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Original article

Direct proof of the deglycating effect of rosmarinic acid on a glycated protein

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Abstract

***Purpose*:** It was shown in a previous work that rosmarinic acid is able to break AGE cross-links. This study focuses on the early stage of glycation: the objective is to highlight the ability of rosmarinic acid to deglycate glycated lysozyme and see to what extent the protein is recovered in its original state.
***Basic procedures*:** Purified lysozyme is glycated with ribose. After elimination of ribose by purification, glycated lysozyme is treated with rosmarinic acid. Glycation and deglycation are monitored over time by ESI-MS analysis.
***Main findings*:** After 168 hours, the glycation leads to the binding of at least three molecules of ribose to the amino radicals of lysozyme forming Schiff bases. The deglycation test shows that glycation is a naturally reversible reaction. The presence of rosmarinic acid leads to a faster deglycation reaction.
***Principal conclusions*:** This study shows that rosmarinic acid has an important deglycating effect on a glycated protein. Further studies are required to better understand the mechanism of the deglycation process involving glycated lysozyme and rosmarinic acid.

***KEY WORDS*:** glycation, deglycation, rosmarinic acid, lysozyme

Introduction

Glycation is a non-enzymatic reaction between carbohydrates and molecules with free amino groups, such as proteins. At first, a Schiff base is produced while a molecule of water is discarded. Later, the irreversible isomerization of the Schiff base leads to more stable products: the Heyns-Carson products which are formed from ketoses and the Amadori products formed from aldoses [1].

At later stages, additional reactions occur on Amadori products, such as cleavage or cyclization. Those reactions lead to the formation of a heterogeneous group of compounds called Advanced Glycation End products (or AGEs) [1]. This process involves several oxidative phenomena [2].

AGEs can be found in all body tissues and fluids. They are able to cross-link with other proteins, altering their functionalities [3]. They can also alter gene expression, intracellular signaling and provoke complications related to diabetes or to aging such as Alzheimer's disease [4], cardiovascular disease [5] or osteoporosis [6].

Research in AGE prevention has led to the discovery of several AGE inhibitors. Some of these inhibitors are antioxidant [7,8] and carbonyl trapping agents [9,10]. Beyond AGE inhibitors, other molecules have been claimed to be able to reverse glycation such as thiazolium compounds [11] or rosmarinic acid [12]. Concerning the latter, a previous study suggested its ability to disrupt AGE cross-links as well as its potential use in preventing functional and structural damage to proteins [13].

Lysozyme is an antimicrobial enzyme essential to the innate immune system [14]. Its small molecular weight makes it better suited to analyze by mass spectrometry. Several reports evaluated the dissociation of lysozyme from dimer to monomer as AGE degradation [15]. But to our best knowledge, no one has studied deglycation in the initial stage.

In this work, a mass spectrometry method was developed to study the ability of rosmarinic acid to reverse glycation at its early steps and to what extent it could return glycated lysozyme back to its original state.

Materials and methods

Reagents and materials are as follows: lysozyme from chicken egg white (Sigma Aldrich, reference 62971), ribose (Sigma Aldrich, reference R7500), acetic acid (Sigma Aldrich, reference 45754), ammonium hydroxide (Sigma Aldrich, reference 221228), deionized water, sephadex® G-50 (Sigma Aldrich, reference G5080) and rosmarinic acid (Sigma-Aldrich, reference 536954).

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Preparation of glycated lysozyme at pH 6

Step 1: Lysozyme is purified by Reveleris® PREP Purification System on Sephadex® G50 in deionized water. Fractions corresponding to pure lysozyme are detected (see *Analysis*), collected and freeze-dried.
Step 2: A 2.000 % (w/w) solution of lysozyme purified in step 1 and ribose 0.500 % (w/w) is prepared in ammonium acetate buffer at pH 6. The mixture is then left for 168 hours (h) in a drying oven at 37 °C.
Step 3: The glycation reaction is stopped through the elimination of excess ribose by Reveleris® PREP Purification System on Sephadex® G50 in deionized water. Fractions corresponding to glycated lysozyme are detected (see *Analysis*), collected and freeze-dried.

Deglycation test

A 1.000 % (w/w) solution of purified glycated lysozyme in ammonium acetate buffer at pH 6 and a 0.017 % (w/w) solution of rosmarinic acid in MeOH/ammonium acetate buffer at pH 6 (8:92) are prepared.
Blank sample: 1 mL of purified glycated lysozyme solution is added to 1 mL of solvent MeOH/ammonium acetate buffer at pH 6 (8 :92).
Deglycation sample: 1 mL of purified glycated lysozyme solution is added to 1 mL of rosmarinic acid. Mixtures are left for 720 h in a drying oven at 37 °C.

Analysis

Lysozyme glycation and deglycation as well as purification steps are analyzed by mass spectrometry on a Q-Tof-2™ instrument (Micromass, Waters) with an electrospray ion source. Nitrogen is used as the nebulizer gas and argon as the collision gas at 7 bars and 2 bars respectively. Analytical conditions are as follow: The source temperature is 80 °C and 150 °C for the desolvation. The collision energy, cone and capillary voltage are set at 10 eV, 35 V and 2.5 kV, respectively. The acquisition range is 90-3,000 m/z in positive ion mode. Samples are analyzed by direct injection with a syringe pump (AL-300, WPI) at a flow rate of 15 µL/min. Masslynx v4.0 software is used to control the instrument, for data acquisition and processing.

Results and discussion

After the purification of commercial lysozyme (step 1), the ESI-MS spectrum obtained by deconvolution of the multiple ions shows a major pic of m/z = 14,302 (theoretical mass: single-chain 14,300 Da, Mr ~ 14,600) (***Fig. S1***). Then, glycation between lysozyme and ribose was carried out as described above. To confirm that the reaction took place, mass spectrometry analysis is performed to detect the presence of glycated lysozyme. These analyses are performed over time at 0 h, 24 h, 48 h, 72 h and 168 h (***Fig. S2***). The mass spectra show an increase in the number of peaks, corresponding to the covalent attachment of ribose to the different amino sites of lysozyme. After purification step 3, at least three molecules of ribose have attached themselves to the amino radicals of lysozyme forming Schiff bases (***Fig. S3***). For more accurate results, the eightfold ion was used for the calculation of the added mass on the protein. The average added mass is m/z = 132.1691 (theoretical mass of ribose: 150.0578 Da, minus 18.0105 Da, H_2O = 132.0473) (***Table 1***).

After the elimination of ribose (purification step 3) to stop the glycation reaction, a deglycation test is performed. In order to assess the impact of rosmarinic acid on glycated lysozyme, mass spectrometry analyses are carried out over time and compared with the blank sample (***Fig. S4***). The mass spectrum of the blank sample (***Fig. S4-a***) generates ion signals m/z ≈ 1,806, m/z ≈ 1,822 and m/z ≈ 1,839 corresponding to sites of glycated lysozyme. For more accurate results, the ion m/z ≈ 1,806 corresponding to the first site of glycation was chosen. ***Table 2*** shows the evolution of intensity ratios between m/z ≈ 1,806 and m/z ≈ 1,789 (which correspond to glycated lysozyme and initial lysozyme respectively).

In both cases, ratios decrease over time. The blank sample shows that glycation is a naturally reversible reaction. The presence of rosmarinic acid leads to a deglycation rate which is 54.24 % higher than in the blank sample at 192 h (***Fig.1***). At 168 h, regarding the structure, initial protein state is nearly recovered (***Fig. S5***). The recovery of enzyme activity will be studied in future works.

Table 1. Calculation showing the addition of ribose on lysozyme after 168 hours.

Mass from Fig. S3 (Da)	1789.4940	1806.0155	1822.5253
	1806.0155	1822.5253	1839.0574
Mass difference (Da)	16.5215	16.5098	16.5321
Deconvolution (ion octuple) (Da)	132.1720	132.0784	132.2568
Average (Da)	132.1691		
Theoretical mass	132.0473		

Analyzed by mass spectrometry on a Q-Tof-2TM instrument (Micromass) with an electrospray ion source. For measurement conditions, see the "Analysis" section of the text.

Table 2-a. Deglycation test: peak intensity evolution over time for blank sample.

Time (hours)	Intensity of m/z ≈ 1789	Intensity of m/z ≈ 1806	Ratio
0	1813	1856	1.02
24	1862	1275	0.68
48	1752	915	0.52
72	2291	994	0.43
168	2325	609	0.26
192	2156	520	0.24
240	2218	476	0.21
336	1911	357	0.19
408	2487	424	0.17
552	2015	323	0.16
720	1476	211	0.14

Analyzed by mass spectrometry on a Q-Tof-2TM instrument (Micromass) with an electrospray ion source. For measurement conditions, see the "Analysis" section of the text.

Table 2-b. Deglycation test: peak intensity evolution over time for deglycation sample.

Time (hours)	Intensity of m/z ≈ 1789	Intensity of m/z ≈ 1806	Ratio
0	1480	1510	1.02
24	1709	947	0.55
48	1735	606	0.35
72	2068	517	0.25
168	2161	265	0.12
192	2084	230	0.11
240	1623	181	0.11
336	1749	174	0.10
408	1415	159	0.11
552	1473	169	0.11
720	647	86	0.13

Analyzed by mass spectrometry on a Q-Tof-2TM instrument (Micromass) with an electrospray ion source. For measurement conditions, see the "Analysis" section of the text.

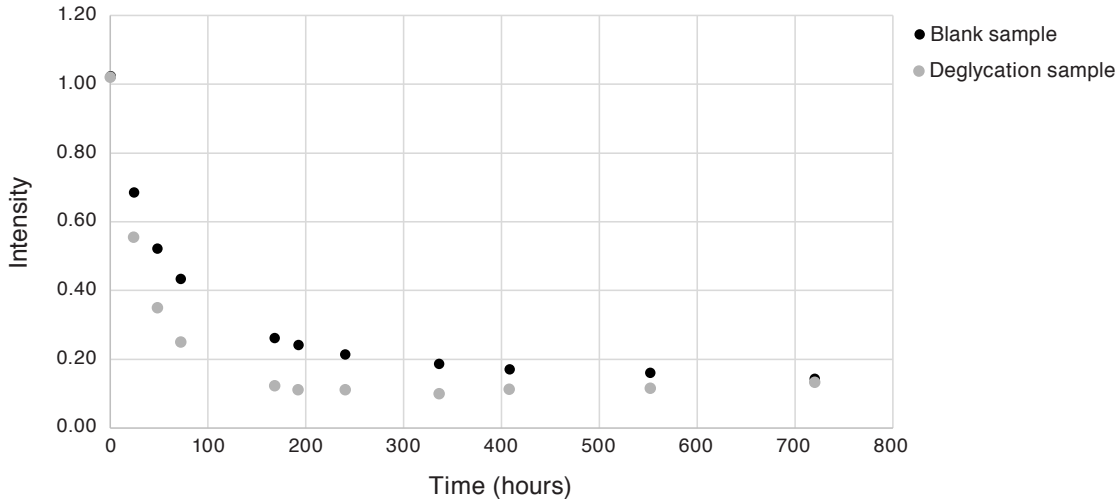

Fig. 1. Deglycation test: Ratios evolution between m/z ≈ 1789 and m/z ≈ 1806 over time.

Analyzed by mass spectrometry on a Q-Tof-2TM instrument (Micromass) with an electrospray ion source. For measurement conditions, see the "Analysis" section of the text.

On the deglycation mass spectra with rosmarinic acid, the ion m/z ≈ 1,834 appears (***Fig.S4-k***). By subtracting the eightfold ion m/z ≈ 1,789 from m/z ≈ 1,834, it leads to m/z ≈ 45. Multiplying this mass by 8 leads to m/z ≈ 360 which correspond to the rosmarinic acid molecular weight. Thereby, the mass m/z ≈ 1,834 might be the eightfold ion of a complex or an adduct between lysozyme and rosmarinic acid. Further studies using chromatographic methods are required to explore this hypothesis.

Conclusion

Using a new simple mass spectrometry method we provide a direct proof of a deglycation reaction on a glycated protein. Indeed, our analysis clearly showcases a reversion of glycation reaching a stationary level after 720 h. Moreover, the impact of rosmarinic acid on the glycated lysozyme is also highlighted: rosmarinic acid leads to a faster deglycation reaction than what naturally occurs. This suggest that rosmarinic acid might play a major role in AGE regulation, going further than previously discovered AGE inhibitors. Our future work will focus on the interaction between glycated lysozyme and rosmarinic acid to better understand the mechanism underlying the deglycation process involving this compound, and on the recovery of enzyme activity.

Conflicts of interest statement

Part of this work was supported by A2P Sciences sas, Lyon, France, as part of its "Glycation and Healthy Longevity" research project.

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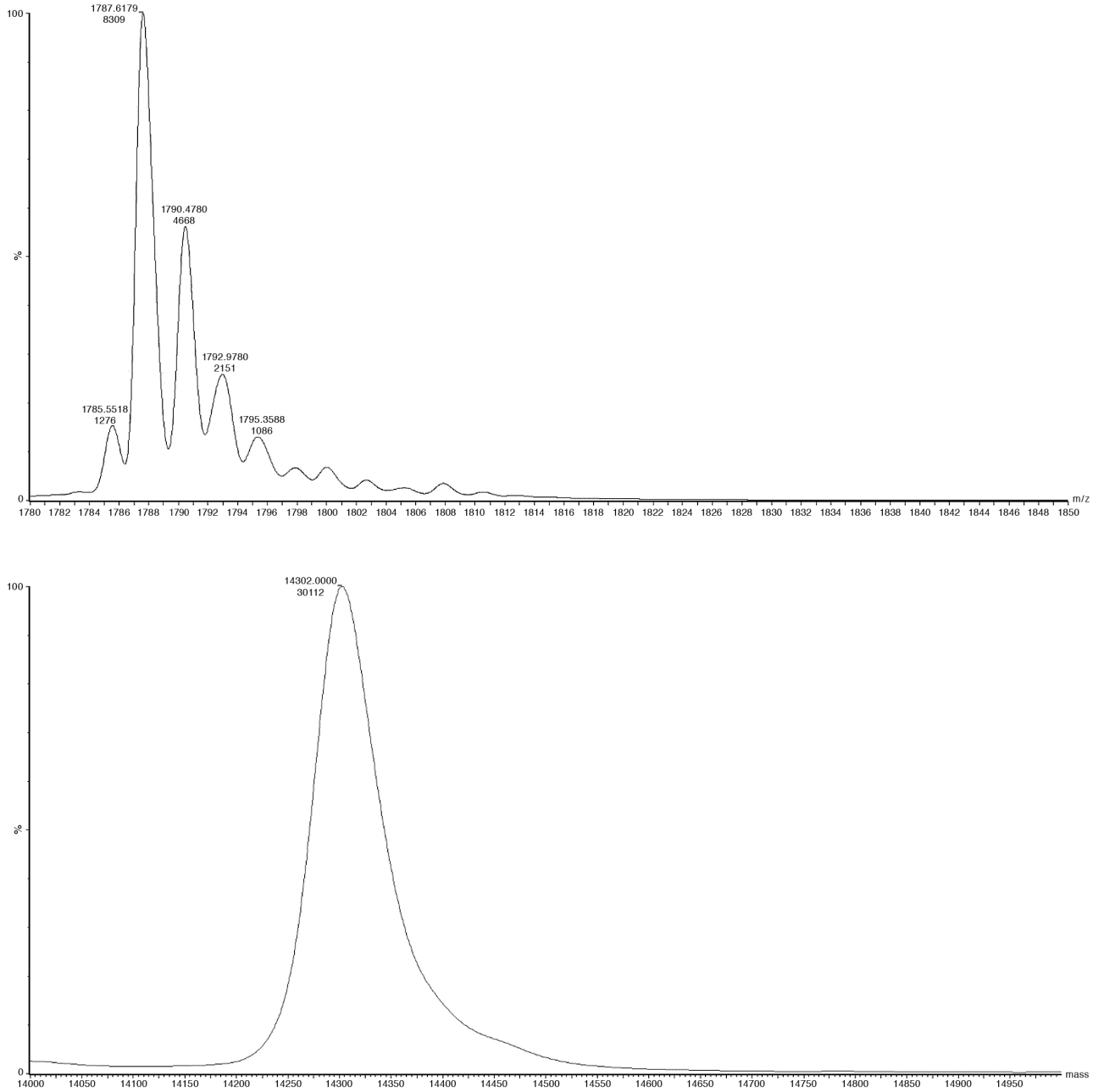

Fig. S1. **ESI-MS spectra (octuple ion) and deconvolved ESI-MS spectra of lysozyme after purification. Molecular weight: 14302.**

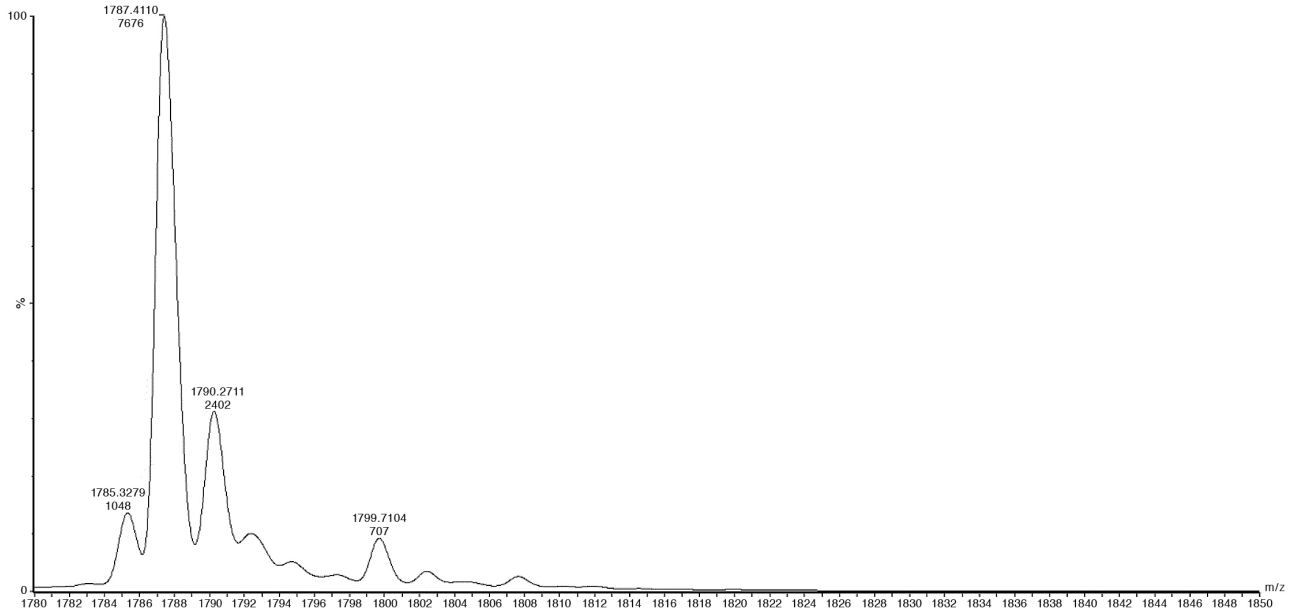

Fig. S2-a. **ESI-MS spectra of lysozyme after reacting with ribose for 0 hour.**

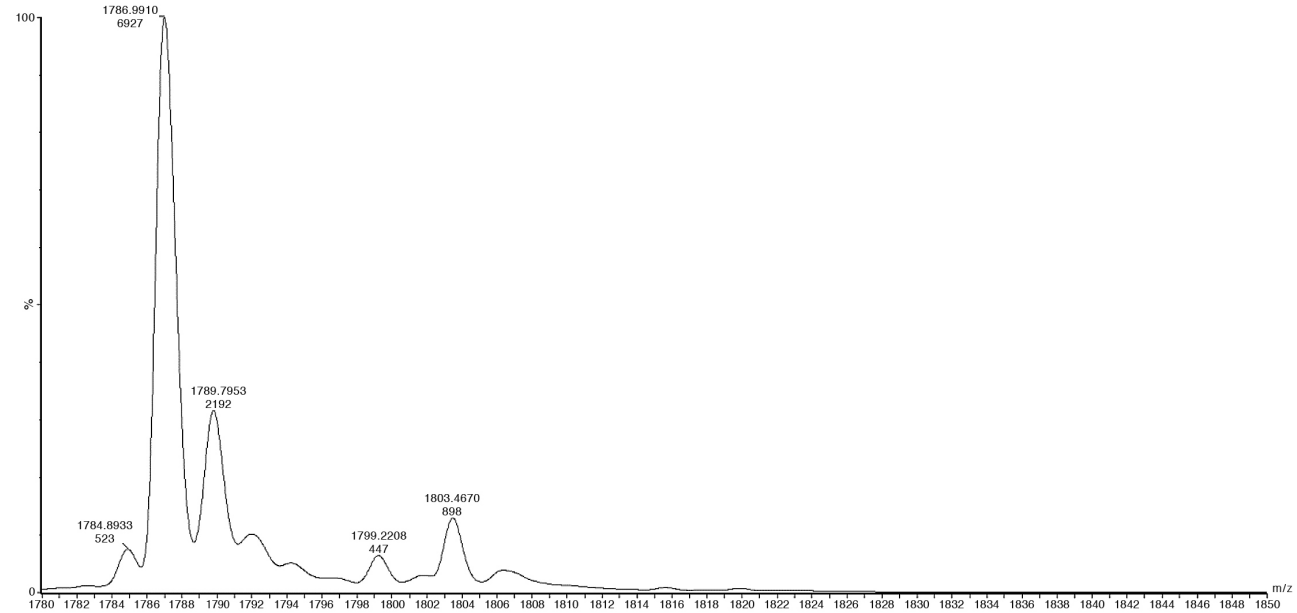

Fig. S2-b. **ESI-MS spectra of lysozyme after reacting with ribose for 24 hours.**

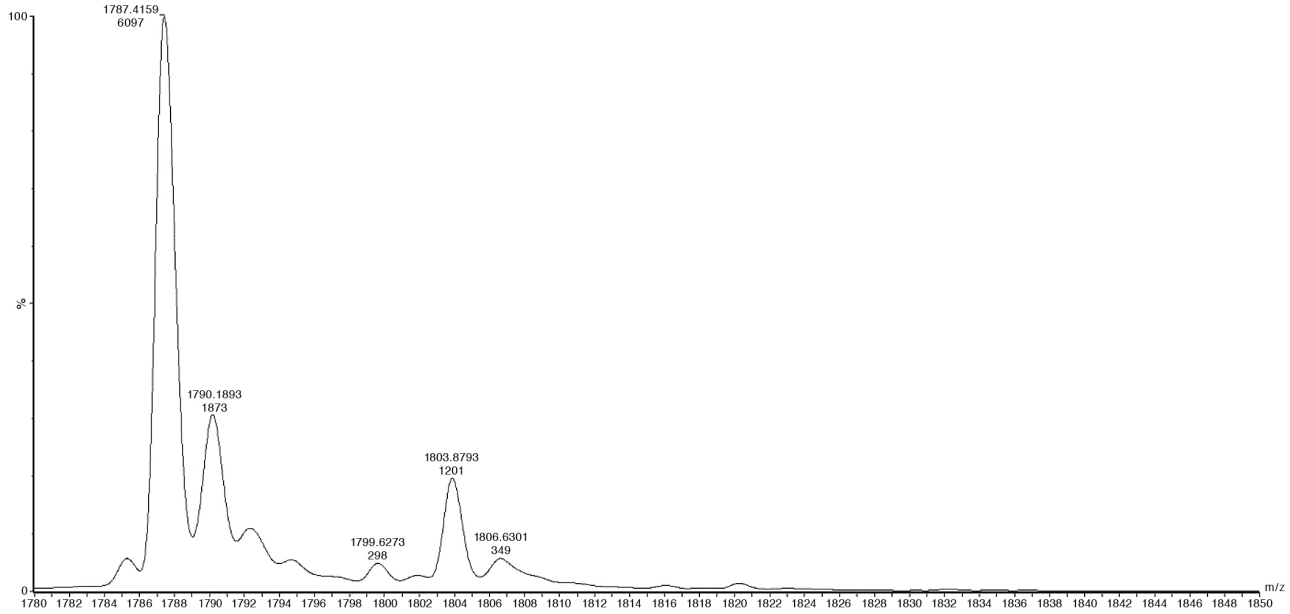

Fig. S2-c. **ESI-MS spectra of lysozyme after reacting with ribose for 48 hours.**

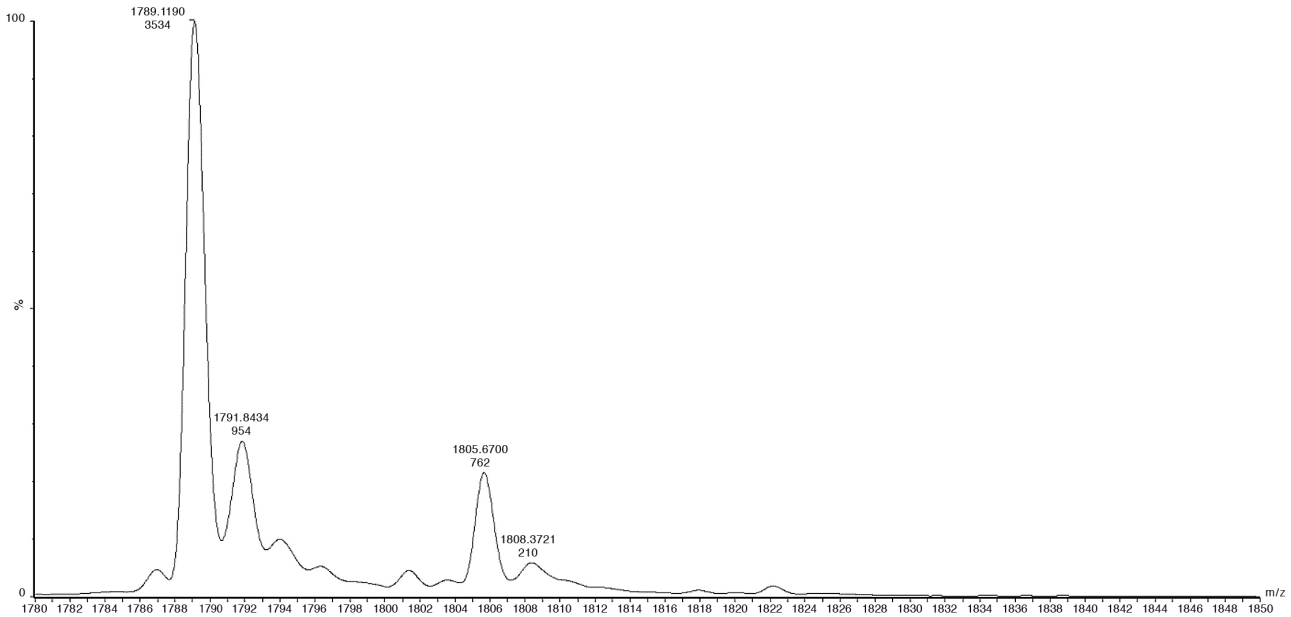

Fig. S2-d. **ESI-MS spectra of lysozyme after reacting with ribose for 72 hours.**

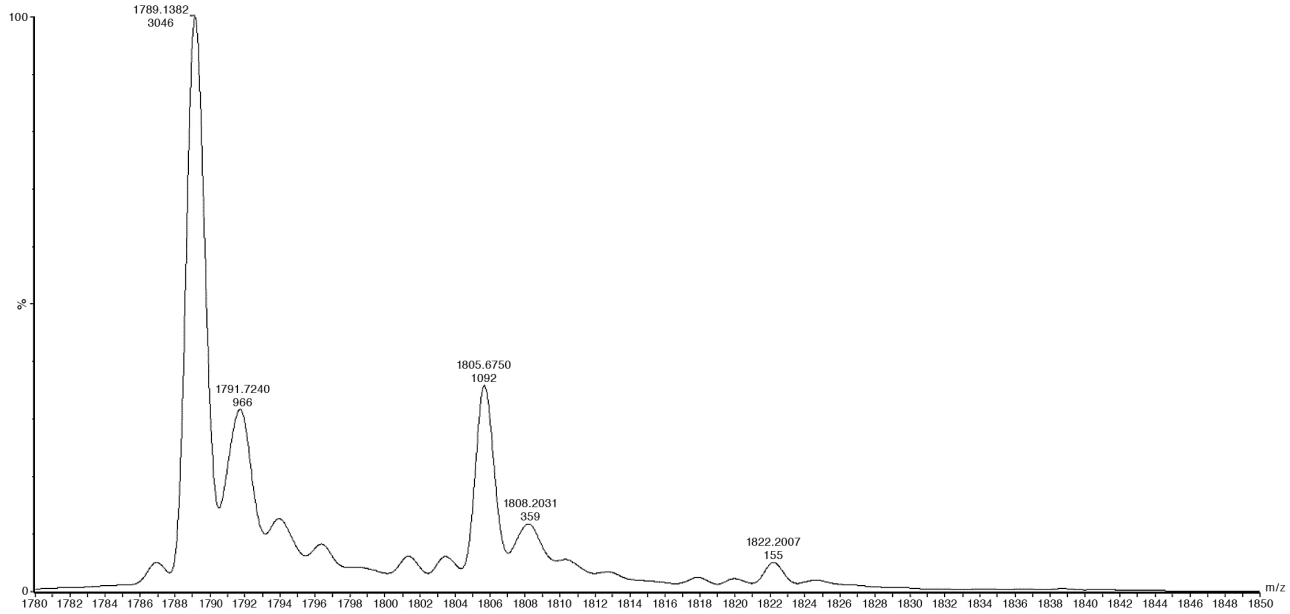

Fig. S2-e. **ESI-MS spectra of lysozyme after reacting with ribose for 168 hour.**

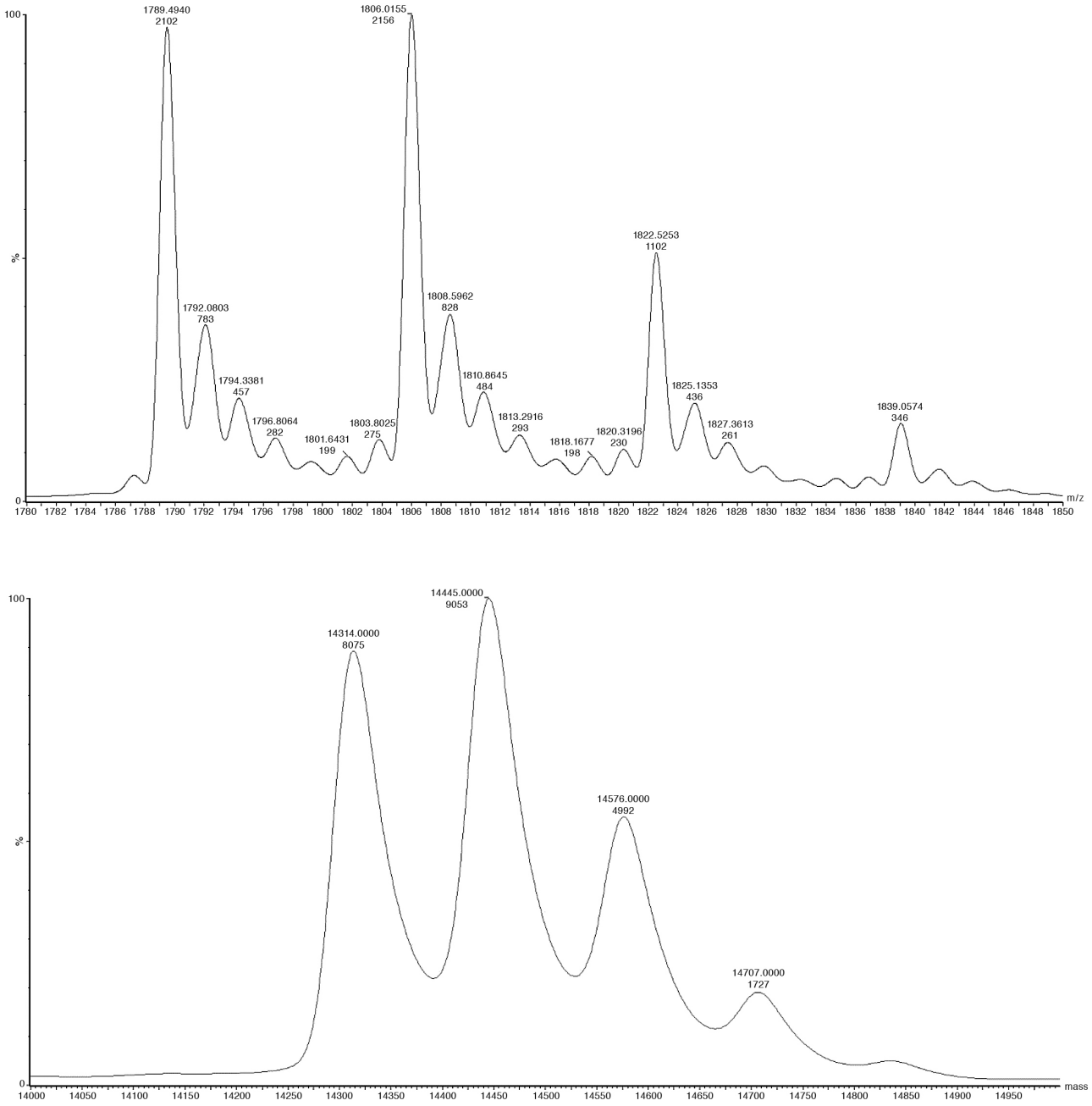

Fig. S3. **ESI-MS spectra (octuple ion) and deconvolved ESI-MS spectra of glycated lysozyme after purification.**

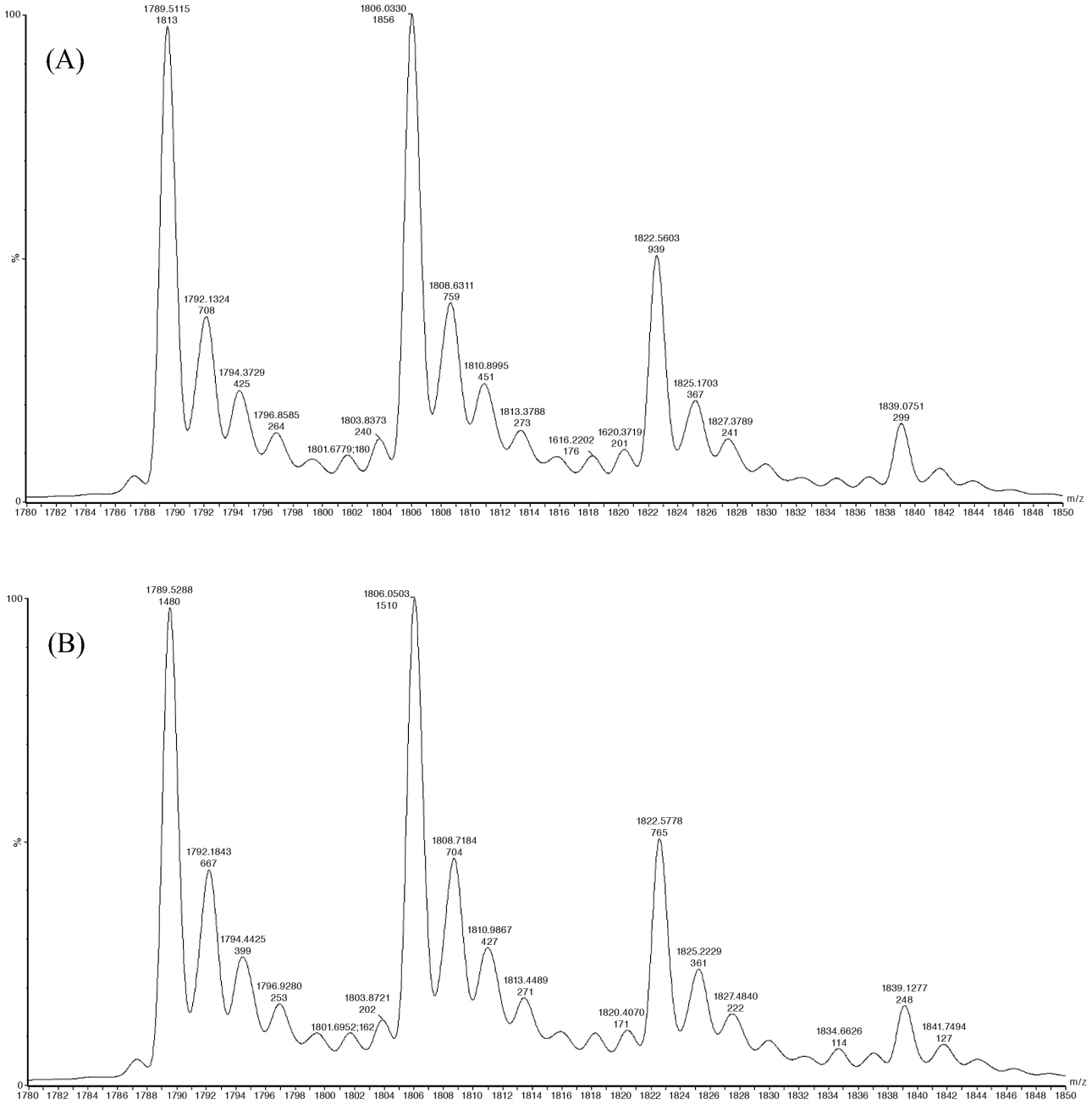

Fig. S4-a. **ESI-MS spectra of deglycation test on glycated lysozyme at 0 hour: (A) blank sample and (B) deglycation sample.**

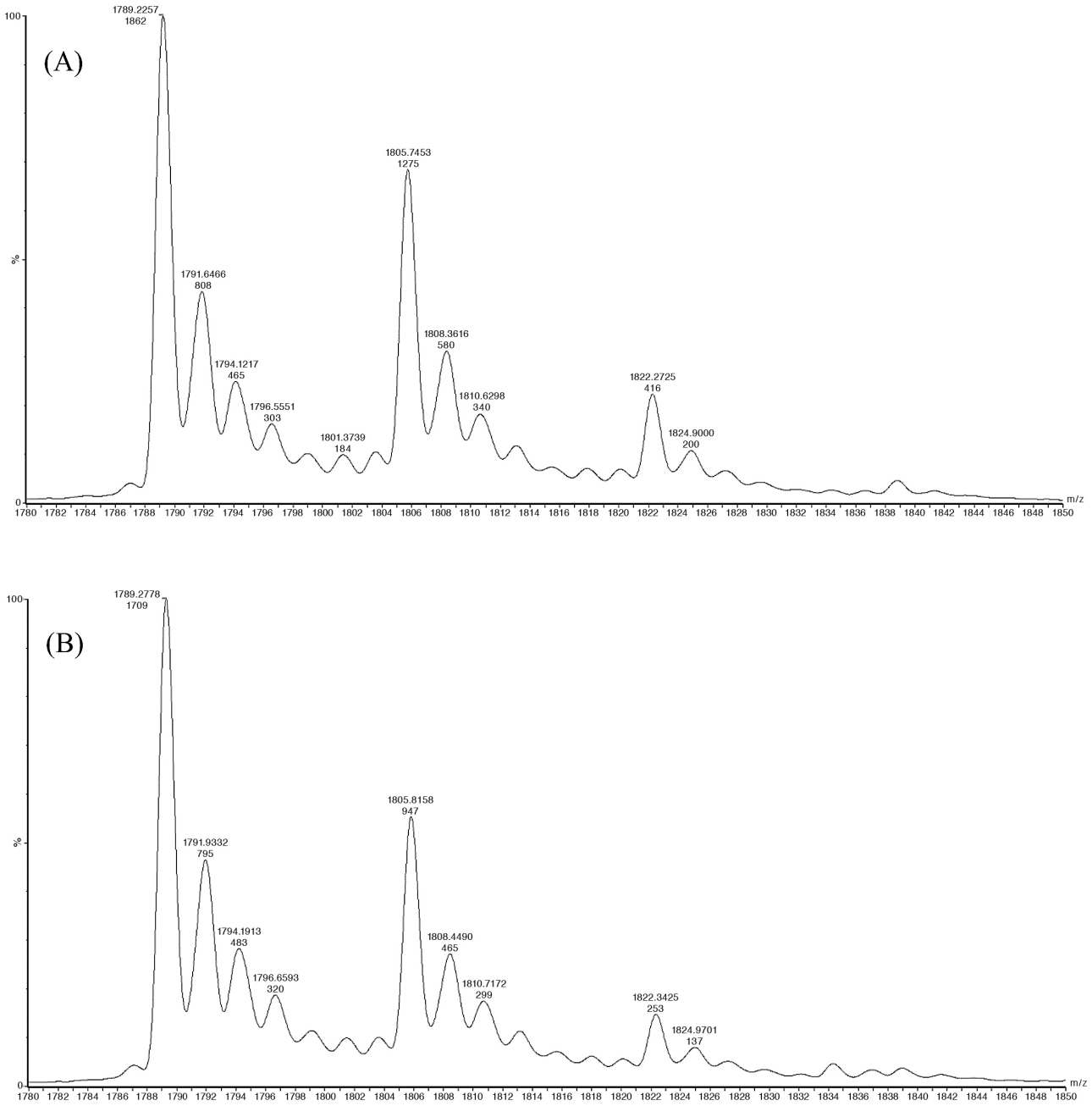

Fig. S4-b. **ESI-MS spectra of deglycation test on glycated lysozyme at 24 hours: (A) blank sample and (B) deglycation sample.**

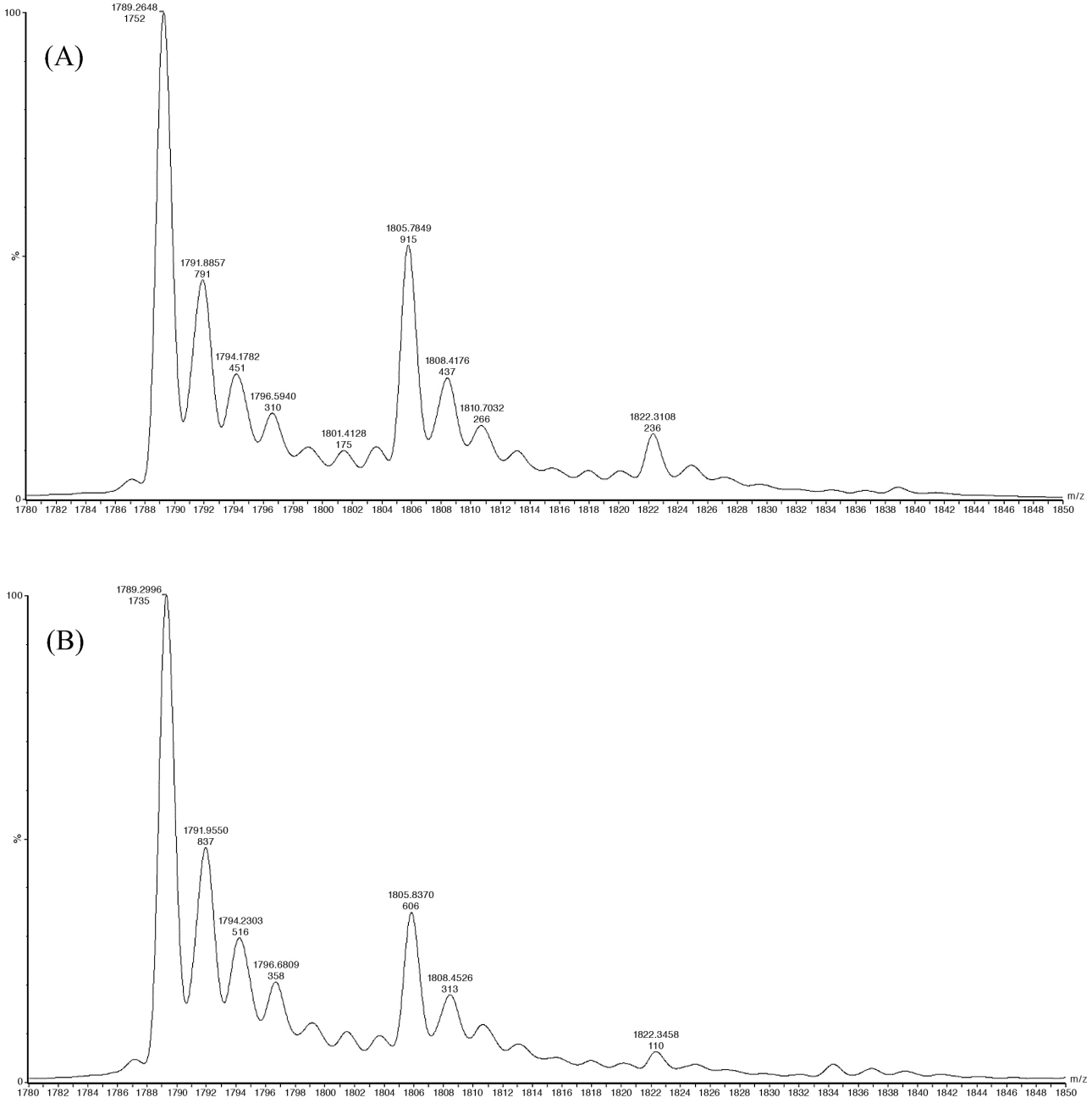

Fig. S4-c. **ESI-MS spectra of deglycation test on glycated lysozyme at 48 hours: (A) blank sample and (B) deglycation sample.**

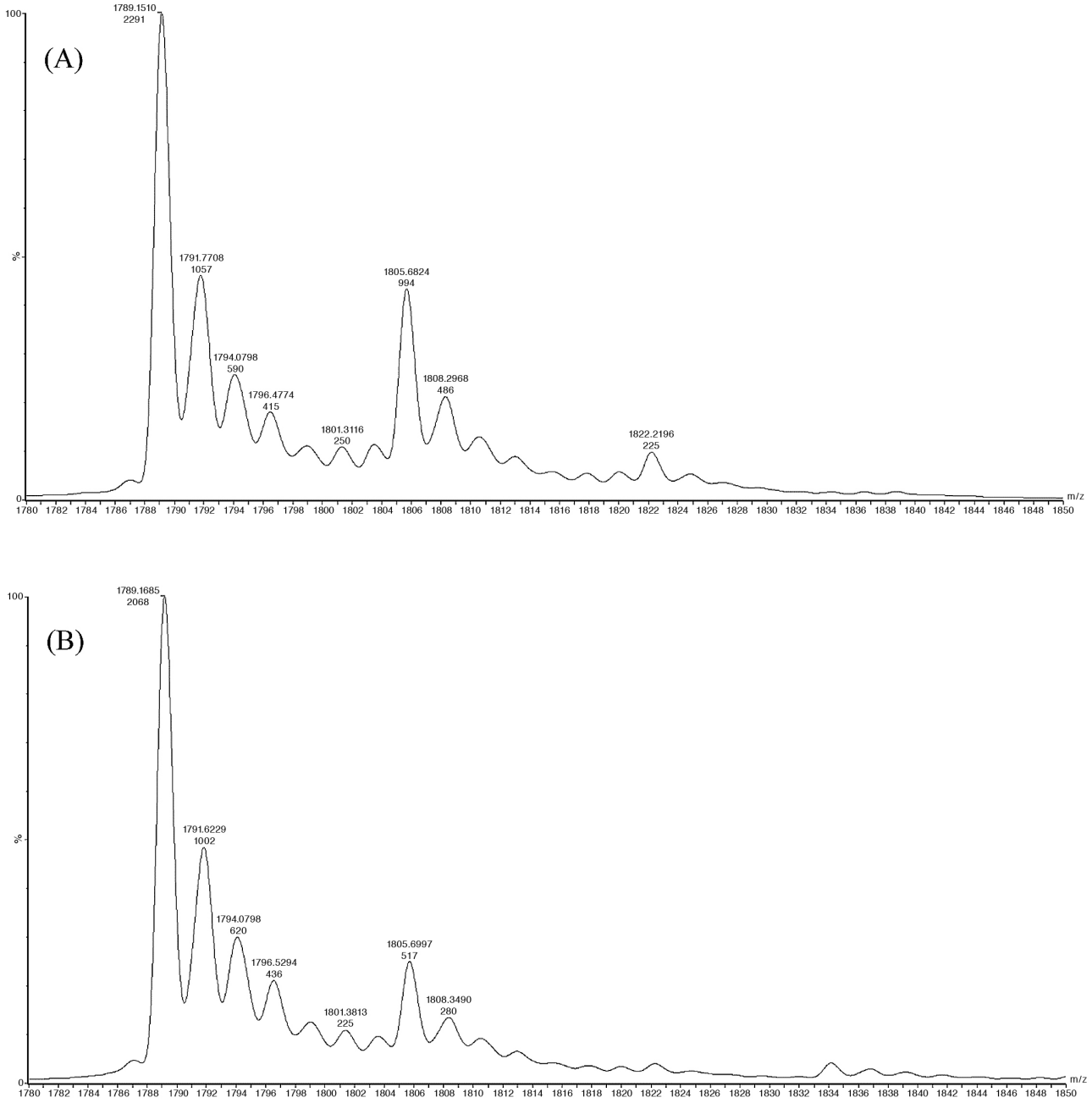

Fig. S4-d. **ESI-MS spectra of deglycation test on glycated lysozyme at 72 hours: (A) blank sample and (B) deglycation sample.**

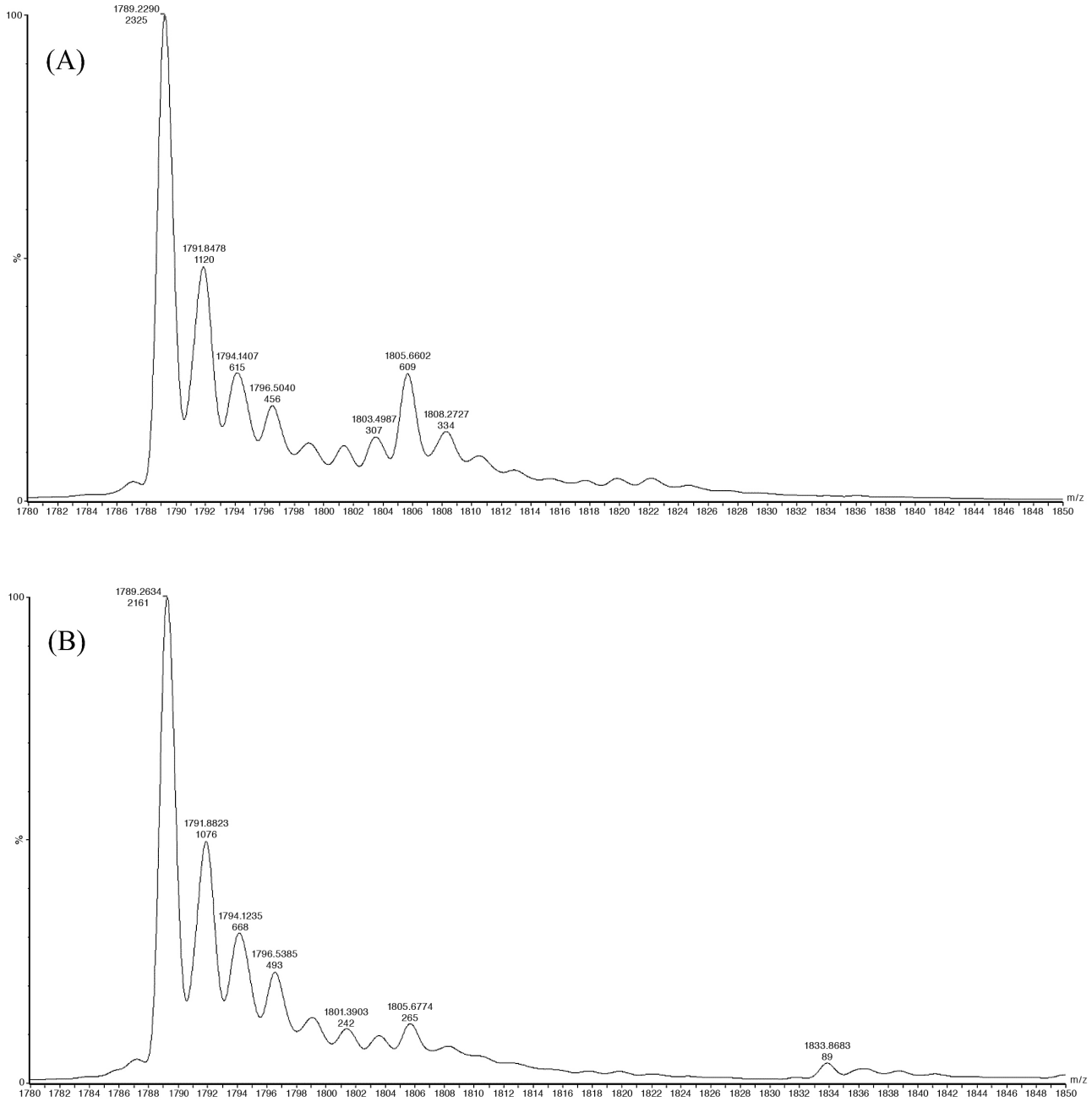

Fig. S4-e. **ESI-MS spectra of deglycation test on glycated lysozyme at 168 hours: (A) blank sample and (B) deglycation sample.**

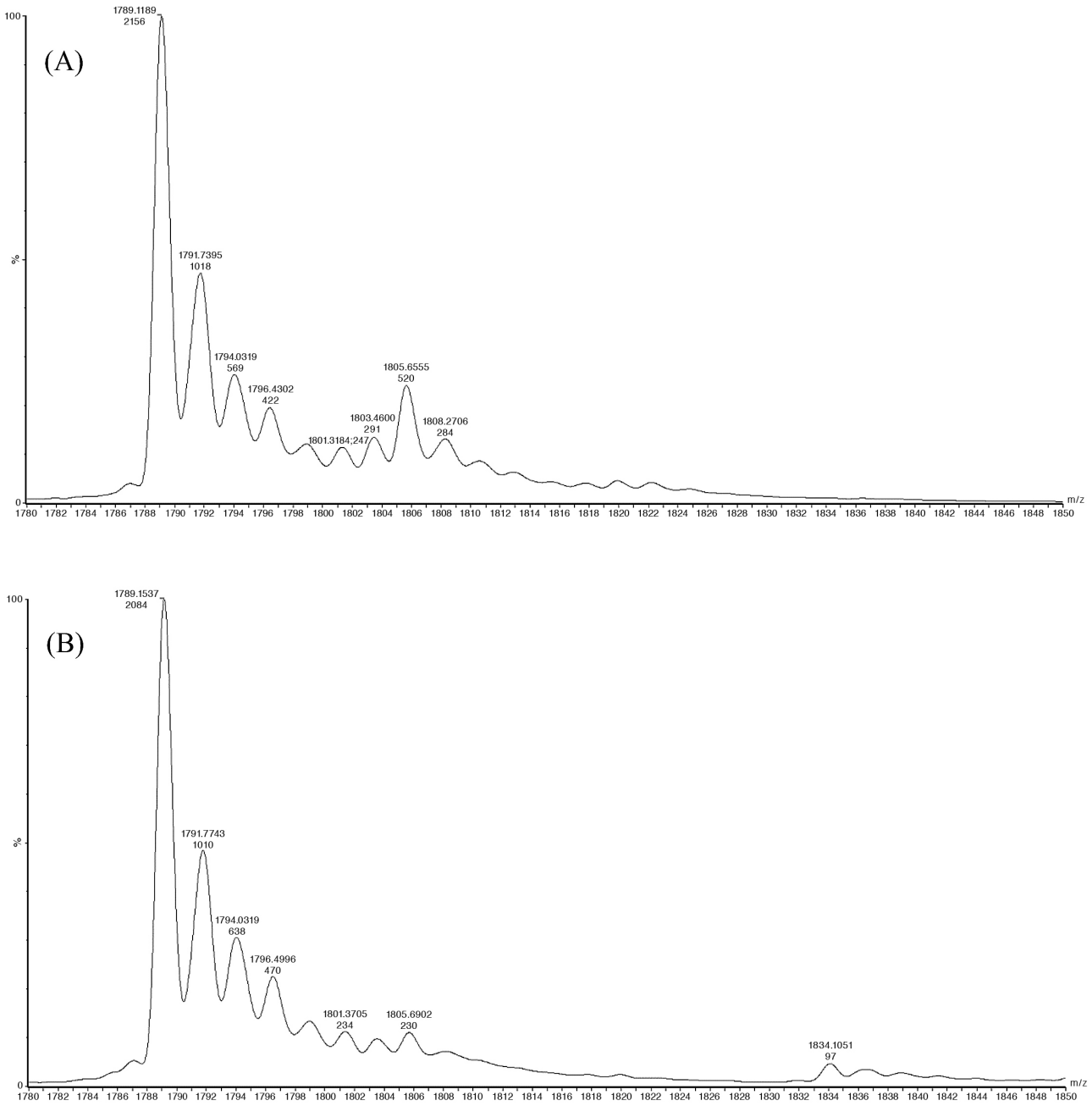

Fig. S4-f. **ESI-MS spectra of deglycation test on glycated lysozyme at 192 hours: (A) blank sample and (B) deglycation sample.**

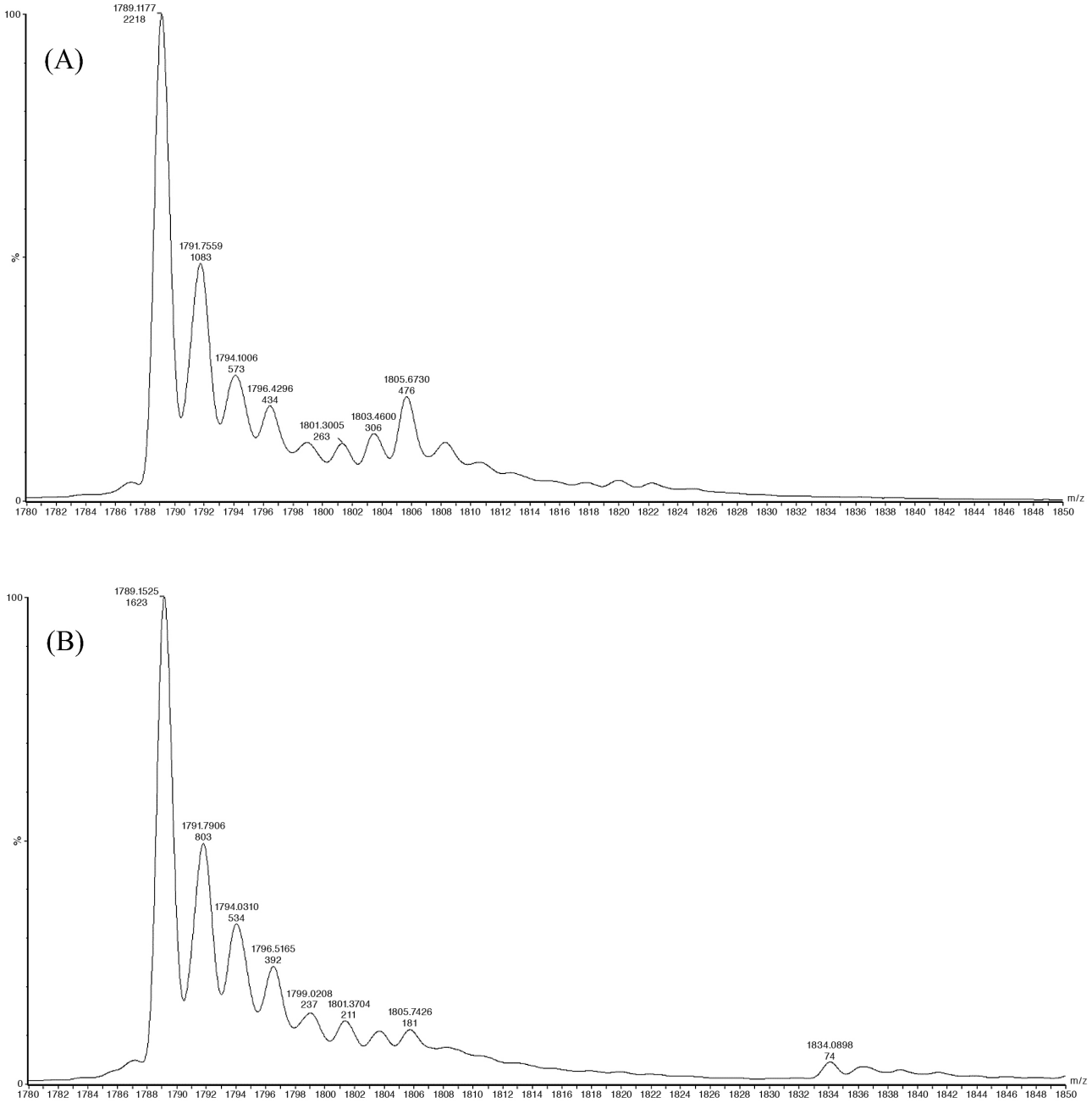

Fig. S4-g. **ESI-MS spectra of deglycation test on glycated lysozyme at 240 hours: (A) blank sample and (B) deglycation sample.**

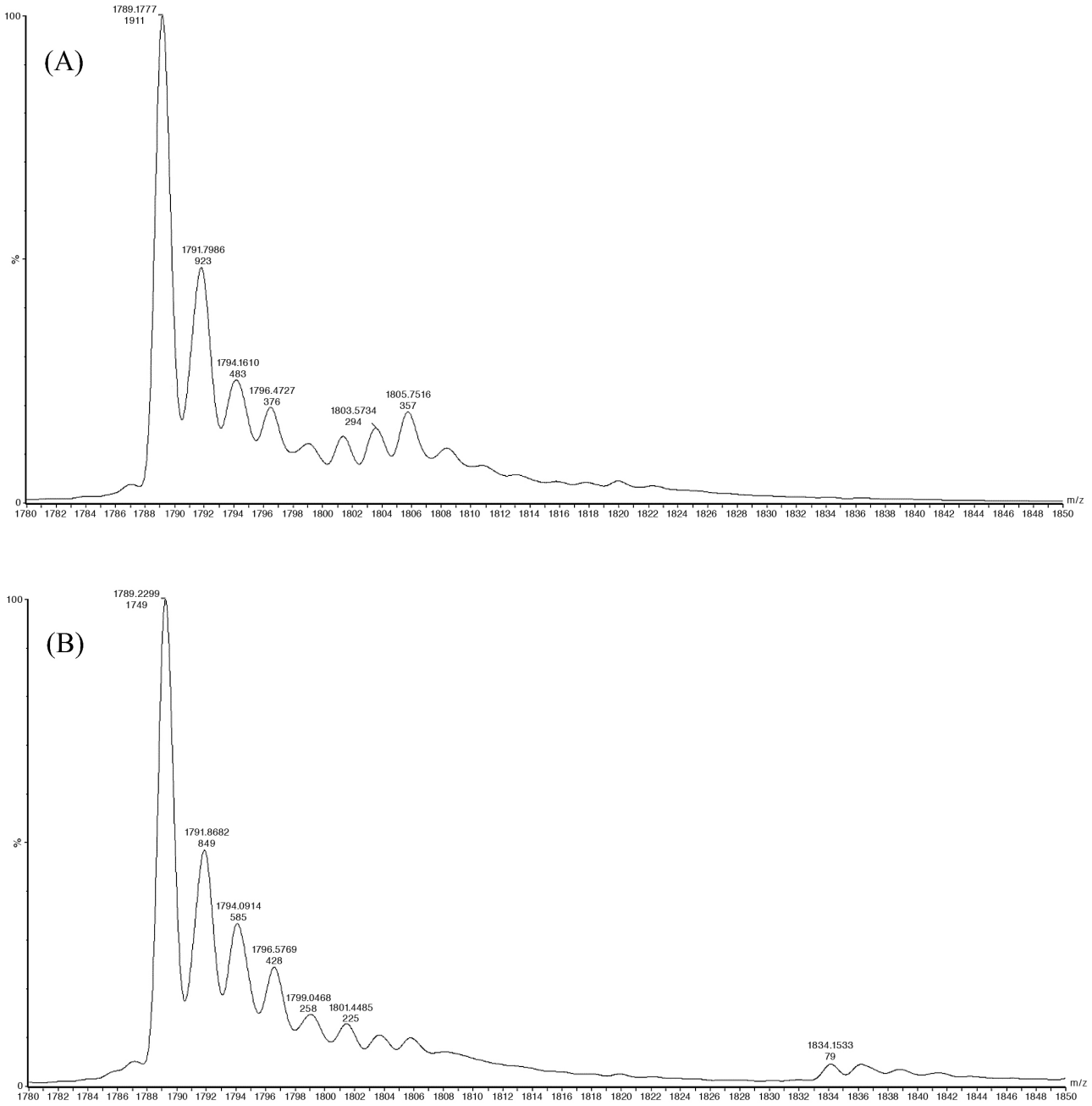

Fig. S4-h. **ESI-MS spectra of deglycation test on glycated lysozyme at 336 hours: (A) blank sample and (B) deglycation sample.**

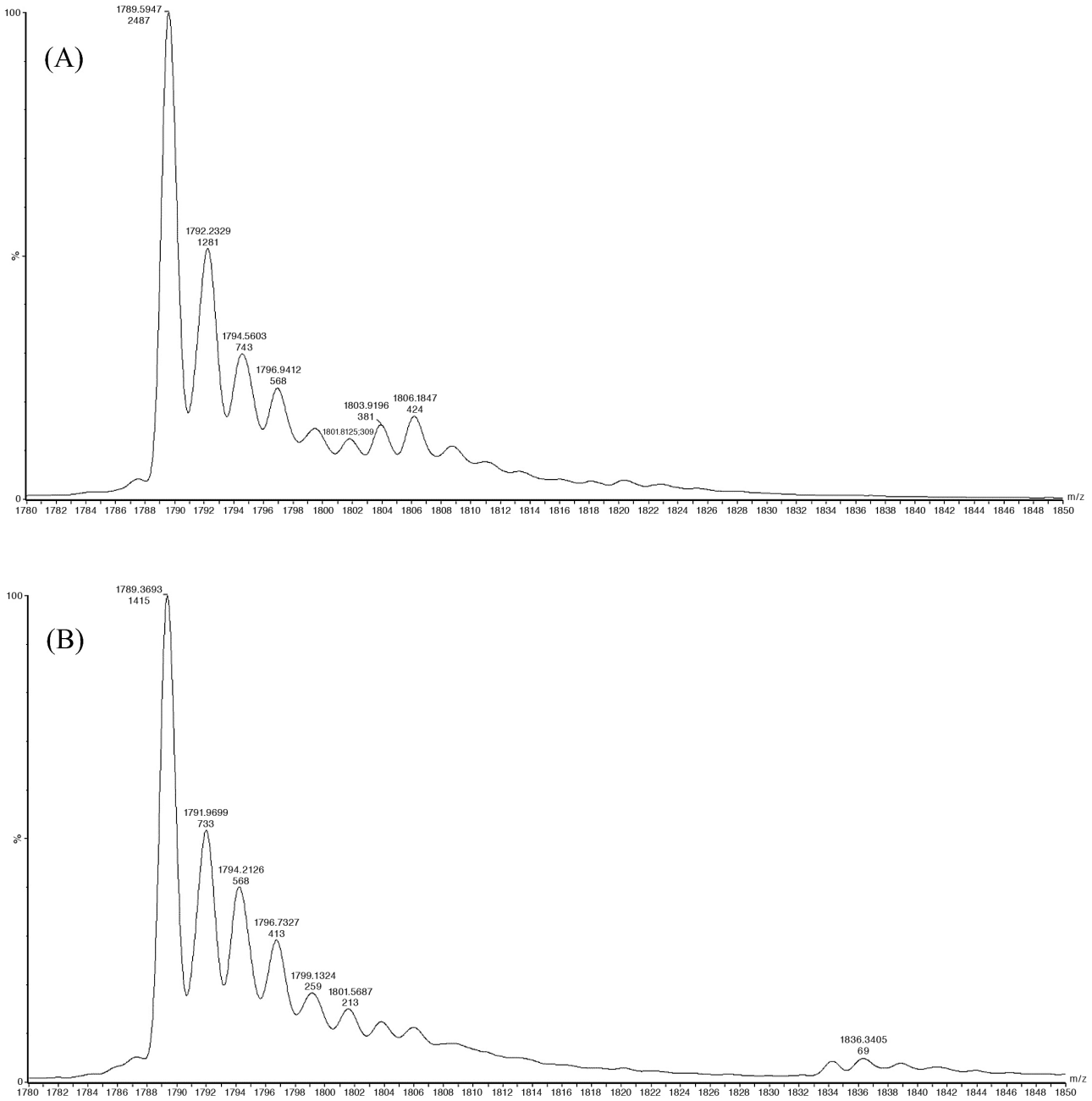

Fig. S4-i. **ESI-MS spectra of deglycation test on glycated lysozyme at 408h: (A) blank sample and (B) deglycation sample.**

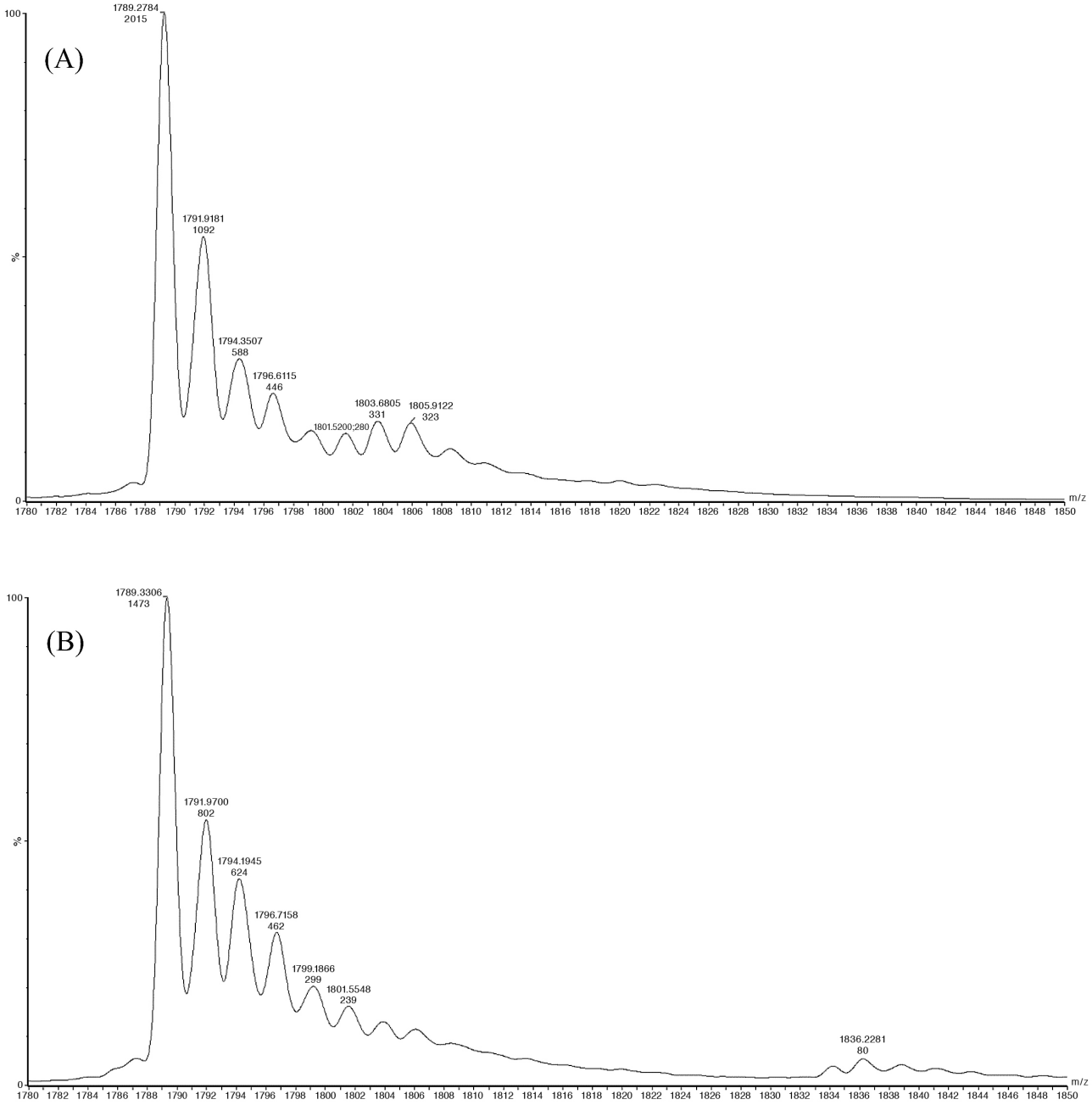

Fig. S4-j. **ESI-MS spectra of deglycation test on glycated lysozyme at 552 hours: (A) blank sample and (B) deglycation sample.**

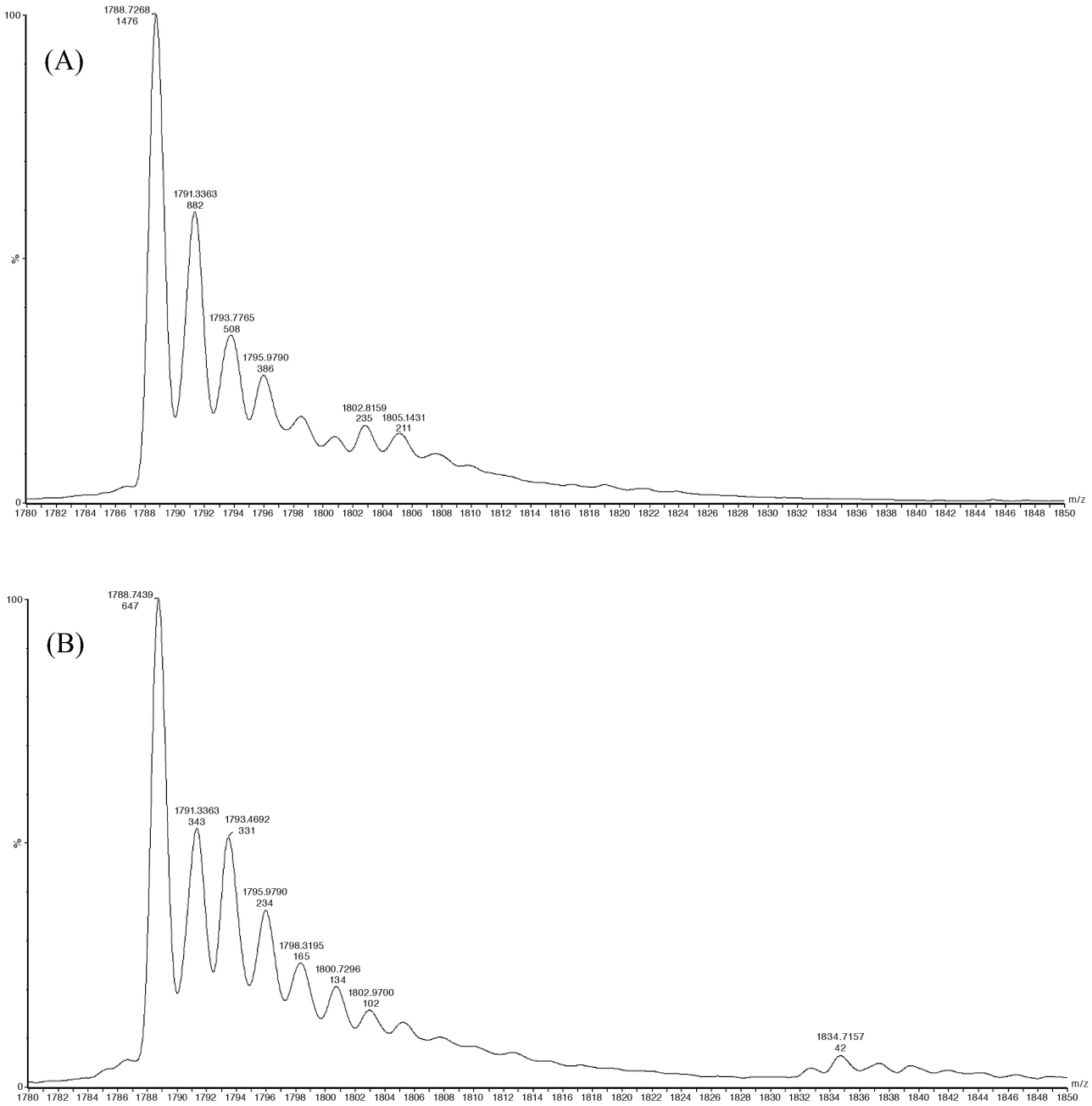

Fig. S4-k. **ESI-MS spectra of deglycation test on glycated lysozyme at 720 hours: (A) blank sample and (B) deglycation sample.**

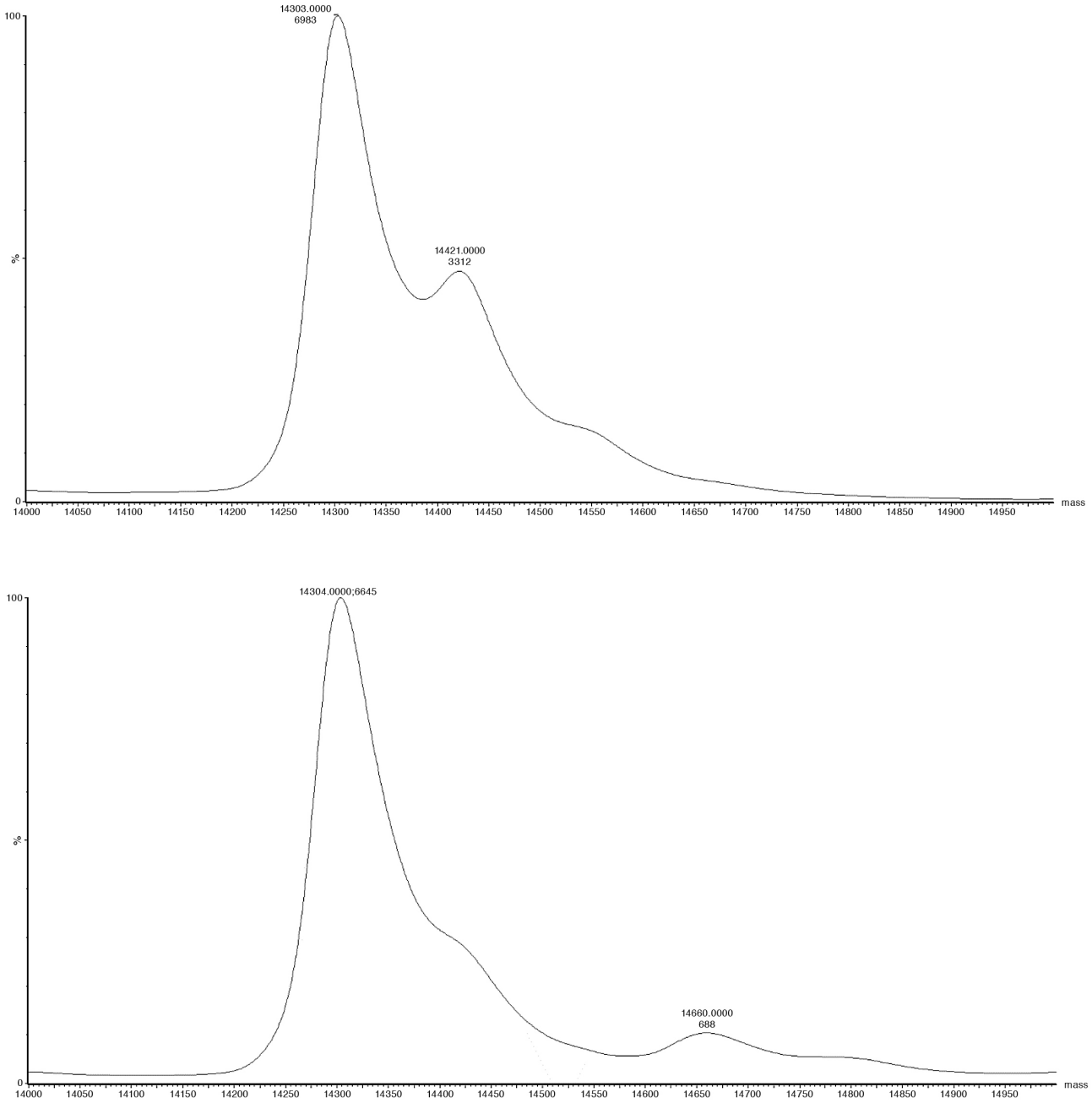

Fig. S5. **Deconvolved ESI-MS spectra of deglycation test on glycated lysozyme at 168 hours: (A) blank sample and (B) deglycation sample.**