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ARTICLE

Role of Pyridoxamine in the Formation of the Amadori/Heyns Compounds and Aggregates during the Glycation of β-Lactoglobulin with Galactose and Tagatose

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The effect of pyridoxamine on the Maillard reaction during the formation of conjugates of β-lactoglobulin with galactose and tagatose under controlled conditions (pH 7, 0.44 a_w, 40 and 50 °C, for 6 days) has been studied, for the first time, by means of the changes in reducing carbohydrates, formation of Amadori or Heyns compounds, and aggregates and browning development. The results showed the formation of interaction products between pyridoxamine and galactose or tagatose either in the presence or in the absence of β-lactoglobulin, indicating that pyridoxamine competes with the free amino groups of β-lactoglobulin for the carbonyl group of both carbohydrates. Thus, a small inhibitory effect of pyridoxamine on the initial stages of the Maillard reaction was pointed out. Furthermore, much lower aggregation and color formation rates were observed in the conjugates of β-lactoglobulin galactose/tagatose with pyridoxamine than without this compound, supporting its potent inhibitory effect on the advanced and final stages of the Maillard reaction. These findings reveal the usefulness of food-grade inhibitors of the advanced stages of the Maillard reaction, such as pyridoxamine, that, in combination with mild storage conditions, could lead to the formation of safer neoglycoconjugates without impairing their nutritional quality.

KEYWORDS: β-Lactoglobulin; galactose; tagatose; pyridoxamine; initial and advanced stages of the Maillard reaction

INTRODUCTION

The Maillard reaction (MR) is well recognized as one of the most important and complex network of reactions that spontaneously occur between reducing sugars and the amino groups belonging to peptide and protein chains in living organisms or heat processed and stored foods. During the progress of this reaction, very different compounds are produced, which can even react with the initial reagents, giving rise to peptide-bound adducts irreversibly formed from the early products (Amadori or Heyns compounds), protein cross-linking, and the formation of brown and fluorescent polymeric materials (*1*). The MR progressing to the formation of advanced glycation end-products (AGEs) may play an important role in the pathogenesis of chronic diseases such as diabetes, atherosclerosis, renal failure, premature aging, and Alzheimer's disease (*2*).

However, over the past few years there has been an increased interest in deliberately promoted MR in a dry-state to obtain glycoconjugates with improved functionalities in relation to the initial proteins. Thus, a number of works have demonstrated the impact of the conjugation via the MR of mono-, di-, oligo-, and polysaccharides to proteins from different origin on their charge and/or conformation and, consequently, on their technological and biological functionalities. Particularly, β-lactoglobulin (β-lg), the most abundant protein in whey and the one responsible for its functionality, has been one of the most widely studied (*3*−*11*). In general, the initial steps of the MR are preferred since hardly any color formation is produced during the formation of conjugates with increased calcium complexing activity, protein solubility, and heat stability, and improved foaming and emulsifying properties. Likewise, it has been described that by controlling the extent of polymerization and protein cross-linking associated with the advanced stages of the MR, it is possible to obtain glycoconjugates with increased viscosity and improved gelling properties but without excessive color formation (*12*).

In this respect, depending on the functional property, it is of paramount importance to carry out an exhaustive control of the reaction, with temperature, reaction time, initial composition of the system, water activity, and pH, the most key parameters to control the progress of the reaction. Nevertheless, considering the complexity of the MR, it is still needed to gain further insight into those parameters that allow a more thorough control of this reaction. In this sense, the use of inhibitors of the advanced glycation reactions, such as pyridoxamine (PM), a natural intermediate of vitamin B_6 metabolism, may help to control the progress of this reaction. Thus, PM has been demonstrated to be an adequate in vitro and in vivo inhibitor of the transformation of the Amadori compounds to AGEs acting on several pathways of the MR (*13*−*20*). While all of these studies were carried out in solution simulating in vivo physiological conditions using aldoses, scarce data have been reported on the usefulness of PM as an inhibitor of MR in food systems and its impact on protein

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aggregation. Very recently, Arribas-Lorenzo and Morales (*21*) studied the effect of PM on acrylamide formation in a glucose/asparagine aqueous model system, and they attributed its effect to the capacity of PM to trap the colored compounds and precursors formed during the degradation of sugars in the MR.

Furthermore, although it is normally accepted that aldoses are intrinsically more reactive than ketoses, the latter can give rise to greater protein aggregation through covalent cross-linking than aldoses (*11*, *22*−*28*), which is also of interest to investigate the effect of MR inhibitors when ketoses are used to glycate food proteins. Finally, despite the fact that PM has a complex pH-dependent tautomeric equilibrium in solution (*29*), it exhibits a very high proportion of a reactive form at physiological pH in solution. In this sense, it is noteworthy to investigate the inhibitory capacity of PM on the MR during heating in a dry-state in order to broaden its applications.

Therefore, the aim of this work has been the study of the impact of PM on deliberately promoted MRs during the glycation of β-lg with galactose (aldose) and tagatose (ketose) in an attempt to obtain neoglycoconjugates in a dry-state with specific functionalities depending on the progress of this reaction.

MATERIALS AND METHODS

Preparation of β-Lactoglobulin−Galactose/Tagatose Conjugates. Galactose (Gal) or tagatose (Tag) and β-lactoglobulin (β-lg) (mixture of A and B variants) (Sigma-Aldrich, St. Louis, MO), and pyridoxamine in a weight ratio of 1:1:0.24, respectively, were dissolved in 0.1 M sodium phosphate buffer, pH 7 (Merck, Darmstadt, Germany) (*17*). Moreover, with the purpose of studying the effect of PM as an inhibitor of the MR, simultaneous storage was carried out in the absence and presence of such an inhibitor. Then, samples were frozen at −20 °C and lyophilized. The protein−carbohydrate powders were kept at 40 and 50 °C under vacuum in a desiccator equilibrated at a_w of 0.44, achieved with a saturated K_2CO_3 solution (Merck, Darmstadt, Germany). Storage was carried out over a maximum period of 6 days, taking samples at 1, 2, 4, and 6 days in order to obtain products with different glycation and aggregation levels. In addition, control experiments were performed under the same conditions of a_w, time, and temperature with β-lg stored without reducing sugars (control heated β-lg) and with carbohydrates stored in the absence of β-lg (control heated Gal/Tag), with and without PM in both cases. All incubations were performed in duplicate, and all analytical determinations were performed at least in duplicate.

After incubation, the products were reconstituted in distilled water to a protein concentration of 1 mg/mL. To remove free carbohydrates and PM in excess, 2 mL-portions were ultrafiltered through hydrophilic 3 kDa cut off membranes (Centricon YM-3, Millipore Corp., Bedford, MA) by centrifugation at 1548*g* for 2 h. Finally, the retentates were reconstituted in distilled water to a protein concentration of 1 mg/mL, and both, retentates and permeates, were kept at −20 °C for further analysis.

Evaluation of the Progress of the Maillard Reaction. MALDI-TOF-MS analyses of β-lg:Gal/Tag conjugates were performed using a Voyager DE-PRO mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a pulsed nitrogen laser (λ = 337 nm, 3 ns pulse width, and 3 Hz frequency) and a delayed extraction ion source. Ions generated by the laser desorption were introduced into the flight tube (1.3 m flight path) with an acceleration voltage of 25 kV, 93% grid voltage, 0.05% ion guide wire voltage, and a delay time of 350 ns in the linear positive ion mode. Mass spectra were obtained over the m/z range 10−35 ku. Myoglobin (horse heart) and carbonic anhydrase were used for external calibration and sinapinic acid (10 mg/mL in 0.3% trifluoroacetic acid/acetonitrile, 70:30, v/v) as the matrix. Samples were mixed with the matrix at a ratio of approximately 1:15, and finally, 1 μL of this solution was spotted onto a flat stainless-steel sample plate and dried in air.

The initial steps of the MR (formation of the Amadori (tagatosyl-lysine) and Heyns (galactosyl-lysine) compounds in the absence and presence of PM) were monitored by ion-pair RP-HPLC after acid hydrolysis of the dry-heated products. Briefly, 400 μL of 8 N HCl was added to 2 mg of β-lg in hydrolysis tubes and heated at 110 °C for 23 h under inert conditions (helium), followed by the addition of 2 mL of 8 N HCl and filtration through Whatman number 40 filter paper. The filtered hydrolysate (500 μL) was applied to a previously activated Sep-Pak C18 cartridge (Millipore Corp., Billerica, MA, USA). Compounds retained in the cartridge were eluted with 3 mL of 3 N HCl, and 50 μL was used for injection (*30*). Analysis was carried out via an ion-pair RP-HPLC method using a C_8 (Alltech furosine-dedicated; Alltech, Nicolasville, KY) column (250 × 4.6 mm i.d.) and a variable wavelength detector at 280 nm (LDC Analytical, SM 4000, Salem, NH). Operating conditions were as follows: column temperature, 35 °C; flow rate, 1.2 mL/min; solvent A, 0.4% HPLC grade acetic acid in double-distilled water; solvent B, 0.3% KCl in solvent A (*31*). Calibration was performed by using known concentrations (0.52 to 5.2 mg/L) of a commercial pure standard of furosine (Neosystem Laboratories, Strasbourg, France). Data were expressed as mg per 100 mg of protein.

Carbohydrate determination was carried out according to the colorimetric method of anthrone−sulfuric acid using 96-well microtitration plates (Microtest Plate 96-Well. Sarstedt, Inc. Newton, NC) (*32*). Briefly, 40 μL of water (blank), standard (0.05, 0.1, 0.25, 0.5, and 1.0 g/L galactose), or sample (control heated Gal/Tag and β-lg:Gal/Tag conjugates incubated with and without PM for 6 days at 40 and 50 °C) was added to individual wells of the microtitration plate, which was maintained in an ice bath at 4 °C throughout the reaction. Then, 0.1 mL of anthrone solution (2 g/L anthrone solution in concentrated sulfuric acid), freshly prepared, was added to each well; then, the plate was vortex-mixed gently but thoroughly and incubated at 92 °C in a nonshaking water bath. After 3 min, the plate was transferred to a nonshaking water bath at room temperature for 5 min to stop the reaction and then placed into an oven at 45 °C for 15 min to dry off. Absorbance at 630 nm was read using a PowerWave XS microplate spectrophotometer (Bio-Tek, Winooski, VT). A linear curve was obtained within the concentration range used for the standards (0.05−1 g/L) represented by the equation $y = 3.03x + 0.0825$, with 0.999 as the coefficient of determination (R^2).

Browning of control heated Gal/Tag and β-lg:Gal/Tag conjugates (1 mg/mL in double-distilled water) incubated in the absence and presence of PM for 6 days at 40 and 50 °C was measured at room temperature by absorbance at 420 nm in a Beckman DU 70 spectrophotometer (Beckman Instruments Inc., Fullerton, CA), as an index of the brown polymers formed in more advanced stages of nonenzymatic browning (*33*).

Determination of Interaction Products of Pyridoxamine with Galactose or Tagatose. Control heated Gal/Tag and permeates of β-lg:Gal/Tag conjugates incubated in the presence or absence of PM for 6 days at 40 and 50 °C were analyzed by RP-HPLC-UV using the method described by Adrover et al. (*34*). RP-HPLC separations were carried out with a Tracer Excel 120 ODSB (25 × 0.46 cm, 5 μm) (Teknokroma, Barcelona, Spain) at room temperature. Operating conditions were as follows: volume sample loaded, 75 μL; solvent A, 50 mM potassium phosphate buffer (pH 6.0); solvent B, HPLC grade acetonitrile (Scharlau Chemie, Barcelona, Spain). The elution was performed as follows: (i) 0−15 min, 0% solvent B in isocratic mode; (ii) 15−19 min, a linear gradient by increasing the concentration of solvent B from 0% to 2%; (iii) 19−30 min, 2% solvent B in isocratic mode; (iv) 30−35 min, a linear gradient by increasing the concentration of solvent B from 2% to 5%; (v) 35−40 min, a linear gradient by increasing the concentration of solvent B from 5% to 10%; (vi) 40−60 min, a linear gradient by increasing the concentration of solvent B from 10% to 11%. The flow rate was maintained at 0.5 mL/min from 0 to 20 min and at 1 mL/min from 20 to 60 min. Detection was performed at 321 nm to provide the greatest sensitivity for the ionic forms of PM present at pH 6.0.

Determination of the Aggregation State of β-Lactoglobulin−Galactose /Tagatose Conjugates. SEC of β-lg:Gal/Tag conjugates incubated with and without PM for 6 days at 40 and 50 °C was carried out under nondenaturing conditions (0.05 M sodium phosphate buffer, pH 7.3, containing 0.15 M NaCl) using a 24 mL Superdex 75 column HR 10/30 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) on an FPLC system. One hundred microliters of a 1 mg/mL sample was applied to the column at room temperature. Elution was achieved in isocratic mode at 0.8 mL/min for 30 min, and detection of eluting proteins was performed at 214 nm. The standard proteins used for calibration were human serum albumin (67 kDa), ovalbumin (43 kDa), α-chymotrypsinogen (25 kDa), and ribonuclease A (13.7 kDa)

Table 1. Number of Gal and Tag Molecules Bound to Glycated β-lg in the Absence and Presence of PM Estimated by MALDI-TOF-MS

		β-lg:Gal conjugates		β-lg:Tag conjugates	
temperature	incubation time	average number of Gal adducts in the presence of PM	average number of Gal adducts in the absence of PM	average number of Tag adducts in the presence of PM	average number of Tag adducts in the absence of PM
40 °C	1 day	10	12	3	5
	4 days	15	17	10	14
	6 days	18	18	13	15
50 °C	1 day	15	16	8	11
	2 days	17	18	12	14
	6 days	20	22	17	20

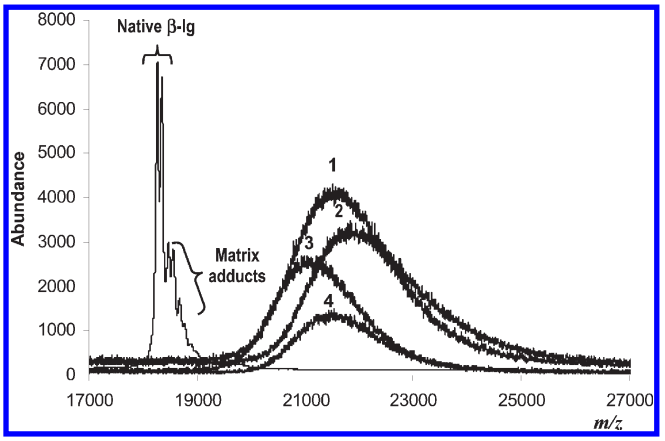

Figure 1. MALDI-TOF-MS spectra of native and incubated β-lg with Gal and Tag at 50 °C for 6 days with and without PM. **1**, β-lg:Gal + PM; **2**, β-lg:Gal; **3**, β-lg:Tag + PM; **4**, β-lg:Tag.

(GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The void volume was determined with Blue Dextran 2000.

RESULTS AND DISCUSSION

Effect of Pyridoxamine on the Initial Stages of the Maillard Reaction during the Glycation of β-Lactoglobulin with Galactose and Tagatose. With the aim of determining the exact number of molecules of carbohydrate linked covalently to the β-lg both in the absence and in the presence of PM, we analyzed the glycoconjugates stored in parallel with and without PM by MALDI-TOF-MS. Mass spectra of such Maillard complexes were characterized by a broad Gaussian peak shape without good resolution because of the great heterogeneity of the glycated forms of β-lg as has previously been described (*11*, *35*, *36*) (**Figure 1**). Considering the maximum of intensity of the Gaussian peaks, the average number of Gal or Tag molecules bound to β-lg after glycation with and without PM was estimated (**Table 1**). In presence of PM, the average number of Gal or Tag bound to β-lg was slightly lower than that in the absence of PM at both temperatures.

To corroborate these results, Amadori and Heyns products were indirectly determined and quantified by ion-pair RP-HPLC following acid hydrolysis, as early indicators of the MR. **Figure 2** shows the evolution of the furoyl (from galactose) and furyl (from tagatose) compounds derived from the Amadori and Heyns products, respectively, upon storage with and without PM at 40 (**A**) and 50 °C (**B**) for 6 days of the β-lg:Gal/Tag conjugates. As expected, faster furoyl and furyl compound formation was observed at 50 than at 40 °C. Moreover, Gal had a higher and faster initial rate of utilization of ε-amino groups of lysine residues than Tag with and without PM at both temperatures. Furthermore, the results showed lower formation rates of Amadori and Heyns products in the presence than in the absence of PM at both temperatures, confirming the MALDI-TOF-MS data. These results could suggest that PM, in addition to its well-described and potent inhibitory capacity of the formation of post-Amadori AGEs, might also have a slight influence on the formation of the Amadori and Heyns compounds. This fact could probably be due to a competitive effect between the primary amino group of PM and the primary amino groups of β-lg for the carbonyl group of Gal or Tag during the early stages of the MR, leading to the delay of Schiff base formation and, therefore, of the corresponding Amadori or Heyns compounds.

In order to demonstrate this competitive effect, the loss of total carbohydrates present in the reaction environment during incubation of control Gal or Tag and β-lg glycated with both carbohydrates in the absence and presence of PM for 6 days at 40 and 50 °C was determined (**Figure 3**). As expected, the decrease in the carbohydrate amount was higher at 50 than at 40 °C because of the acceleration of reaction with the temperature, which is in good agreement with the furosine and MALDI-TOF-MS data. The final concentration of Gal or Tag incubated either with or without β-lg was lower in the presence than in the absence of PM, indicating that the primary amino group of PM would be able to interact with the carbonyl group of Gal or Tag in the presence and in the absence of β-lg. Adrover et al. (*34*), in a kinetic study carried out with PM and glucose under physiological pH and temperature conditions, found that the reaction rate between PM and glucose is similar to previously reported values for reactions in model systems between glucose and several amino acids, indicating, therefore, the possibility of the reaction of PM with reducing sugars. Very recently, Adrover et al. (*37*) indicated in aqueous model systems simulating physiological conditions that PM could exerts its inhibitory action by scavenging carbonyl compounds, exhibiting competitive inhibition with nucleophilic groups in protein side chains. Moreover, our results showed a higher consumption of Gal than Tag throughout the reaction, confirming a greater reactivity of Gal with PM and β-lg as compared to Tag because the aldehyde group of the acyclic form of Gal is more electrophilic than the keto group of the acyclic form of Tag (*11*).

To gain more insight into this subject, the formation of the interaction products between PM and Gal or Tag in the presence of β-lg was confirmed by RP-HPLC-UV (**Figure 4**). The chromatographic profiles of β-lg incubated with PM at 40 and 50 °C in the absence of carbohydrates consisted of a main double peak of a retention time of 8.7 corresponding to PM. However, the profiles of the glycated β-lg in the presence of PM showed several chromatographic peaks corresponding to the products resulting from the interaction between PM and Gal or Tag in the presence of β-lg at both temperatures. Likewise, the peaks corresponding to the interaction products of PM with Gal were more intense than those with Tag, confirming a greater reactivity of Gal than

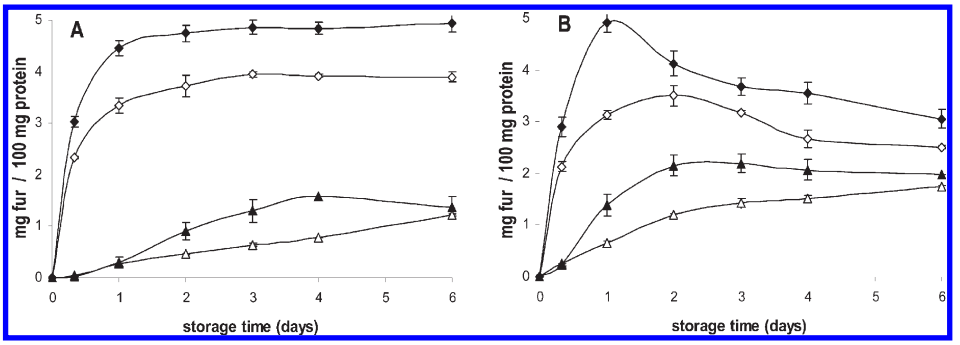

Figure 2. Evolution of the content of 2-furoyl/furyl-methyl-amino acid derived from the Amadori and Heyns products during storage for 6 days in the absence and presence of PM at 40 °C (**A**) and 50 °C (**B**). −◆−, β-lg:Gal; −◇−, β-lg:Gal + PM; −▲−, β-lg:Tag; −△−, β-lg:Tag + PM.

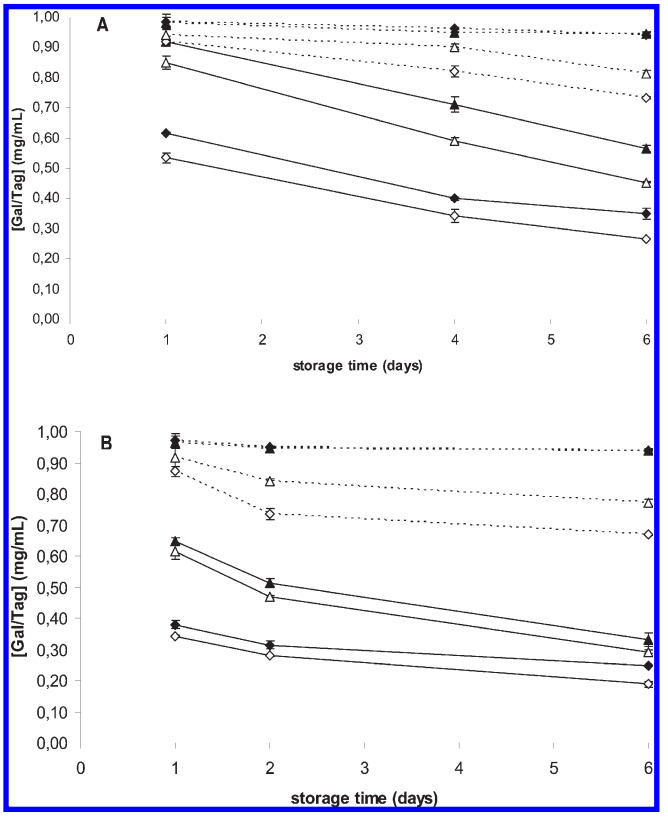

Figure 3. Determination by the anthrone−sulfuric acid assay in a microplate of the loss of carbohydrates during the incubation of the control Gal/Tag and β-lg glycated with Gal/Tag in the absence and presence of PM for 6 days at 40 (**A**) and 50 °C (**B**). --◆--, Gal; --◇--, Gal + PM; --▲--, Tag; --△--, Tag + PM; −◆−, β-lg:Gal; −◇−, β-lg:Gal + PM; −▲−, β-lg:Tag; −△−, β-lg:Tag + PM.

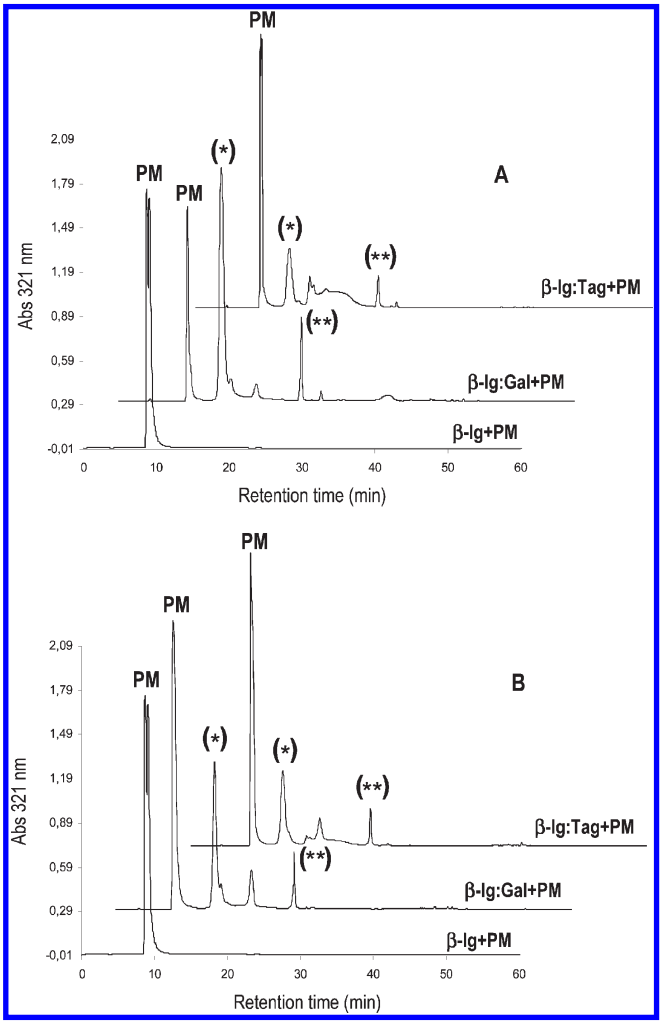

Figure 4. RP-HPLC-UV chromatograms for the reaction between pyridoxamine (PM) and Gal or Tag in the presence of β-lg for 1 day at 40 °C (**A**) and 50 °C (**B**). Labeled peaks are described in the text.

Tag toward PM. These interactions products were also observed when PM was incubated with both carbohydrates in the absence of β-lg under identical reaction conditions (profiles not shown). These compounds have recently been purified and identified by Adrover et al. (*20*, *34*) in several studies on the kinetics of the interaction between PM and various aldoses (hexoses and pentoses) under physiological conditions of pH and temperature. The mechanism of interaction of PM-aldohexoses was elucidated by Adrover et al. (*20*) as follows. Initially, the carbonyl group of reducing sugar in the acyclic form undergoes a nucleophilic attack from the primary amino group in PM, giving rise to a Schiff base, which may evolve in three different ways depending on the nature of the aldohexose: (a) decomposition, by transamination; (b) a double internal cyclization of the carbohydrate portion, giving rise to the tentative structure of the peak marked with a double asterisk in **Figure 4**; and (c) rearrangement into an Amadori compound which, finally, can in turn decompose to give the tentative structure of the peak marked with a single asterisk (**Figure 4**). Last, chromatograms obtained for the reactions of PM with Gal and Tag were qualitatively very similar, suggesting that both reactions might take place via the same mechanism.

Effect of Pyridoxamine on the Advanced Stages of the Maillard Reaction during the Glycation of β-Lactoglobulin with Galactose and Tagatose. In order to evaluate the effect of PM on protein aggregation, the polymerization degree of native, control heated, and glycated β-lg with Gal or Tag in the absence and presence of

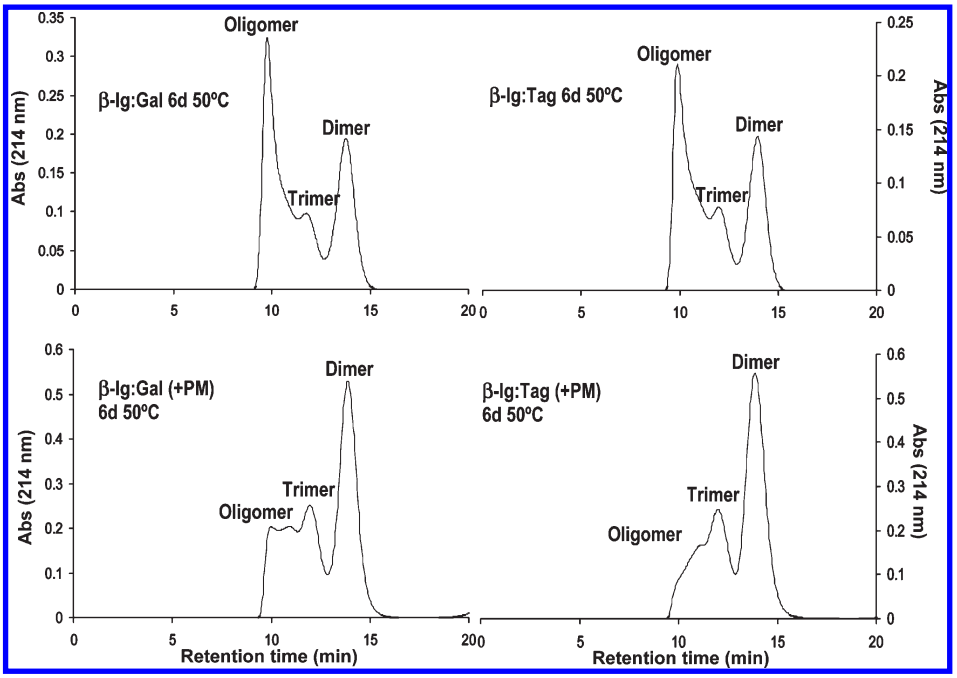

Figure 5. Chromatograms of β-lg glycated with Gal/Tag for 6 days at 50 °C in the absence and presence of PM obtained after its analysis by size exclusion chromatography under nondenaturing conditions.

Table 2. Polymer (Trimeric + Oligomeric Forms)/Dimer Ratios of Native, Control Heated, and Glycated β-lg with Galactose and Tagatose Incubated at 40 and 50 °C for 6 Days in the Absence and Presence of PM, Estimated after Their Analysis by Size Exclusion Chromatography under Nondenaturing Conditions

temperature	sample	storage time (days)	ratio polymer/dimer with PM	ratio polymer/dimer without PM
control	native β-lg	0	0.04 ± 0.003	0.042 ± 0.003
	heated β-lg	4	0.09 ± 0.01	0.13 ± 0.005
		6	0.11 ± 0.004	0.21 ± 0.03
40 °C	β-lg:Gal	1	0.11 ± 0.01	0.19 ± 0.04
		4	0.25 ± 0.04	0.69 ± 0.05
		6	0.43 ± 0.04	0.73 ± 0.08
	β-lg:Tag	1	0.10 ± 0.01	0.19 ± 0.02
		4	0.22 ± 0.04	0.98 ± 0.03
		6	0.40 ± 0.03	0.99 ± 0.01
50 °C	heated β-lg	2	0.05 ± 0.01	0.23 ± 0.06
		6	0.09 ± 0.001	0.30 ± 0.008
	β-lg:Gal	1	0.27 ± 0.05	0.72 ± 0.02
		2	0.49 ± 0.004	1.18 ± 0.03
		6	1.08 ± 0.01	2.46 ± 0.07
	β-lg:Tag	1	0.18 ± 0.01	0.66 ± 0.05
		2	0.37 ± 0.004	1.04 ± 0.06
		6	0.82 ± 0.06	2.18 ± 0.02

PM for 6 days at 40 and 50 °C was investigated by means of SEC under nondenaturing conditions (**Table 2**). As expected, native and control heated β-lg eluted mainly as a dimer with some minor aggregated forms at neutral pH (chromatograms not shown). β-lg:Gal/Tag conjugates incubated in the absence of PM eluted predominantly as a dimer upon storage at 40 and 50 °C for 1 day; however, with increasing incubation time, several peaks corresponding to the trimeric and oligomeric forms appeared, indicating that glycation of β-lg promoted its polymerization, particularly at 50 °C (*11*). In contrast, β-lg:Gal/Tag conjugates incubated in the presence of PM displayed a much lower polymerization degree, suggesting that PM strongly inhibits the cross-linking reactions associated with the advanced stages of the MR. This effect of PM was especially noticeable upon storage at 50 °C for 6 days, which are conditions that strongly favor the protein aggregation (**Figure 5**). Likewise, **Table 2** shows the polymer (trimeric and oligomeric species)/dimer ratios of native, control heated, and glycated β-lg incubated at 40 and 50 °C in the absence and presence of PM. These ratios were much lower for β-lg:Gal/Tag conjugates incubated in the presence of PM than in the absence of PM at both temperatures, indicating, therefore, a lower aggregation degree in the former. Comparing the behavior of the inhibitor with both carbohydrates, as can be seen in **Table 2**, a higher inhibition was detected in the case of the assays carried out with tagatose, particularly at 40 °C. To the best of our knowledge, the present results are the first evidence showing that PM efficiently inhibits the formation on post-Heyns aggregates following protein glycation.

Other compounds such as aminoguanidine (*38*) and D-penicillamine (*39*) have been shown to possess an inhibitory effect on the advanced stages of the MR during the glycation of collagen and ribonuclease A, respectively, under physiological pH and temperature conditions. In these cases, the inhibition of protein aggregation was attributed to the interaction between the amino

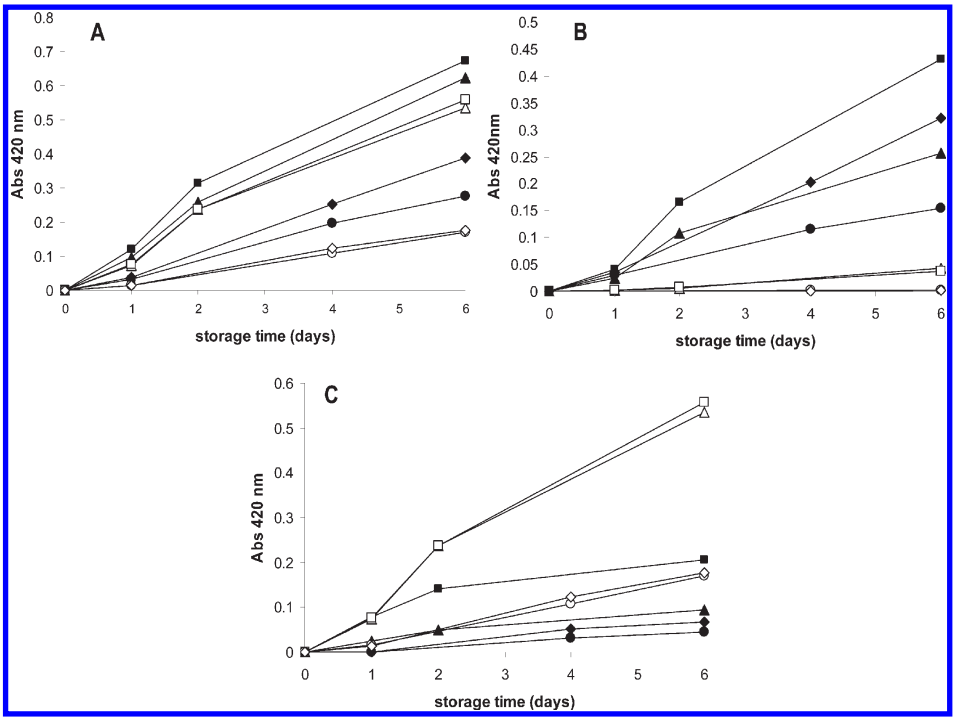

Figure 6. Evolution of absorbance at 420 nm of (**A**) β-lg:Gal/Tag conjugates with and without PM, −■−, β-lg:Gal + PM 50 °C; −▲−, β-lg:Tag + PM 50 °C; −◆−, β-lg:Gal + PM 40 °C; −●−, β-lg:Tag + PM 40 °C; −□−, β-lg:Gal 50 °C; −△−, β-lg:Tag 50 °C; −◇−, β-lg:Gal 40 °C; −○−, β-lg:Tag 40 °C; (**B**) control heated Gal/Tag with and without PM, −■−, Gal + PM 50 °C; −▲−, Tag + PM 50 °C; −◆−, Gal + PM 40 °C; −●−, Tag + PM 40 °C; −□−, Gal 50 °C; −△−, Tag 50 °C; −◇−, Gal 40 °C; −○−, Tag 40 °C; and (**C**) β-lg:Gal/Tag conjugates with and without PM minus the absorbance due to the interaction of PM:Gal/Tag. (**A** − **B** = **C**).

group of inhibitors and the reactive carbonyl intermediates, which act as precursors of aggregate compounds formed during the advanced and final stages of the MR. In addition, according to Adrover et al. (*20*, *34*, *37*), some of the degradation products of the Amadori compounds derived from the interaction of PM-aldoses possess free carbonyl groups that could also react with free amino groups of proteins, preventing additional cross-linking reactions and, hence, the formation of aggregates of high molecular weight.

Strikingly, absorbance at 420 nm of glycoconjugates obtained after incubation with PM was higher than that of conjugates incubated in the absence of PM with both carbohydrates and at both temperatures (**Figure 6A**). This could be attributed to the formation of colored compounds derived from the degradation of PM:Gal/Tag adducts since, as can be observed in **Figure 6B**, control heated Gal/Tag incubated with PM displayed a color development much higher than that of control heated Gal/Tag incubated without PM, which hardly developed color. Likewise, samples of Gal + PM showed higher browning than samples of Tag + PM, confirming the previously mentioned greater reactivity of Gal than Tag with the primary amino group of PM. Thus, **Figure 6C** shows the absorbance at 420 nm of glycoconjugates obtained after incubation with PM minus the color due to PM:Gal/Tag adducts. The browning of conjugates incubated with PM was lower than that of conjugates incubated in the absence of PM, demonstrating, therefore, the inhibitory effect of PM on the advanced and final stages of the MR.

Last, it is well described that protein digestibility and, consequently, amino acid bioavailability may be reduced following glycation, especially when protein cross-linking takes place during the advanced stages of the MR (*40*). In this sense, the use of powerful protein-aggregation inhibitors during the promotion of the MR to obtain glycated food proteins could allow the formation of neoglycoconjugates possessing good functional properties without notably impairing their nutritional quality. Since the advanced stages of the MR can lead to a decrease of safety in many food products, mild processing conditions and food additives may be required in order to suppress the MR in diverse food items. However, up to date, food additives which are considered to be safe for human consumption and can prevent carbonyl reactions in foods are very scarce (*41*).

To conclude, results of the present work showed that PM competes with the free amino groups of β-lg for the carbonyl group of Gal/Tag, slightly delaying the formation of the Schiff base and, therefore, the corresponding Amadori or Heyns compounds. Most significantly, PM also prevents additional cross-linking reactions and, hence, the formation of aggregates with high molecular weight, following glycation with both monosaccharides, particularly with Tag at 40 °C. To the best of our knowledge, this is the first time that PM is proposed to control the glycation of food proteins such as β-lg and that products resulting from the interaction between PM and a ketose, concretely Tag, have been studied. These results are of particular importance in the deliberately promoted glycation of food proteins as a tool to obtain neoglycoconjugates as food ingredients with an improved functionality. Finally, the use of food-grade inhibitors of the advanced stages of the MR, such as PM, combined with the application of relatively mild conditions for protein glycation could lead to the formation of safe neoglycoconjugates with high nutritional value.

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