deuterium at the adsorption site. These differences in deuterium uptake had disappeared after 10 min, indicating that the adsorption of the HSA molecule at the AWI at 1 min was reversible, i.e., an adsorption/desorption process was occurring. The 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 surface tension measurements showed that HSA was adsorbed on the AWI at 1 min; however, given that structural changes result in very slow desorption or irreversible adsorption,[3] the interfacial structural changes were negligible or did not occur at 1 min. Therefore, there were no significant differences in the H/D exchange rate that could be related to structural perturbation at 1 min.

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

Fig. 3c, is another adsorption site and the peptide S480-R521, which includes E492-T506, showed higher deuterium uptake in the foam state; however, there was no difference in the deuterium uptake of E492-T506 at 90 min. The peptide E492-T506 forms a loop with few hydrogen bonds between the main chains, and therefore deuterium exchange in E492-T506 cannot be used to determine any structural changes because deuterium exchange is sensitive to changes in hydrogen bonding in secondary structures, such as α-helix and β-sheet, in which most of the amides form hydrogen bonds with carbonyl groups.[40,41] Thus, the difference in the deuterium uptake is likely to be related to structural changes in the region S480-R521, excluding E492-T506, (i.e., S480-L491 and/or F507-R521).

Aggregates of HSA were generated by the foaming and could prevent the deuterium uptake of peptides located at the interface of the aggregates. Although this aggregation could lead to an underestimation of the increase in deuterium uptake, and there was a possibility that some structurally perturbated peptides could be overlooked, we focused on the peptides that showed significantly higher deuterium exchange rates because these peptides are likely to be the characteristic peptides that are structurally perturbated by foaming. Furthermore, the presence of aggregates could lead to an overestimation of the decrease in deuterium uptake; however, there were no peptides that showed significantly 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
378 components, such as polyphenols, in food products.[11,12] While it is difficult to elucidate
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**
385 The secondary structure of HSA before and after defoaming was examined using CD
386 spectroscopy. The CD spectrum of HSA before foaming showed a helix-specific pattern,
387 which was consistent with previously reported spectra.[42] The CD spectra after foaming
388 for 1, 30, 60, and 90 min were measured immediately after defoaming. As shown in Fig.
389 4, there were no obvious differences in the spectra before and after defoaming. The
390 secondary structure composition was estimated from each spectrum from 200 to 250 nm
391 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

410 deuterium uptake in the defoamed condition could be those found at the interfaces of
411 aggregates. There is another possibility that another type of structural change, which was
412 not detected by CD spectroscopy, could occur after defoaming. However, in either case,
413 these results indicated that the structural changes at the foam AWI are likely to be
414 reversible. These results emphasized the advantage of in situ structural analysis of
415 proteins in the foam state.
416
417 **Influence of hydrophobicity and surface charge**
418 The AWI is hydrophobic,[1] and thus the hydrophobic moieties of a protein are generally
419 thought to adsorb to the AWI. Positively charged amino acids are also potential
420 interaction sites with the AWI via electrostatic interactions].[25] The hydrophobicity and
421 surface charge of the HSA surface were estimated using eF-site (Fig. 6).[43,44] The surface
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

Abbreviations

HDX, hydrogen/deuterium exchange; MS, mass spectrometry; AWI, air-water interface; HSA, human serum albumin; CD, circular dichroism; MRE, mean residue ellipticity; SEC, size exclusion chromatography.

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Author contributions

Conceptualization, K.E., T.T., and S.U.; Investigation, K.E., T.T., J.M., and R.T.; Data curation, K.E., T.T., J.M., and R.T.; Writing – original draft, K.E., T.T., and S.U.; Writing – review & editing, K.E., T.T., and S.U.; Supervision, T.T. and S.U.

Supporting information

Figures S1–S3

Tables S1 and S2

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631 **Figures captions:**

632

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 ± 0.16 (HSA solution) and 70.8 ± 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).
664