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Evaluation of high temperature glycation of proteins and peptides by electrospray ionization mass spectrometry★✪

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Recently Boratyński & Roy (*Glycoconjugate J.*, 1998, 15, 131) described a fast and convenient procedure for the synthesis of glycoconjugates. In the present study we used ESI-MS and circular dichroism as tools to analyze non-enzymatic glycation products of proteins and peptides. We discuss influence of reaction conditions on the rate of glycation of lysozyme. We analyze for the first time collision induced dissociation spectra of the obtained peptide conjugates.

Electrospray mass spectrometry (ESI-MS) is currently a widely accepted method for the characterization of the primary structure of proteins and other biomolecules. For proteins, molecular masses can be measured within an accuracy of 0.01%. This is sufficient to confirm the identity of a protein and to determine its purity. ESI-MS method also allows the detection of posttranslational and chemical modifications as long as the modification alters the molecular mass of the molecules.

In this paper we applied the ESI-MS technique to evaluate high temperature glycation of proteins and peptides. This is a key reaction in the process of the food products, in which mixtures containing high concentrations of

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Abbreviations: CID, collision induced dissociation; ESI, elestrospray ionization; MS, mass spectrometry; CD, circular dichroism.

proteins and carbohydrates are exposed to high temperature or microwaves. Recently Boratyński and Roy described a fast and convenient procedure for the synthesis of glycoconjugates [1, 2].

MATERIALS AND METHODS

All compounds were glycated according to the procedure reported [1, 2]. Briefly: aqueous solution containing peptide and glucose was frozen in liquid N_2 and after lyophilization samples were heated at 70–100°C for 20–30 min. In the case of LA (linear analogue of cyclolinopeptide A N,N-dimethylformamide (DMF) was used as a cosolvent. Mass spectrometric analysis was performed on a Finnigan TSQ-700 instrument equipped with an electrospray ion source. Samples were dissolved in water/methanol/acetic acid mixture (50:49:1, v/v) to the final concentration of 0.1 mg/ml for low molecular mass compounds and 1 mg/ml for samples of glycated lysozyme and infused into the ion source at a flow rate of 2 μl/min. Fragmentations were performed in the ion source on samples purified by HPLC using declustering potential of 6–20 V.

RESULTS AND DISCUSSION

Glycation of lysozyme

Lysozyme was glycated according to the Boratyński's procedure at various temperatures. Mass spectra of the obtained products are presented in Fig. 1. Panel A shows unmodified lysozyme used as a standard. Panel B – lysozyme glycated at 77°C and C – glycated at 100°C. Figure 2A shows a typical pattern observed for pure, homogenous protein. Multiple peaks at *m/z*: 1021.9, 1100.7, 1192.5, 1300.9, 1431.0, and 1591.2 correspond to the molecules with charge: +14, +13, +12, +11, +10, and +9, respectively. In Figs. 1B and 1C groups of peaks are observed (for example in Fig 1B, for charge +11 a group of peaks at *m/z* 1300.9, 1315.5, 1330.1 and 1344.7). These peaks correspond to the adducts with different numbers of glucose moieties (0, 1, 2 and 3, respectively). The most abundant peak for the sample glycated at 77°C corresponds to the protein modified with two residues of the sugar. The sample glycated at higher temperature (100°C) does not contain the peak of unmodified protein. The peak at the lowest *m/z* corresponds to the protein glycated with three moieties of glucose; the most abundant peak is the result of the addition of five residues of glucose. Incubation of a sample of lysozyme at a high temperature without glucose does not

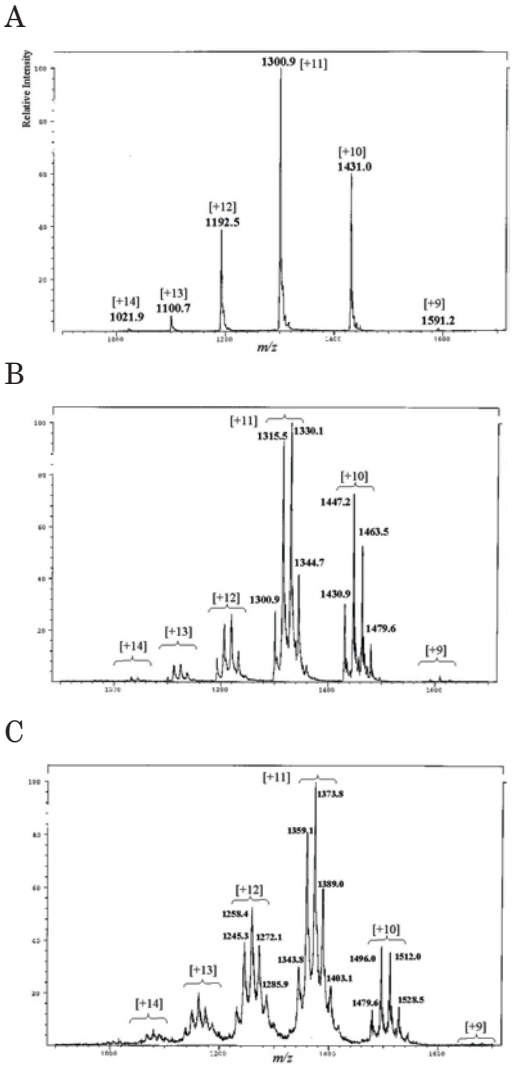

Figure 1. ESI-MS spectra of glycated lysozyme.

A, unmodified protein; B, sample glycated at 77°C; C, sample glycated at 100°C.

influence the ESI spectrum. This result suggests that there is no covalent modification of this protein during short heating of the sample in the absence of carbohydrates.

Our results clearly show that the glycation process is strongly temperature dependent.

The level of glycation is comparable to that obtained in previous studies [3] (using low temperature) but the homogeneity of material produced by Boratyński's procedure is significantly higher. Another advantage of Boratyński's method is the relatively short time required for glycation. However, the ESI-MS method provided data on the total level of glycation only; answering the question which groups underwent the Maillard reaction requires additional studies (enzymatic hydrolysis and peptide mapping).

Conformational changes of lysozyme in the course of glycation were evaluated on the basis of CD and ESI-MS spectra. It has previously been found that the net charge of a protein molecule is sensitive to protein conformation [4]. Disordered proteins tend to have a larger net charge than native proteins. According to the spectra presented in Figs. 1A–1C the highest abundance was observed for peak +11 for unmodified lysozyme and for all glycated forms as well. This result suggests that there is no significant difference in conformation of glycated and unmodified lysozyme. Also a comparison of CD spectra of the glycated material and a lysozyme standard does not show any essential differences. Influence of glycation on the conformational properties of lysozyme seems to be negligible.

On the other hand, it should be noted that results obtained for lysozyme can not be generalised for proteins with higher molecular masses and less compact tertiary structure.

Glycation of peptides

The possibility of application of the high temperature procedure for glycation of peptides was tested using the following model systems: Gly-Gly, LA (Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe-Leu) and free phenylalanine. Products of glycation were analyzed directly by the ESI-MS technique. Representative spectra are presented in Figs. 2A, B and C. In each case abundant peak of glycated product (mass increased by 162 Da) was found. In Fig. 2A, showing an ESI-MS spectrum of glycated Gly-Gly, the peak of conjugate is the most abundant. This spectrum also shows a peak of intact Gly-Gly, and a peak of the dehydrated conjugate. The linear analogue of CLA (Fig. 2B) seems less reactive, but even this peptide was significantly glycated. Glycation of Phe (Fig. 2C) gave a peak of the expected conjugate, it was not the predominant one.

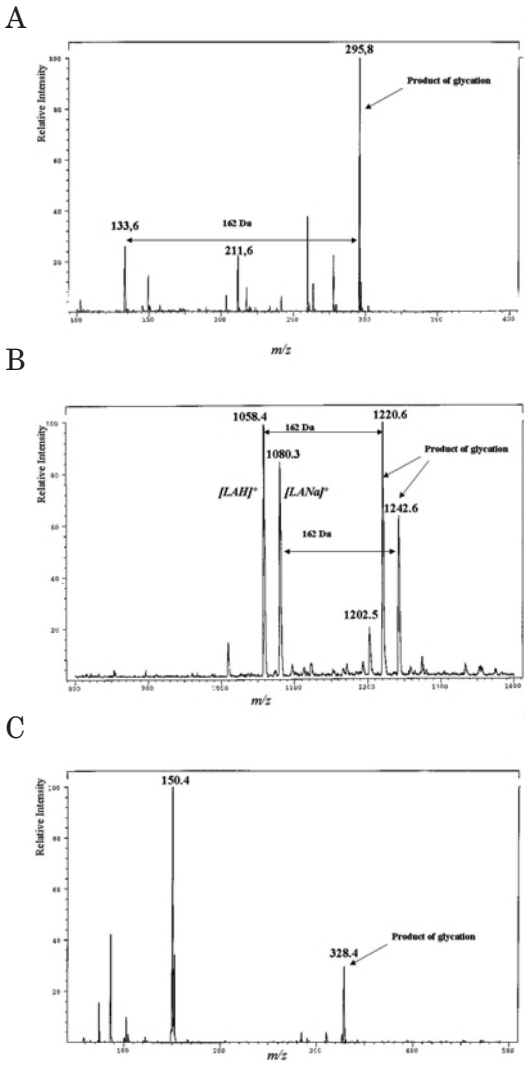

Figure 2. ESI-MS spectra of glycated peptides.

A, Gly-Gly; B, Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe-Leu; C, Phe.

The most intense peak was observed at *m/z* 150.4 and its origin has not been identified yet. The peak corresponding to unmodified phenylalanine (at *m/z* 166.2) was not observed. All the compounds tested gave products of glycation at the high temperature conditions, but there are distinct differences in the reactivities of particular compounds.

CID spectra were recorded for the conjugate of Gly-Gly with D-glucose (Fig. 3). The results of a CID experiment presented in Fig. 3 shows that dehydration is the basic

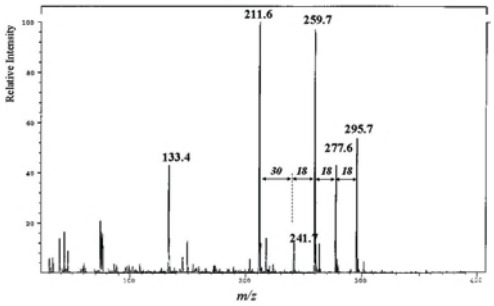

Figure 3. CID spectrum of glycated Gly-Gly.

Fragmentation was performed in the ion source by using declustering potential of –6 V, on the sample purified by HPLC.

process of fragmentation for this compound. Neutral loss of three molecules of water gives peaks at *m/z* 277.6, 259.7 and 241.7, then a neutral loss of 30 Da (which corresponds to a CH_2O molecule) produces peak at *m/z* 211.6. The proposed mechanism of fragmentation of glycated glycylglycine presented in Fig. 4 is similar to the fragmentation of lactolated peptides described by Mole *et al.* [6]. In both cases the bond between the amino group of the peptide and the sugar moiety is quite stable and fragmentation takes place mainly at the sugar moiety (dehydration for glycated peptides, dehydration and cleavage of the O-glycosidic bond for lactolated peptides).

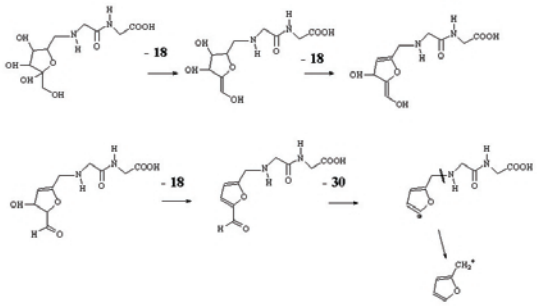

Figure 4. Proposed mechanism of fragmentation of glycated Gly-Gly.

CONCLUSION

According to our results the procedure described by Boratyński gives products with a high level of glycation without significant dehydration of the attached sugar moiety. Our procedure led to more homogenous (less dehydrated and oxidized) conjugates in comparison to preparations of glycated lysozyme characterized by Yeboah *et al.* [3]. CD studies did not indicate essential conformational changes of lysozyme after glycation.

Our experiments showed that Boratyński's procedure is not limited to proteins only. Modification of peptides gives high yields of glycated products with small quantities of dehydrated compounds.

The high temperature method of glycation of proteins and peptides provides a model system for studying proceses taking place during food processing. Answering the question whether conjugates obtained during the mentioned process can be used as models for products of *in vivo* Maillard reaction needs additional studies.

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