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Studies on the Effect of Amadoriase from *Aspergillus fumigatus* on Peptide and Protein Glycation In Vitro

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Amadoriase I is a fructosyl amine oxidase from *Aspergillus fumigatus* that catalyzes the oxidation of Amadori products (APs) producing glucosone, H_2O_2, and the corresponding free amine. All the enzymes of this family discovered so far only deglycate small molecular weight products and are inactive toward large molecular weight substrates, such as glycated BSA or ribonuclease A. Therefore, they cannot be used to reverse protein glycation occurring in diabetes or in foods. In this paper, the effect of Amadoriase I added during the in vitro reaction between glucose and peptides having different polarities or proteins with molecular weights ranging from to 5 to 66 kDa was tested. The formation of APs was monitored by ESI-MS of intact glycated protein or peptides and by measuring the N^{ϵ}-(1-deoxy-D-fructos-1-yl)-L-lysine and furosine concentrations. Results showed that the formation of APs is reduced up to 80% when peptides and glucose are incubated in the presence of Amadoriase. The effect is more evident for hydrophobic peptides. In protein−glucose systems, the effect was dependent on the molecular weight and steric hindrance being negligible for BSA and at a maximum for insulin, where the formation of APs was reduced up to 60%. These findings indicate new potential applications of Amadoriase I as an efficient tool for inhibiting protein glycation in real food systems.

KEYWORDS: Amadori products; protein glycation; browning; Amadoriase I

INTRODUCTION

The Maillard reaction (MR) involves carbonyl compounds and free amino groups of amino acids, peptides, or proteins (*1*). This reaction occurs during processing, cooking, and storage of foods causing both deterioration or enhancement of food quality (*2*, *3*). Through a series of reversible reactions, the sugar carbonyl moiety forms a Schiff base with an amino acid or with the ϵ-amino group of the protein lysine residues. The Schiff base, through rearrangements, gives the Amadori product (AP), which is a stable aminoketose compound (*4*). The APs have been isolated from several processed foods and found in tissues of the human body, especially in diabetics (*5*). APs can undergo dehydration and fragmentation reactions to form stable covalent adducts of proteins called advanced glycation end products (AGE). The corresponding final products of the MR in foods are called melanoidins or melanoproteins (*6*, *7*).

In the medical field, the prevention of in vivo protein glycation is an important research challenge aiming at reducing some of the protein malfunctions observed in diabetes mellitus. In this respect, the availability of deglycating enzymes would be of great interest (*8*). About 10 years ago, some enzymes able to use APs as substrates were described (*9*, *10*). Until now, there were three types of enzymes capable of acting on fructosamines: Amadoriases, fructosamine-3-kinase, and the system constituted by fructoselysine-6-kinase plus fructoselysine-6-P deglycase (*11*). Two deglycating enzymes were isolated from *Aspergillus* spp., and they were named Amadoriase I and II. These enzymes catalyze the hydrolysis of APs producing free amine, glucosone, and H_2O_2 (*12*, *13*).

The substrate specificity and kinetic parameters of the different fructosyl amine oxidases have been largely investigated (*14*). It was concluded that enzymes from different sources, as well as the different isoforms from the same sources, could have different substrate specificities varying according to their size and charge: some Amadoriases prefer anionic substrates, while others prefer more hydrophobic APs (*15*). Until now, all studies have shown that intact glycated proteins are not substrates of

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fructosyl amino oxidases, which means that the enzymes are not able to revert protein glycation (*16*). It is very likely that glycated proteins are not good substrates for these enzymes because of the steric hindrance exerted by the polypeptide on the APs. In other words, the presence of the protein backbone physically does not allow the enzymes to reach their substrate.

Despite these hurdles, Amadoriases still have many potential applications both in vivo and in foods. Recently, Hirokawa and co workers (*17*, *18*) characterized a new fructosyl amino oxidase that is able to react with a glycated hexapeptide released from glycated hemoglobin by endoproteinase Glu-C. The authors proposed the use of this enzyme in clinical diagnostics for the measurement of hemoglobin glycation. Similarly, Kouzuma et al. (*19*) developed an enzymatic method using a ketoamino oxidase to determine glycated albumin in blood samples after extensive proteolysis. A commercial kit using fructosyl amino oxidase is now available to measure the extent of protein glycation (*20*).

In a previous work (*21*), we have demonstrated that Amadoriase I is able to hydrolyze APs formed on di-, tri-, and tetrapeptides incubated with glucose, showing that the affinity of the enzyme for the glycated substrates slightly decreases with the increase of the molecular weight of the substrates.

In this paper, we aimed at characterizing the Amadoriase effect when it is added during the in vitro reaction between glucose and either peptides of different polarities or proteins with molecular weights ranging from to 5 to 66 kDa. The results showed that AP formation is reduced up to 80% by Amadoriase activity. In peptide systems, the effect is more evident for hydrophobic peptides, while in protein systems, it depends on the molecular weight being negligible for BSA and at a maximum for insulin.

MATERIALS AND METHODS

Materials. Solvents and reagents were obtained from Merck. Buffers for the extraction and purification of the enzyme were obtained from Novagen. A TLC 25 DC-Platten 20 cm × 20 cm RP_{18} F_{254s} column from Merck and a Bond Eluate Column C_{18}, 500 mg, 3 mL, 50/PK were supplied by Varian. *o*-Phenylendiammine (OPD) was purchased from Aldrich. Plasmid coding for Amadoriase I from *Aspergillus fumigatus* was a kind gift of Prof. J. Gerrard (Department of Biochemistry, University of Canterbury, New Zealand).

Gene Expression, Protein Production, and Purification. *Escherichia coli* BL21(DE3)PLYsS (Novagen) transformed with the pET-15b-Amadoriase I construct (amp +) was grown on LB agar and 50 ppm ampicillin at 37 °C overnight. The clone was inoculated in 5 mL of LB agar and 50 ppm ampicillin medium and incubated at 37 °C for 16 h with constant stirring. Then, the culture was completely transferred to 100 mL of LB agar and ampicillin medium incubated at 37 °C until the absorbance at 600 nm reached 1 unit, added to 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), and incubated at 37 °C for 3 h.

One gram of cells (wet pellet) was resuspended in 5 mL of BugBuster and added to 25 units of Benzoase Nuclease and 1 KU lysozyme per milliliter of BugBuster. After 20 min, the insoluble cell debris was removed by centrifugation at 16 000*g* at 4 °C for 20 min.

The His-Bind Purification Kit (Novagen) was used to purify the recombinant protein following the procedure indicated by the manufacturer.

The obtained proteins were analyzed by 12% SDS-PAGE (*22*), and the gels were stained by SimplyBlue safe Coomassie G-250 (Invitrogen). Concentration of pure Amadoriase I was quantified by a DC protein assay kit 1 following the protocol proposed by the producer (Bio-Rad).

Enzymatic Activity of Amadoriase I. The enzymatic activity was determined according to Takahashi (*12*), monitoring the release of glucosone from the Amadori compound, which was measured by a colorimetric reaction with OPD. The reaction mixture contained 20 mM sodium phosphate, pH 7.4, 10 mM OPD, 10 mM substrate, and 100 μL of Amadoriase I (30 μg mL^{-1}) in a final volume of 1 mL. After incubation at 37 °C for 120 min, the absorbance at 320 nm was monitored. A calibration curve was constructed using pure 3-deoxyglucosone (3-DG) for quantification. 3-DG was prepared according to a previously published procedure (*23*).

Synthesis of N^{ϵ}-(1-Deoxy-D-fructos-1-yl)-L-lysine (FL). The synthesis of FL was carried out according to a previously published procedure with small modifications (*24*). N^{α}-*t*-Boc-lysine (0.75 mmol) added to D-glucose (9.15 mmol) in methanol (31 mL) reacted at 80 °C for 4 h. The methanol was evaporated, and the dried residue was dissolved in 3 mL of methanol and purified on a C_{18} cartridge. After elution, the Boc group was removed with 1 M HCl at room temperature for 3 h. The formation of FL in a purity of ∼95% was verified by ESI-MS under the conditions described next.

Synthesis of Peptide- and Protein-Bound APs. The synthesis of the APs on four lysine-containing dipeptides was performed as recently described (*25*). The dipeptides Lys-Phe, Lys-Ser, Ac-Phe-Lys, and Ac-Gly-Lys were reacted with glucose at a molar ratio of 1:4 in methanol at 64 °C for 2 h. The APs present in the reaction mixture were purified using a silica gel column and TLC using different developing systems (*25*). The identity of the compounds obtained was verified by LC-MS as described next.

To prepare the protein-bound APs, a solution containing 25 mg mL^{-1} BSA, insulin, β-lactoglobulin (Sigma), isolated whey protein (Alacen, New Zealand), and isolated soybean protein (Prodotti Gianni) was incubated in PBS with 250 mM glucose at 37 °C for 14 days. Protein solutions were extensively dialyzed using a 12 kDa cutoff tube, and glycation was assessed by ESI-MS as described next.

Glycation Experiments in the Presence of Amadoriase I. The effect of the Amadoriase I on the glycation reaction between glucose and four lysine-containing dipeptides (Lys-Phe, Lys-Ser, Ac-Phe-Lys, and Ac-Gly-Lys) and between glucose and three different proteins (BSA, insulin, and β-lactoglobulin) was assayed. The dipeptides (10 mg mL^{-1}) and proteins (25 mg mL^{-1}) were mixed with glucose (250 mM) in 20 mM phosphate buffered saline (PBS) pH 7.4. The final volume was in all cases 1 mL. To avoid bacterial contamination during the incubation time, all solutions were sterilized by 0.22 μm filtration, and all operations were performed under a sterile hood. Reaction systems were incubated at 40 °C for up to 14 days (peptides) or up to 28 days (proteins). A total of 3 μg mL^{-1} Amadoriase I (100 μL of a solution containing 30 μg mL^{-1} enzyme) was added or not added at each system. Aliquots of 100 μL of samples were taken at regular intervals, and the formation of the AP was checked by mass spectrometry of intact glycated products, determination of FL, and furosine analysis.

Mass Spectrometry Analysis of Glycated Peptides and Protein. Glycated peptide determination was performed by ESI-MS using a mass spectrometer API 100 model (Sciex) equipped with an electrospray ion source. The peptide samples were injected directly into the ion source at a flow of 5 μL min^{-1}. Acquisition was made in positive ion mode in the range of 200−1000 amu with a dwell time of 1 ms and a step size of 0.1 amu. A probe voltage of 4.8 kV and a declustering potential of 60 V were used. The area of peaks corresponding to glycated and non-glycated peptides was used to estimate the extent of glycation.

For glycated insulin and β-LG analysis, the conditions reported by Fogliano et al. (*26*) were used with a probe voltage of 4.8 kV and a declustering potential of 90 V. The analysis was performed by injecting directly into the ion source at a flow of 10 μL min^{-1} a 0.1 mg mL^{-1} solution of protein. Data acquisition was made in the positive ion mode with a dwell time of 0.5 ms and a step size of 0.2 amu. The instrument mass-to-charge ratio scale was calibrated with the ions of the ammonium adducts of polypropylene glycol (PPG). Data were processed through the Bio Multi View software (Sciex).

Determination of FL. The quantification of FL on the glycated proteins incubated or not incubated with Amadoriase I was performed by LC-MS-MS after extensive enzymatic hydrolysis as described by Henle and co-workers (*27*), by comparison with a calibration curve built up with standard FL synthesized as previously described.

LC analysis was performed using a system consisting of a series 200 binary pump (PerkinElmer) and an API 3000 triple-quadrupole tandem mass spectrometer (Applied Biosystems). The mass spectrometer was equipped with a Model 11 syringe pump (Harvard Apparatus). All the analyses were performed using an electrospray source (ESI) in positive ion mode, and the collision induced dissociation was performed using nitrogen as the collision gas. The analysis was performed using a Luna column (5 μm C_8 250 mm $\times$ 4.60 mm (Phenomenex)). The solvent system was composed of water and 0.1% formic acid (solvent A) and acetonitrile and 0.1% formic acid (solvent B). The linear solvent gradient was 0−5 min 80% A and 20% B, 5−8 min 80% A and 20% B, 5−8 min 100% A, 8−12 min 100% A, and 12−15 min returning to initial conditions. The acquisition has been carried out by an MRM (multiple reaction monitoring) system, in positive mode, monitoring the transition of parent and product ions specific for FL with a dwell time of 500 ms. To promote ionization of the precursor ion, the voltage applied was 5500 V, and the collision energy (CE) and collision cell exit potential (CXP) were optimized for each transition. Data acquisition and processing were performed using Analyst software 1.4.

Furosine Analysis. FL was indirectly determined by quantifying the 2-furoyl-methyl-lysine, also called furosine analysis. Furosine was obtained from FL by acid hydrolysis at 110 °C for 24 h in a Pyrex screw-cap vial with PTFE-faced septa. High purity helium gas was bubbled through the solution for 2 min. The hydrolyzate was filtered with a medium grade paper filter. A 0.5 mL portion of the filtrate was applied to a Sep-pack C_{18} cartridge (Millipore) activated with 5 mL of methanol and 10 mL of water (Millipore Corp.). 2-Furoylmethyl-amino acids (2-FM-AA) were eluted with 3 mL of 3 N HCl. RP-HPLC analysis was performed employing a C_8 column (250 mm $\times$ 4.6 mm i.d.) (Alltech Biotechnology, furosine detected) at 37 °C, with a linear binary gradient and a variable wavelength detector at 280 nm (LCD Analytical SM 4000). Operating conditions were as follows: flow rate 1.2 mL/min; solvent A, 0.4% HPLC grade acetic acid in double distilled water; and solvent B, 0.3% KCl in solvent A. The quantitative analysis was performed by the external standard method, using a commercial standard of pure 2-FM-Lys (furosine) (Neosystem Laboratoire). The data were the mean values expressed as mg/100 g of protein.

RESULTS AND DISCUSSION

Production of Amadoriase I and APs. Amadoriase I was obtained as a fusion protein containing an amino-terminal His-Tag. Purity of the recombinant protein was analyzed by SDS-PAGE analysis, which showed a single band with a molecular mass of 53 kDa. A yield of 5 mg of pure Amadoriase I per liter of bacterial culture was achieved. The synthesis of FL gave 245 mg of the compound with a yield of 75%. FL was used as a pure standard substrate for measuring the enzymatic activity. Other APs bound to lysine-containing dipeptides were obtained in quantities ranging from 10 to 50 mg with a yield of 8−67%.

Amadoriase I Affinity for Glycated Dipeptides. Figure 1 reports the time course of the deglycation reaction showing the absorbance at 320 nm monitored for 120 min. The enzymatic activity of Amadoriase I was measured by recording the amount of glucosone released from the glycated products (*12*). **Table 1** summarizes the amount of glucosone released from the glycated lysine-containing dipeptides by Amadoriase I activity after 120 min of reaction and the corresponding values of k_{cat} and K_m.

Data indicated that Amadoriase I possesses a high affinity for hydrophobic dipeptides. This result confirms previous data (*12*, *15*) reporting a K_m of 3.1 mM for N^{α}-*t*-Boc-FL and of 0.023 mM for fructosyl propylamine. Interestingly, the two N^{α} acetylated dipeptides showed lower K_m than that of FL, suggesting that the hydrophobicity has an important role on enzyme affinity. On the other hand, it cannot be ruled out that the differences in the K_m values are related to the different type of APs that are formed on the various substrates: only on the ϵ amino group for acetylated peptides and on both the ϵ and the α amino groups for the non-acetylated ones.

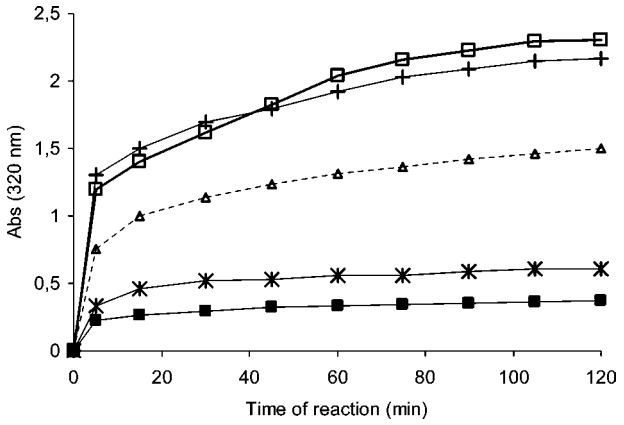

Figure 1. Time course of the release of glucosone by Amadoriase I from different dipeptides. △, FL; □, AcPhe-Lys; +, AcGly-Lys; *, Lys-Phe; and ■, Lys-Ser.

Table 1. Biochemical Parameters of Amadoriase I toward FL and Four Different Lysine-Containing Dipeptides

substrate	glucosone at 120′ (mM)	K_M (mM)	k_{cat} (s^{-1})
N^{ϵ}-(1-deoxy-D-fructos-1-yl)-L-lysine (FL)	0.147	4.2	1.1
deoxy-D-fructosyl-Lys-Phe	0.094	8.6	0.5
deoxy-D-fructosyl-Lys-Ser	0.080	10.3	0.3
deoxy-D-fructosyl-AcGly-Lys	0.187	1.7	1.4
deoxy-D-fructosyl-AcPhe-Lys	0.195	1.5	1.8

Amadoriase I Affinity for Glycated Proteins. The initial research on Amadoriase was boosted by the potential application to revert the in vivo protein glycation occurring in diabetic subjects. Unfortunately, it is now firmly established that the various isoforms of Amadoriase are not able to hydrolyze APs formed on whole protein, particularly on BSA. This was confirmed from the data shown in **Figure 2**. The amount of glucosone released from pure glycated proteins incubated with Amadoriase I up to 7 days, independent of the protein size, is negligible in reaching a maximum of 0.2 mmol/mmol of proteins. Data of **Figure 2** demonstrated that also APs formed on a very small protein such as insulin, having a molecular weight of about 5700 Da, are not a good Amadoriase substrate. Similarly, a very small amount of glucosone was released by the enzyme from glycated whey and soybean proteins. The same results were obtained on a long time-glycated β-LG sample (21 days) or monitoring FL formation instead of glucosone release (data not shown).

Inhibitory Effect of Amadoriase I during Peptide Glycation Reaction. At body or room temperature, MRs take place over a relatively long time (weeks or months). In the following set of experiments, we aimed at investigating the effect of the Amadoriase I when it was added during the time of reaction with glucose. Under these conditions, the enzyme was quite stable, retaining 50% of initial activity both in a peptide−glucose or in a protein−glucose system after 4 weeks of incubation at 40 °C.

Amadoriase I and 0.25 M glucose were simultaneously added to peptides and proteins at time zero of the incubation period. In **Figure 3**, the reduction rate of the APs formation caused by Amadoriase in peptide−glucose systems is shown. Both after 8 and after 14 days of incubation, the rate of APs formation was strongly reduced for all peptides tested. The reduction was more evident for the acetylated peptides according to the

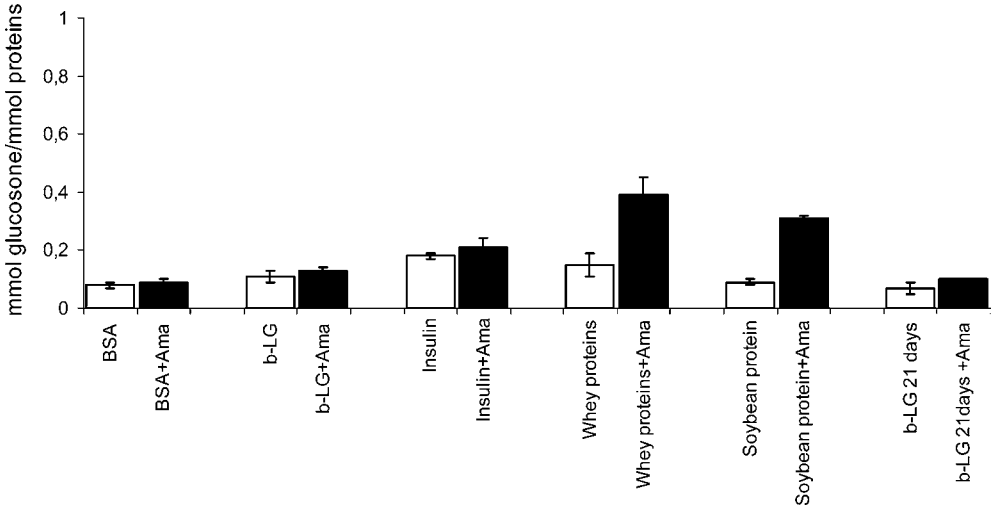

Figure 2. Release of glucosone from glycated proteins after 7 days of incubation with and without Amadoriase I addition. For one of the β-LG samples, the incubation was prolonged up to 21 days.

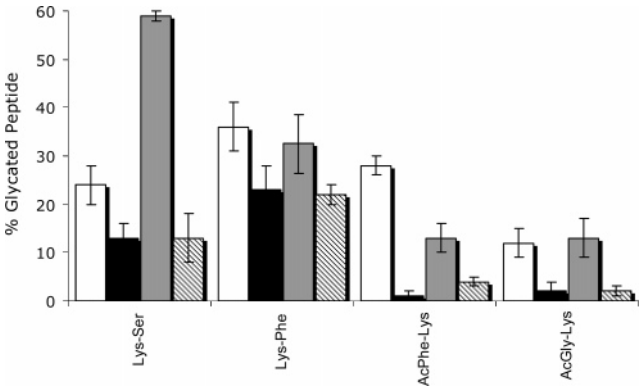

Figure 3. Effect of Amadoriase I on the glycation of different dipeptides in peptide–glucose systems. Peptide glycation was monitored by ESI-MS. White bars, 8 days of incubation; black bars, 8 days of incubation added to Amadoriase I; gray bars, 14 days of incubation; and shaded bars, 14 days of incubation added to Amadoriase I.

observation of the higher affinity of the enzyme for the hydrophobic substrates.

The results of the peptides were expected considering the previously shown ability of the enzyme to hydrolyze, with different efficacies, the corresponding APs. However, it is worth noticing that Amadoriase is able to work for several weeks in conditions that resemble conventional food storage. This result suggests the potential application of Amadoriase for the inhibition of the MR during long term storage in products containing elevated concentration of hydrolyzed proteins, such as formulas for enteral nutrition, infant formulas, or foods containing high concentrations in free amino acids.

Inhibitory Effect of Amadoriase I during Protein Glycation Reaction. Amadoriase I was also added to systems of glucose and proteins during a glycation reaction simulating physiological conditions. The incubation was carried out at 37 °C for different times, and the extent of the glycation was evaluated in three different ways: by ESI-MS of intact glycated proteins, by measurement of FL bound to glycated proteins after enzymatic digestion, and by furosine analysis.

In **Figure 4**, the ESI-MS spectra obtained from the mixture of insulin glycated in the presence (panel **A**) and in the absence (panel **B**) of Amadoriase I are shown. Comparing the peaks from to the intact proteins with those derived from the monoglycated form, it is quite clear that the formation of glycated insulin is reduced in the presence of Amadoriase I. In fact, the native insulin was almost absent in the sample without Amadoriase I (panel **B**), while it is still clearly detectable in the presence of the enzyme (panel **A**). This result confirms preliminary evidence obtained with the same techniques using β-lactoglobulin (*21*). As this data do not give a quantitative measurement of the effect caused by Amadoriase, FL and furosine concentrations were measured to quantify the extent of the glycation achieved in the mixtures.

Table 2. Amount of FL (mmol of FL/mmol of Proteins) Formed during Glycation of Proteins and 0.25 M Glucose with or without Addition of Amadoriase I for 28 Days

protein	control	Amadoriase	% of reduction
BSA	7.69 ± 0.39	7.20 ± 0.54	6.4
β-LG	1.15 ± 0.12	0.75 ± 0.12	34.7
insulin	0.62 ± 0.09	0.35 ± 0.04	43.5

The determination of FL was performed by LC-MS−-MS after extensive enzymatic digestion of three different glycated proteins. The analytical system described in the Materials and Methods is based on the detection of the transition of parent and product ions specific for FL. In fact, the molecular ion of FL $[MH]^+$ *m*/*z* 309.5 gave three main fragments at *m*/*z* 273.1, 225.2, and 145.5 corresponding to losses of two H_2O molecules (−36 amu), three H_2O and one CHOH molecules (−84 amu), and one $C_6H_{10}O_5$ molecule (−162 amu). The MS−MS method proves to be highly specific and reproducible, and it allowed the unique identification of FL in the systems studied.

Quantitative FL data confirmed the ability of Amadoriase to inhibit protein glycation. In **Table 2**, the concentration of FL measured after 6 weeks of incubation is reported. The amount of detectable FL in the sample added to Amadoriase was 34.7 and 43.5% lower than those measured in controls consisting of β-LG and insulin, respectively. No significant effects were observed on BSA. These data, showing a large reduction for insulin intermediate for β-LG and negligible for BSA, confirmed that small molecular weight proteins are better substrates for the Amadoriases than high molecular weight polypeptides. It is likely that the action of the enzyme does not solely depend on the molecular weight, as the protein conformation and position of the carbohydrate-modified Lys in the polypeptide chain are also relevant. In this respect, the effect of Amadoriase on insulin could be also facilitated by the presence of a Lys residue on the C-terminus of the protein.

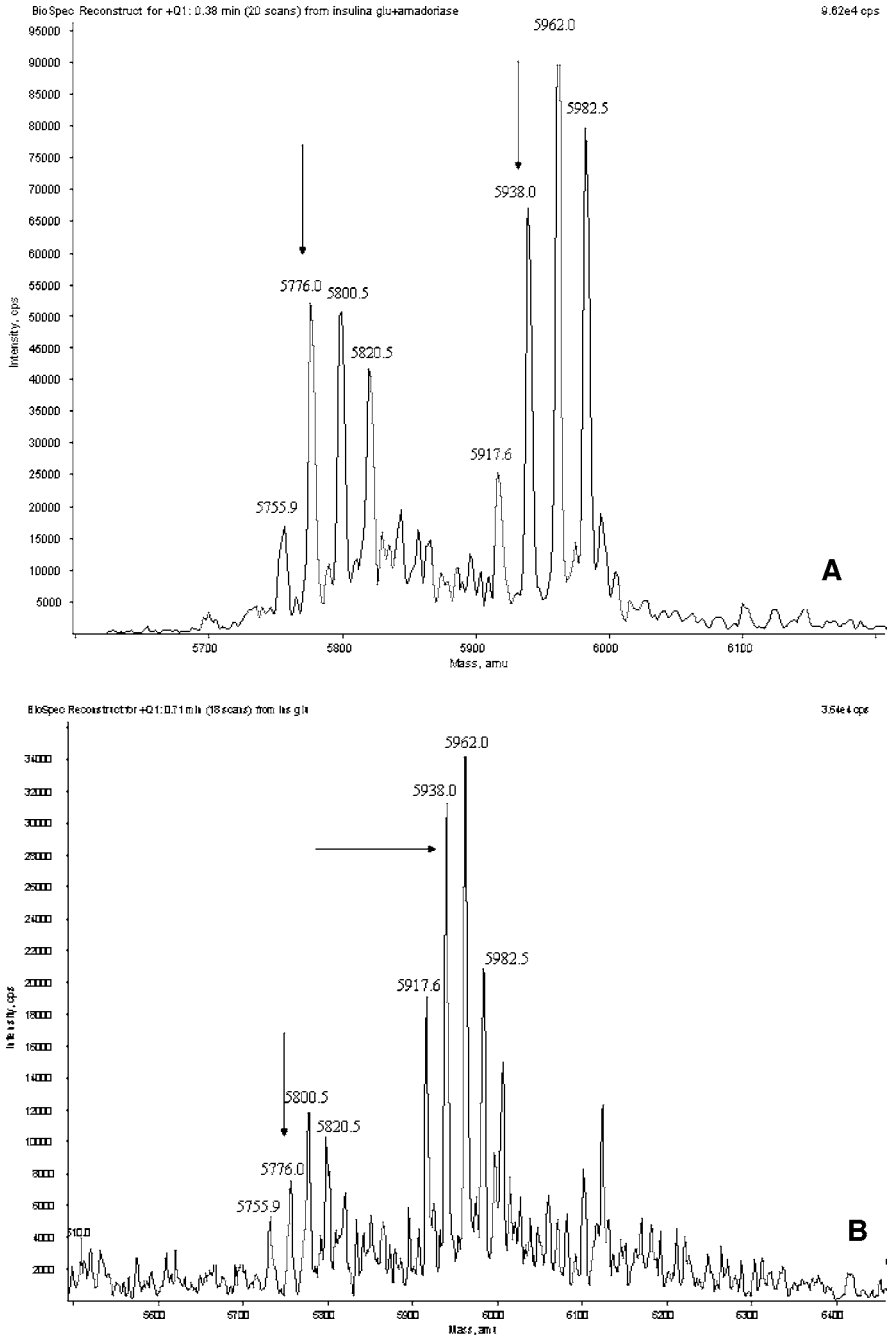

Figure 4. ESI-MS spectra of glycated insulin. (**A**) Sample incubated in the presence of Amadoriase and (**B**) control (without Amadoriase). The group of peaks at low molecular weights (between 5756 and 5820) are the non-glycated forms of the proteins, while those peaks with a higher molecular weight (between 5918 and 5982), a difference of 162 a.m.u, are due to the monoglycated forms of insulin.

Table 3. Furosine Concentration (mg/100 g of Protein) in Glycation Mixtures Consisting of Proteins and 0.25 M Glucose with and without Addition of Amadoriase

protein	control 2 weeks	Amadoriase 2 weeks	control 4 weeks	Amadoriase 4 weeks	control 6 weeks	Amadoriase 6 weeks
BSA	670.2	659.3	1398.5	1355.1	2230.6	2278.8
insulin	147.1	59.3	243.4	156.0	256.0	221.2
β-LG	795.6	535.4	1388.3	987.3	1380.7	1010.6
denatured β-LG	748.5	452.1	1405.1	1046.7	1378.9	1323.7

The effect of Amadoriase on protein−glucose systems was also measured by the well-known method of using furosine. A time course over 6 weeks for the glycation reaction of BSA, β-LG, and insulin was performed. Data are shown in **Table 3**, and they substantially agreed with those of FL. The glycation reaction progressed at different rates for the three proteins studied. Insulin glycation was inhibited by 59.7, 35.9, and 13.6% after 2, 4, and 6 weeks of incubation, respectively. Glycation rates of β-LG decreased by 32.7 and 26.8% after 2 and 6 weeks of reaction, respectively. On the other hand, BSA glycation was scarcely prevented by enzyme addition (reduction not higher than 3% at all times). Furosine data confirmed that the inhibitory

effect of Amadoriase I is related to the molecular size of the protein undergoing glycation. It is worth noticing that, under the studied conditions, a maximum inhibition of the furosine concentration was achieved after 2 weeks of incubation. From all these data, it can be definitively concluded that Amadoriase I is able to reduce the formation of APs on some proteins undergoing a nonenzymatic glycation with glucose in vitro.

This result is of great interest, and it is unexpected because Amadoriase is not able to oxidize the APs bound to intact protein chains (ref *14* and **Figure 2**). According to the literature, this lack of activity is mainly due to steric hindrance reasons, namely, Amadoriase is physically not able to reach most of the APs formed on the lateral chains of amino acids belonging to complex polypeptides. Therefore, it can be hypothesized that during the glycation reaction, conformational changes of the protein might occur favoring Amadoriase activity. In other words, the temporary unfolding of the protein region involved in the binding of the carbohydrate allows Amadoriase to reach its target and oxidize APs.

To check this hypothesis in the last experiment, heat-denatured β-LG was incubated in the same conditions previously described in the presence of Amadoriase. Results shown in the last line of **Table 3** indicate that the concentration of furosine was quite similar to that detected using native β-LG and that the effect of Amadoriase is slightly higher after 2 weeks but decreases during incubation time. Therefore, we can conclude that Amadoriase activity is not favored by the thermal denaturation of β-LG. Further research, under more controlled conditions, is needed to find out any link between the degree of folding of the glycated protein and the Amadoriase activity.

Until now, the possible use of Amadoriase was limited to the measurement of the glycation level of human blood proteins. The quantification of glucosone and hydrogen peroxide produced by adding this enzyme to extensively proteolyzed glycated proteins is an indirect indication of the extent of protein glycation (*19*). The data shown in this paper demonstrated that this enzyme is able to inhibit the glycation of peptides and of some proteins when it is present during the glycation reaction with glucose. AP formation is reduced up to 80% in the presence of Amadoriase. Hydrophobic peptide deglycation is favored in comparison with others peptides studied in the present paper, while the effects on proteins depends on the molecular weight being negligible for BSA and at a maximum for insulin.

Further studies should be carried out to elucidate critical factors for enzyme activity such as maximum length of the protein chain, shelf life of the enzyme in the different reaction media, and influence of the neighboring amino acids. For example, it has been demonstrated that the affinity of Amadoriase for glycated proteins increases when the carboxylated group of the protein was amidated (*14*). Enzymatic protein deglycation, although difficult, is theoretically possible, and protein engineering can be very helpful to improve the stability and activity of Amadoriase in real systems (*28*).

In this framework, we believe that the data here reported open the possibility for a reliable use Amadoriase I as a tool to efficiently inhibit protein glycation in food systems particularly where MR during storage affect sensorial and nutritional quality of the final product.

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