Study of Lysozyme Glycation Reaction by Mass Spectrometry and NMR Spectroscopy

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The Advanced Glycation End Products (AGEs) are the causative substances of lifestyle-habit illness. To elucidate the glycation mechanism of the protein, the reaction of lysozyme with D-glucose was analyzed by the fluorescence, TOF-MS, and ^{13}C-NMR spectroscopy under the physiological condition. The fluorescence intensity of lysozyme in the glycation solution increased proportionally with a reaction time of ten weeks. The MALDI-TOF-MS spectra of the reaction solution after two weeks showed a peak at *m*/*z* 15066, which indicated the presence of a larger molecule than the native lysozyme (*m*/*z* 14331), and new peaks at *m*/*z* 30105 (dimer) and 45000 (trimer) were also observed. The spectral analysis supported the assumption of a continuous glycation reaction of D-glucose with lysozyme and a 30% transformation of lysozyme to the dimeric form during ten weeks. The ^{13}C-NMR spectra of lysozyme showed six [^{13}C]-labeled signals by the glycation reaction with [^{13}C]-glucose after two weeks of reaction. The combined analysis of TOF-MS and ^{13}C-NMR spectra uncovered that first products of the glycation reaction of lysozyme with D-glucose can be observed already three hours after starting the reaction and that nine D-glucose units are attached during ten weeks at 37°.

Introduction. – In recent years, the preventive medicine has been in the spotlight from the anti-aging standpoint of adult and elderly persons, because many of them have lifestyle-habit illnesses such as arteriosclerosis, diabetes, and the melanism of the skin. A major factor of such illnesses is the generation and storage of Advanced Glycation End Products (AGEs) during the long-range life. The AGEs are artificial substances that are produced between the constituent protein in the body and the reducible sugars taken from the food. It is known that the glycation reaction progresses according to a nonenzymatic reaction (glycation = non-enzymatic glycosylation; *Scheme*). The initial stage of this reaction proceeds *via* the *Schiff* base formation of the aldehyde group of glucose with the amino group of the amino acid residue in the protein such as albumin, and it is then transformed to the *Amadori* compound by the *Amadori* rearrangement. In the late stage of the reaction process, the *Amadori* compound is changed to AGEs by oxidation, dehydration, and polymerization; the brownish AGEs having a cross-linked structure are fluorescent [1–3]. The AGEs are age-dependently accumulated in skin, lenses, erythrocyte membranes, *etc.*, and the storage is especially prominent in lesioned places such as in affeted regions by *Alzheimer* disease, in vessel walls of the arteriosclerosis, and in the erythrocytes of patients with diabetic complications [4]. Therefore, the development of an AGE decomposer or an AGE generation inhibitor is

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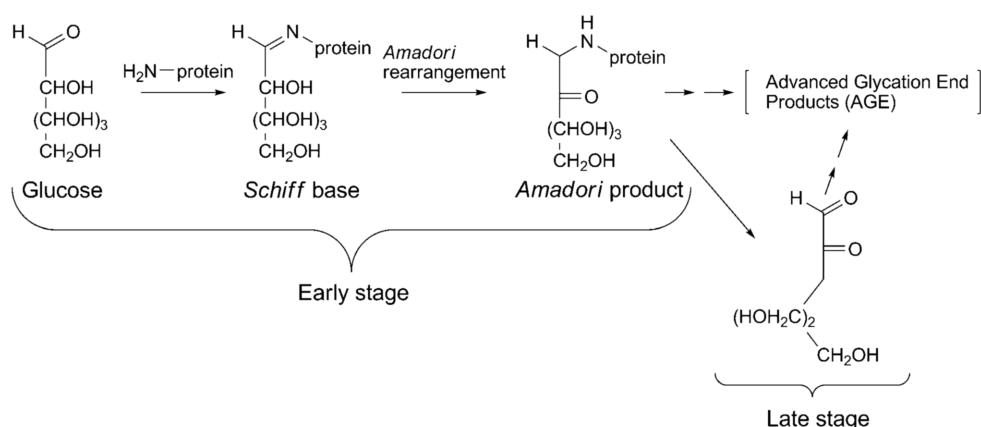
useful for the prevention or the improvement of the lifestyle-habit illness. Although aminoguanidine or OPB-9195 have been utilized as a medical supplement, an effective inhibitor for the AGE formation is not available yet [5][6]. The present study was performed to increase the knowledge of the glycation reaction, with the hope to become able to design a drug with the capacity to suppress AGE generation. Although the glycation reaction of the protein has been mainly investigated in the field of food chemistry [7–9], structural research under physiological conditions is quite lacking. Thus, the present results on the glycation reaction of lysozyme and D-glucose in a neutral buffer (pH 7.4) at 37° are valuable.

Scheme. *Reaction Scheme of the Generation of Advanced Glycation End Products* (AGEs)



Results and Discussion. – *Fluorescence Spectroscopy.* As AGEs are fluorescent, it is possible to monitor the AGE formation process by fluorescence spectroscopy. In general, the estimation of the amounts of AGEs generated has been performed by fluorescence intensity [10–12]. However, it is doubtful whether such analysis reflects the produced quantity of the AGE exactly, as the structure of the glycated protein has not yet been established precisely. To investigate the reaction process between protein and aldose under physiological conditions, the production of AGE was measured as a function of reaction time by fluorescence spectroscopy. The glycation reaction was investigated using a solution of lysozyme (chicken egg white) and D-glucose (glc) in a phosphate buffer (pH 7.4) at 37°. The results are given in the *Table.* As shown in *Fig. 1*, a linear relationship was observed between the fluorescence intensity and reaction time in the range of 1–56 days, and the fluorescence intensity reached a plateau 60 days after the start of the experiment, indicating a 'saturation' of the glycation reaction.

MALDI-TOF-MS Spectroscopy. Recently, the MALDI-TOF-MS method has been used for the analysis of the glycation products [8][13–16]. Thus, in selected intervals, a fraction of the reaction solution was subjected to the MALDI-TOF-MS analysis to investigate the reaction progress of the glycation; the results are shown in *Fig. 2* and given in the *Table.* In the starting solution, a peak at *m/z* 14331, corresponding to the molecular weight of native lysozyme, is observed (*Fig. 2,a*). After two weeks of

Table. *Fluorescence Intensity,* m/z *Values of glc-Lysozyme Adducts in the Reaction Solution, Numerical Values of the Covalent-Bonded* D-*Glucose, and the Relative Ratio* [%] *between the Monomer and Dimer of Glycated Lysozyme*

Reaction duration [d]	FI[a])	MS Data				
		Monomer			Dimer	
		m/z	Numerical value of D-glucose[b])	Peak integral [%]	*m/z*	Peak integral [%]
0	20	14331	0	100	–	0
0.1	21	14331	0	100	–	0
		14497 (s)[c])	1			
0.3	24	14331	0	100	–	0
		14497 (s)	1			
1	44	14331	0	100	–	0
		14504 (s)	1			
		14656 (s)	2			
3	1554	14331 (s)	0	100	–	0
		14495 (s)	1			
		14652	2			
		14824 (s)	3			
		14992 (s)	4			
14	720	15066	4.5	80.0	30105	20.0
21	910	15252	5.6	76.5	30416	23.5
28	1053	15349	6.2	76.8	30670	23.2
35	1141	15522	7.2	73.3	31121	26.7
42	1287	–	–	–	–	–
49	1411	15629	7.9	73.5	31254	26.5
56	1714	–	–	–	–	–
63	1702	15839	9.1	70.0	31630	30.1
70	1731	–	–	–	–	–

[a]) FI = Intensity of fluorescence; represented as mean ± SD = 4%. [b]) Numerical value of D-glucose = increased *m/z* value/[180.1(MW of glc) – 18]). [c]) (s) = shoulder peak.

reaction (*Fig. 2,b*) the peak at *m/z* 14331 had disappeared, and a new broad peak centered at *m/z* 15066 was observed. The increase of *m/z* 735 indicates the covalent-bond formation between glc and lysozyme and corresponds to 4.5 molecules of glc: this number can be estimated by the division of the molecular weight of glc (MW 180) and the subtraction of H_2O(18). After nine weeks, the starting peak at *m/z* 14331 had shifted toward *m/z* 15839, indicating the linkage of nine glc units to lysozyme. The time-dependent reaction process and the covalent bonding of glc to lysozyme are summarized in the *Table*.

The mass spectra also showed a peak close to *m/z* 30000, which appeared first in the sample collected after two weeks, which could be due to a dimer form of $(glc)_n$-lysozyme. The *m/z* value and the integrated area of this peak increased with the progress of the reaction. The comparison with the integrated peak areas suggested a dimer formation of about 30% in nine weeks, although an exact ratio could not be estimated due to differing ionization efficiencies.

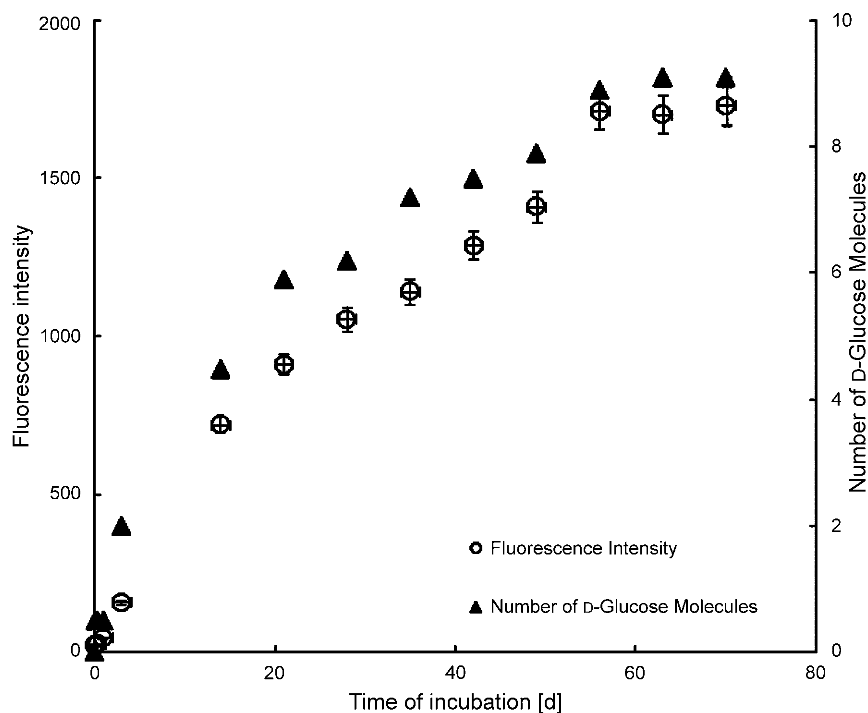


Fig. 1. *Relationship between the reaction time and the fluorescence intensity, as well as the reaction time and the calculated number of bound glc units determined by MS data*

To examine the starting mechanism of the glycation reaction, the changes in the MS data within the first 72 h were studied (*Fig. 3*), and the covalent-bonding process was analyzed with the help of the corresponding fluorescence intensity changes (*Table*). The MS of the sample collected after 3 h showed a new peak of a glc-lysozyme adduct as a shoulder peak of the native lysozyme peak (*Fig. 3,a*). The peak profile remained unchanged during the first 3–9 h of the reaction. The MS spectrum of a sample taken after 24 h showed three peaks of lysozyme, glc-lysozyme, and $(glc)_2$-lysozyme, and subsequently, the n value of the $(glc)_n$-lysozyme adduct increased to 4 in the 24–72 h range of the reaction progress (*Fig. 3,d*).

^{13}C-NMR Analyses. The structure of the $(glc)_n$-lysozyme was examined by ^{13}C-NMR spectroscopy. In the glycation reaction, the aldehyde group at C(1) of glc (*Fig. 4*) participates in the reaction and is subjected to a series of reactions such as oxidation and transition (*Scheme*). The C(6) atom of glc is the sole CH_2 group in the molecule and seems to be least affected group in the glycation reaction. We decided to monitor the glycation reaction with D-[6-^{13}C]-glucose (^{13}C-glc; 10% ^{13}C-enriched), the enriched rate of which is *ca.* tenfold higher than that of the natural C(6) of glc (1.11% abundance). The glycation reaction between ^{13}C-glc and lysozyme was performed under the same conditions as described above. The ^{13}C-NMR spectrum for the sample collected after three weeks of reaction is shown in *Fig. 4*. Six ^{13}C-NMR signals were

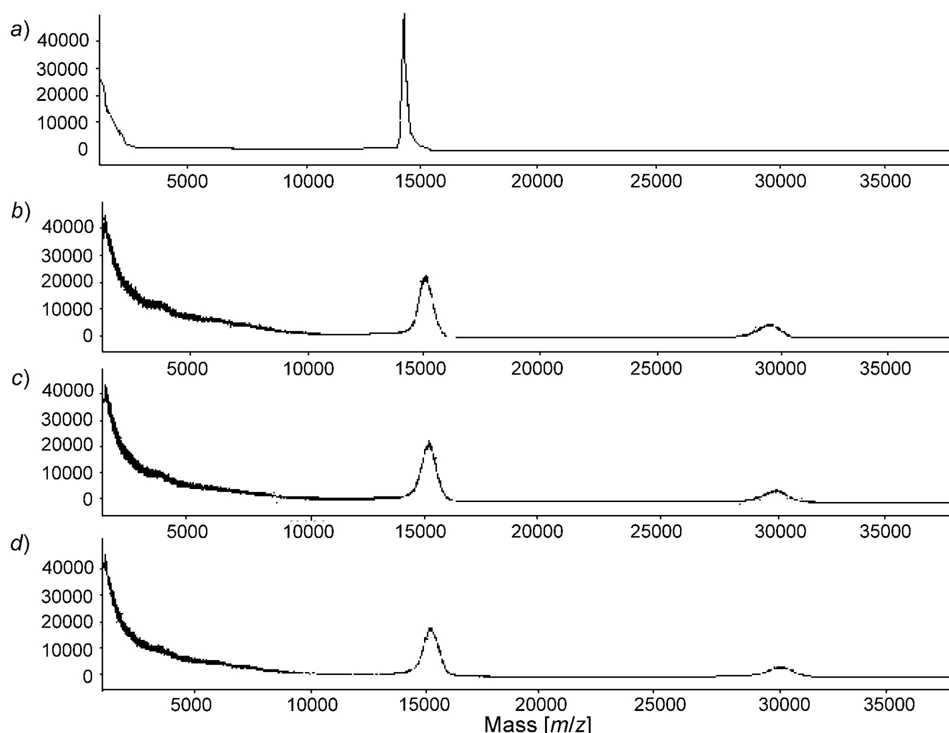


Fig. 2. *MALDI-TOF-MS Spectra of the reaction soln. of glucose and lysozyme* a) *at the beginning,* b) *after two weeks,* c) *after three weeks, and* d) after four weeks

observed in the region of 60–80 ppm and confirmed to be CH_2 groups by the DEPT spectral measurements (*Fig. 4,b* and *c*). Six CH_2 signals, 71.92, 69.72, 64.53, 64.15 (×2), and 60.63 ppm, of the $(glc)_n$-lysozyme adducts can be assigned to be CH_2 groups of ^{13}C-glc bound to different amino groups of lysozyme. This number of glc agrees with the result of the MALDI-TOF-MS experiments. The chemical shift of C(6) of the unreacted ^{13}C-glc is 64.70 ppm. It is obvious that the chemical shifts of the CH_2 groups can vary widely depending on the binding environment. This means that the structural environment of the glc in the AGE adduct of lysozyme could be estimated by these changes.

Conclusions. – It is known that the amino groups of lysine (Lys), arginine (Arg), and histidin (His) form *Schiff* bases with the aldehyde group of glucose [17–19]. The MALDI-TOF-MS spectra showed the AGE formation of nine glcs with lysozyme within nine weeks after starting the glycation reaction under physiological conditions, together with the formation of a dimer and trimer of $(glc)_n$–lysozyme adducts. This means that the agglutination reaction between proteins is interlocked with the protein glycation progress. The different chemical shifts of the $^{13}CH_2(6)$ groups in the $[^{13}C$-$glc]_n$–lysozyme adducts indicate the different spatial environments of amino acid residues that participate in the *Schiff* base formation with glc molecules. A linear

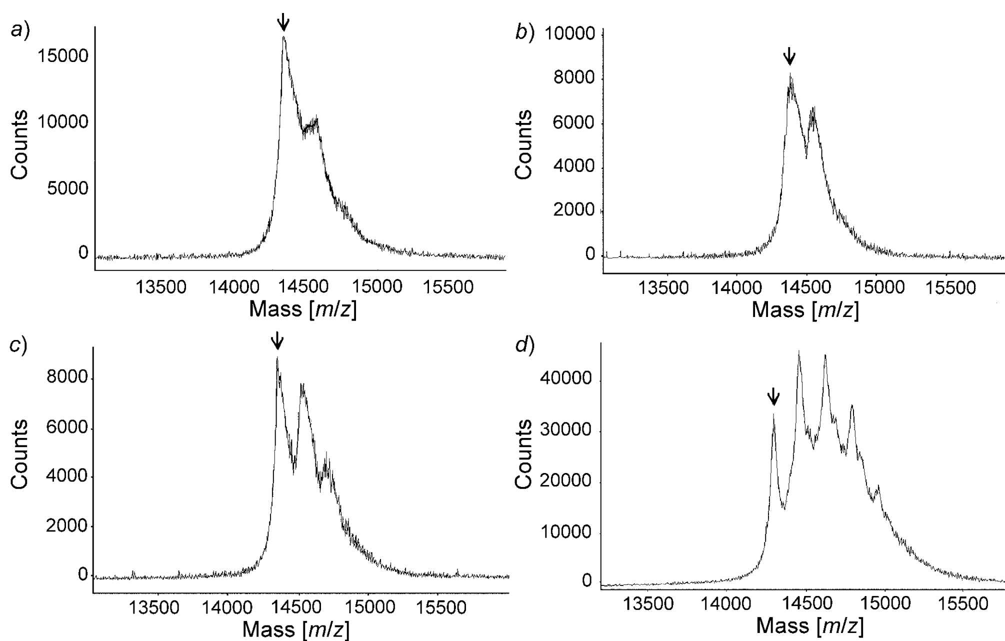


Fig. 3. *Reaction time-dependent MS spectra of glc and lysozyme solution,* a) *3 h,* b) *9 h,* c) *24 h, and* d) *72 h after starting the glycation reaction*. The arrow shows native lysozyme (*m*/*z* 14331).

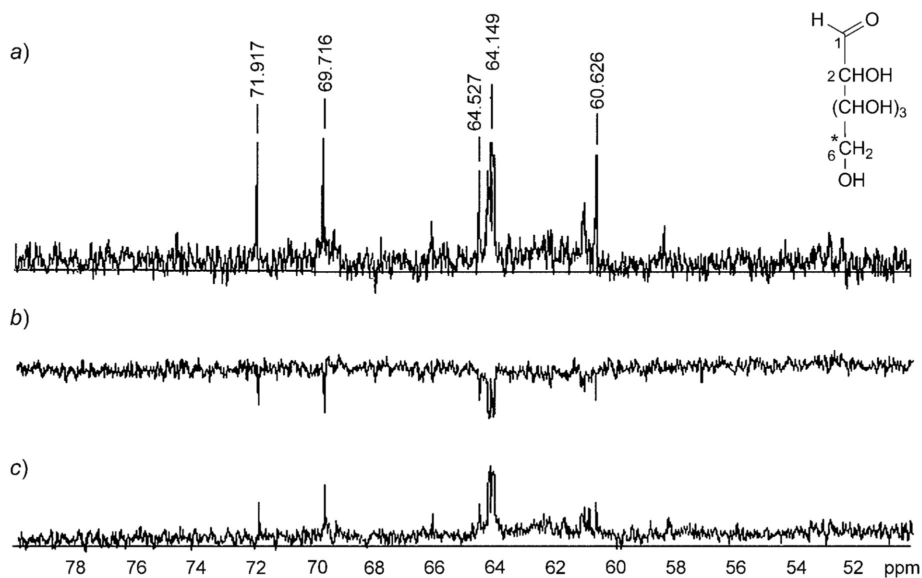


Fig. 4. *Chemical formula of* D-*[6-^{13}C]-glucose and partial ^{13}C-NMR spectra of the (*D-*[6-^{13}C]-glucose)$_n$-lysozyme sample of the glycation reaction collected after three weeks. a*) One-dimensional ^{13}C-NMR, DEPT; *b*) CH_2 down, CH/Me up, DEPT; *c*) all protonated C-atoms.

relation was observed between the increase of the fluorescence intensity and the number of attached glc molecules (*Fig. 1*). This indicates that the increase of the fluorescence intensity is mainly due to the restricted molecular motion of the $(glc)_n$–lysozyme adduct by increasing the sugar number. At present, the X-ray crystal-structure analysis of the glc–lysozyme adduct is in progress to clarify the structural situation at an atomic level and to serve for the design of a drug to suppress AGE generation.

It is believed that the glycation reaction proceeds for a long span of years. However, the present model study has shown for the first time that the glycation reaction progresses within several hours under physiological conditions at 37°. It was also confirmed that the combination of MALDI-TOF-MS and ^{13}C-NMR methods is very useful to analyze the glycation mechanism.

Experimental Part

Materials. Lysozyme (from chicken egg white; *Sigma*, USA), D-glucose, (guaranteed reagent: *Nacalai Tesque*, Japan), and D-[6-^{13}C]glucose (99%, *Cambridge Isotope Laboratories, Inc.*, USA) were used for this experiment. Lysozyme for the spectrum measurement was used after the recrystallization.

Recrystallization of Lysozyme. 1 ml of the aq. soln. of lysozyme (50 mg/ml) and 1 ml of the soln. of precipitant soln. (20 % NaCl and 50 mM acetate buffer) were mixed and let stand for 4 h in 4°. The crystals were then obtained by removing the supernatant.

Fluorescence Measurements of the Glycation Reaction. The formation of AGEs was measured with slight modifications for the operation developed by *Matsuura et al.* [20]. The soln. for the glycation reaction was prepared by dissolving lysozyme (0.4 g) and D-glucose (18 g) in phosphate buffer (49 ml, 50 mM, pH 7.4). The soln. (0.5 ml) was transferred to a micro glass tube and incubated for ten weeks at $37 \pm 0.2°$ using a high-precision metal bath (*MB-2L-U*, *Koike Precision Instruments*, Japan). The reaction soln. (0.2 ml) was diluted with alkaline PBS (1 ml, pH 10) [20]. A very small amount of insoluble precipitation was removed by centrifugal separation (30000*g*, 4 min, 4°). The supernatant soln. was used for the fluorescence measurement. The fluorescence intensity was measured with a *HITACHI F-2000* fluorometer (excitation: 360 nm, emission: 460 nm). The experiments were carried out 2–4 times at $N =$ 5. The AGE generation rate was calculated using the mean value of these experiments.

MALDI-TOF-MS Analysis. Each reaction soln. was purified by column chromatography (CC; *Hi Trap Capto MMC*, *GE Healthcare*, Sweden) in phosphate buffer (50 mM). The reaction soln. desalted by zip-tip (75% MeCN/0.1% TFA) was subjected to MALDI-TOF-MS (*Voyager-DE PRO*) analysis. The measurements were carried out 2–4 times for each sample. The mean value was used for the calculation of the number of bound glc units.

^{13}C-NMR Analysis. The ^{13}C-NMR spectra were measured on *Varian VNMRS-500* (^{13}C: 125.7 MHz). D-[6-^{13}C]glucose (10% enriched) and lysozyme were incubated under the same conditions as those of the glycation reaction, except for H_2O instead of phosphate buffer. The reaction soln. was purified by CC with *Hi Trap Capto MMC* (50 mM phosphate buffer). The protein fraction was then concentrated to 31 mg/ml (2000*g*, 3 h, 4°). The ^{13}C-NMR measurements of the protein (600 µg in 750 µl H_2O) were carried out by adding D_2O (25%) for the field-frequency rock.

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