JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effect of Pyridoxamine on Acrylamide Formation in a Glucose/Asparagine Model System

GEMA ARRIBAS-LORENZO AND FRANCISCO J. MORALES*

Consejo Superior de Investigaciones Científicas, Instituto de Ciencia y Tecnología de los Alimentos y Nutrición (formerly Instituto del Frío), José Antonio Novais 10, 28040 Madrid, Spain

The effect of pyridoxamine (PM) on the reduction of acrylamide (AA) formation in a low-moisture equimolar glucose/asparagine model system was investigated. Formation/elimination kinetics of acrylamide was carried out at temperatures between 120 and 180 °C. Time courses of glucose, asparagine, pyridoxamine, 3-aminopropionamide (3-APA), acrylamide, and browning were measured to get more insight on the mechanism of action of PM. PM exhibited an inhibitory effect on AA formation at all temperatures studied, but became more relevant at 160 and 180 °C (up to 51% reduction). Degradation rates of glucose and asparagine were not significantly affected by PM, but PM was rapidly consumed in the glucose/asparagine system. Browning was significantly suppressed by addition of PM in the system, and formation of 3-APA was increased as compared to control. In comparison with pyridoxal, pyridoxine, and ascorbic acid, PM exerted the highest inhibition activity against AA formation, and a clear dose−response was observed. The nucleophilic aminomethyl group of PM was crucial for the exertion of an inhibition effect more than double those other B6 vitamers. The action mechanism of PM was attributable to its structural features that have the capacity to scavenge intermediary dicarbonyls formed during sugar degradation and advanced stages of the Maillard reaction. These findings open new possibilities for strategies in acrylamide mitigation where formation of reactive dicarbonyls should be carefully considered.

KEYWORDS: Acrylamide; pyridoxamine; Maillard reaction; glucose; asparagine; color; 3-aminopropionamide

INTRODUCTION

Acrylamide (AA) is naturally formed mainly in carbohydrate-rich foods during thermal treatment such as frying, baking, or roasting, and it is largely present in the diet of western countries. Levels of AA in foods suggest potential safety issues relative to its potent mutagenicity and carcinogenity as well as its damages to the central nervous system functions. Thus, the presence of this heat-induced toxicant in foodstuffs has led to public health concern, and intense research is being focused on ways of preventing or minimizing its formation (*1*, *2*).

Since 2002, significant efforts have been conducted to elucidate the chemistry pathways of AA for which the main actors have been identified. Thermal degradation of free asparagine in the presence of reducing sugars or carbonyls following Maillard-type reactions has been proposed as the major route in acrylamide formation (*3*, *4*). It is known that Schiff bases, decarboxylated Amadori compounds, Strecker aldehydes, and glycoconjugates such as *N*-glycosides contribute to the formation of AA (*3*−*5*), via Maillard reaction (MR). Therefore, yields of AA can be reduced by competitive reactions with these key intermediates, suppressing the early steps of the MR. In this way, for instance, it was shown that metal ions inhibited acrylamide formation by impeding the intermediate Schiff base of asparagine, or ferulic acid, from reacting with AA precursors or intermediates; alternative approaches involve adding amino acids that compete with asparagine in the MR such as glycine, reduction by olive oil phenolic compounds, and the addition of bamboo leaves or green tea extracts. Recently, Friedman and Levin (*2*) published a comprehensive review of the different strategies of mitigation applied.

Vitamin B_6 is a water-soluble vitamin that exists in three major chemical forms: pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM). The phosphate ester derivative pyridoxal 5′-phosphate (PLP) is the principal coenzyme form and has the most importance in human amino acid and protein metabolism and red blood cell metabolism; it is mainly present in fruits, vegetables, cereal grains, meat, poultry, and fish (*6*).

In recent years, PM has attracted increasing attention because it is a pharmacological agent used for the treatment of multifactorial chronic diseases, such as diabetes or atherosclerosis complications, by inhibiting the MR and reducing the pathogenicity of carbonyl compounds (*7*). PM also prevents renal and vascular pathology and hyperlipidemia in the Zucker obese nondiabetic rat model (*8*). In addition, as recently reviewed by Friedman and Levin (*2*), it has been pointed out

* Corresponding author (telephone +34 91 549 23 00; fax +34 91 549 36 27; e-mail fjmorales@if.csic.es).

10.1021/jf802870t CCC: $40.75
Published on Web 01/14/2009

that PM might be a possible inhibitor of AA in food, so investigations should be conducted to clarify this point.

Furthermore, PM has been described as a post-Amadori inhibitor capable of inhibiting the formation of advanced glycation end-products (AGEs) in vivo and in vitro (*9, 10*) as well as advanced lipoxidation end-product (ALEs) (*10, 11*), but its reaction mechanisms are yet subject to debate. Its inhibition properties involved in the MR are a consequence of its reactivity due to the phenol and aminomethyl group at pyridinium ring, respectively. In addition, there has been a previous study demonstrating that vitamin B_6 supplementation can delay and reduce the severity of the neurotoxicity caused by acrylamide (*12*).

Previous knowledge supports the idea that PM could act as an inhibitor of AA formation in foods. Its demonstrated inhibitory effects in vivo have not been studied in foods so far. The main purpose of this investigation was, therefore, to evaluate whether this natural intermediate of vitamin B_6 capable of inhibiting AGE formation could mitigate AA formation in foods systems as well. Another objective was to gain information about the action mechanism of PM on reactions that involve acrylamide formation. The inhibition rates of PM were compared with those of PN and PL as well as vitamin C as reference. In the present paper the main changes in glucose, asparagine, and 3-aminopropionamide (3-APA) and the extent of browning as well as the changes in the PM, subjected to rate of disappearance or destruction due to a heating process, were considered.

MATERIALS AND METHODS

Chemicals. Acrylamide (99%), D-(+)-glucose, dansyl chloride (5-[dimethylamino]naphthalene-1-sulfonyl chloride), pyridoxine hydrochloride, pyridoxal hydrochloride, and *o*-phthaldehyde (OPA) were purchased from Sigma (St. Louis, MO). Heptafluorobutyric acid and pyridoxamine dihydrochloride were from Fluka Chemicals (Madrid, Spain). L-Asparagine monohydrate, glycine, β-mercaptoethanol, methanol, and acetonitrile (HPLC grade) were bought from Merck (Darmstadt, Germany). Boric acid, sulfuric acid (96%), formic acid (98%), sodium phosphate monobasic, and sodium bicarbonate were from Panreac (Madrid, Spain). β-Alaninamide hydrochloride (97%) was from ABCR GmbH & Co. KG (Karlsruhe, Germany). Ultrapure water was used (Milli-Q system, Millipore Bedford, MA). Oasis-HLB cartridges (30 mg, 1 mL) were supplied by Waters (Milford, MA). [$^{13}C_3$]Acrylamide (isotopic purity 99%) was from Cambidge Isotope Laboratories (Andover, MA). Glass vials with septum screw caps were supplied by Agilent Technologies (Wilmington, DE).

Preparation of Model Systems. *Kinetic Model. (a) Glucose and Asparagine (Glc/Asn).* Reaction mixtures were prepared with equimolar quantities (1:1) of Glc and Asn (0.3 mmol each). To obtain a homogeneous mixture, both reactants were carefully dry-mixed in a mortar until a powder was obtained. Aliquots of the mixtures (99.1 mg) were transferred to Pyrex hydrolysis tubes (10 cm × 0.9 mm i.d.) with 100 μL of 0.1 M NaH_2PO_4 buffer (pH 6.8), tightly capped, and heated in an oil bath at temperatures of 120, 140, 160, and 180 °C for various times. Temperature (reference temperature ± 1 °C) was previously calibrated, with external thermocouples (type K, 0.1 mm) coupled to a datalogger. The heated samples were cooled immediately in iced water to stop any further reaction. Samples were kept at 4 °C for analysis within the same working day or frozen for further analysis. Experiments were prepared at least in duplicate.

(b) Glucose, Asparagine, and Pyridoxamine (Glc/Asn/PM). The Glc/Asn reaction mixture was modified by adding pyridoxamine (0.03 mmol) dissolved in 100 μL of 0.1 M NaH_2PO_4 buffer (pH 6.8). The heating experiment was worked up as previously described. Experiments were prepared at least in duplicate.

(c) Dose Effect. Glc (0.3 mmol) and Asn (0.3 mmol) were mixed and transferred to Pyrex tubes, and variable concentrations of pyridoxamine, pyridoxine, pyridoxal, and vitamin C were added (0, 5, 10, 20, and 30 μmol) dissolved in 100 μL of 0.1 M NaH_2PO_4 buffer (pH 6.8). The experiment was heated at 140 °C for 30 min and cooled in ice−water. Samples were kept at 4 °C for analysis within the same working day or frozen for further analysis. The experiments were run at least in triplicate.

Acrylamide Analysis. One milliliter of distilled water was added to the heated mixtures, and the reaction solution was gently vortexed and centrifuged for 5 min at 4000 rpm for removing insoluble particles, if any. Appropriate dilution (100-fold) was made using distilled water and then purified on an Oasis-HLB solid phase extraction cartridge. The cartridges were washed with 1.0 mL of methanol and 1.0 mL of water prior to use. The sample dilution (1.0 mL) was loaded on the cartridge, and the first drops were discharged. The eluate was filtered through a 0.45 μm filter and subjected to a liquid chromatography−diode array detector (LC-DAD). The results were confirmed by using liquid chromatography−mass spectrometry (LC-MS).

The quantification of acrylamide was conducted with a Shimadzu HPLC system (Kyoto, Japan) equipped with a LC-20AD pump, a SIL-10ADvp autosampler, a CTO-10ASVP oven, and a DAD (SPD-M20A). The chromatographic separations were performed on an Inertsil ODS-3 column (250 × 4.6 mm, 5 μm). The mobile phase condition was isocratic at 100% ultrapure water at a flow rate of 0.6 mL/min at 32 °C. Acrylamide was detected at 210 nm. The quantification of AA was performed using a calibration curve in the range of 5−1000 μg/L. The limit of quantitation (5 μg/L) was similar to that found by other authors (*13*).

Confirmatory LC-ESI-MS analysis were performed as described by Rufián-Henares et al. (*14*) using an Agilent 1100 HPLC system (Waldbronn, Germany) consisting of a quaternary pump, an autosampler, and a temperature-controlled column oven, coupled to an Agilent 1100 MS detector equipped with an electrospray ionization interface. The analytical separation was performed on an Inertsil ODS-3 column (250 × 4.6 mm, 5 μm; GLC-Sciences Inc., Kyoto, Japan) using an isocratic mixture of 0.2% aqueous solution of formic acid at a flow rate of 0.6 mL/min at 25 °C. Data acquisition was performed, with a delay time of 8 min, in a selected ion monitoring (SIM) mode using the following interface parameters: drying gas (N_2, 100 psig) flow of 12 L/min, nebulizer pressure of 45 psig, drying gas temperature of 350 °C, capillary voltage of 3 kV, and fragmenter voltage of 70 eV. Monitored ions were *m*/*z* 72.1 for acrylamide and *m*/*z* 75.1 for $^{13}C_3$-labeled acrylamide. An acrylamide calibration curve was built in the range of 2−100 μg/L. The accuracy of the procedure was recently demonstrated for potato crisps in an interlaboratory comparison study organized by the Institute of Reference Materials and Measurements, yielding a *z* score of −0.5. The analyses are integrated within the scope of a certified laboratory controlled by AENOR (Spanish Association for Standardization and Certification).

Analysis of 3-Aminopropionamide (3-APA). 3-APA was analyzed according to the procedure reported by Bagdonaite et al. (*15*) with some minor modifications. Samples (200 μL) were dissolved in 200 μL of 0.5 M $NaHCO_3$ (pH ~8) and then derivatized with dansyl chloride. To 100 μL of the mixture was added 100 μL of dansyl chloride solution (5 mg/mL in acetone) in a test tube. The mixture was vigorously mixed for 3 min and left in the dark overnight. Twenty microliters of a glycine solution (100 mg/mL) was added, vortexed for 1 min, and left for 15 min. The extraction of the sample was done with 1 mL of diethyl ether, twice. The combined extracts were dried with nitrogen, and the residue was dissolved in 1 mL of acetonitrile and filtered at 0.45 μm. A Shimadzu HPLC system (Kyoto, Japan) equipped with an LC-20AD pump, an LC-20AD/AT low-pressure gradient former, a SIL-10ADvp autosampler, a CTO-10ASVP oven, and an RF-10AxL fluorescence detector controlled by a CBM-10A communication bus module was used. The chromatographic separations were performed on a Mediterranea-Sea-C18 analytical column (25 × 0.40 cm, 5 μm, Tecknokroma, Barcelona, Spain) using a gradient elution of ultrapure water (phase A) and acetonitrile (phase B) at a flow rate of 0.8 mL/min at 32 °C. Data acquisition was performed by acquiring chromatograms at an excitation wavelength of 320 nm and an emission of 500 nm. The column was equilibrated in 90% phase A and 10% phase B; the gradient was as follows: time 0−2 min, 30% B; time 12 min, 70% B; time 20 min, 70% B; time 21 min, 10%, held until the end of the run (30 min). The quantification of 3-APA was performed using a calibration curve. Stock solution of 3-APA was prepared at a concentration of 10000

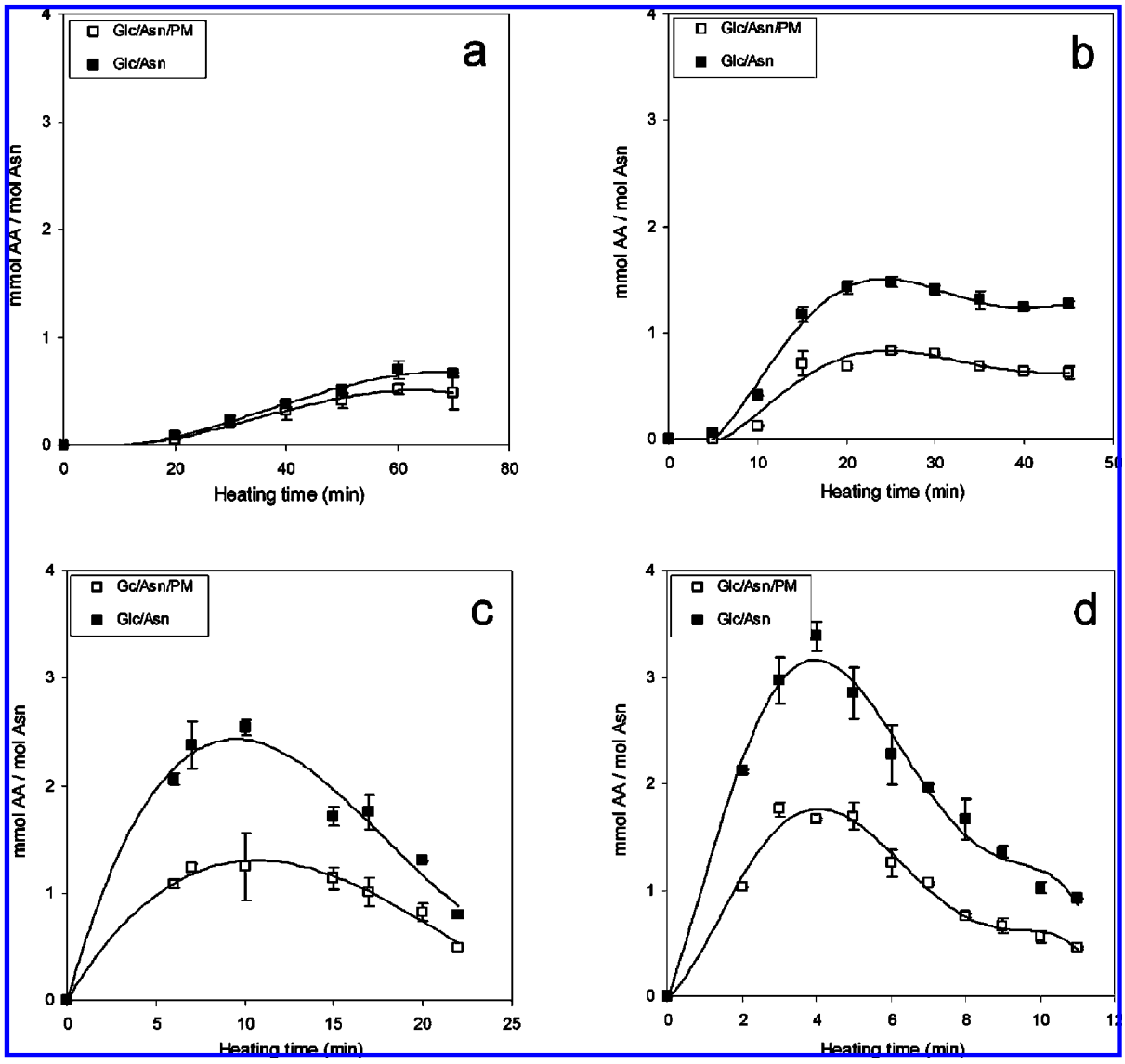

Figure 1. Kinetics curve of acrylamide formation and elimination in glucose/asparagine model system (solid box, control) and in glucose/asparagine/pyridoxamine model system at 120 °C (**a**), 140 °C (**b**), 160 °C (**c**), and 180 °C (**d**).

μg/L dissolved in 0.25 M $NaHCO_3$ (pH 8). Working standards were freshly prepared by diluting the stock solution to concentrations of 100, 200, 300, 400, 500, 750, and 1000 μg/L.

Determination of Glucose (Glc). Samples were diluted (1:10) with distilled water and then analyzed for Glc by HPLC-RI, consisting of an MD-420 pump, an MD-465 autosampler, a refractive index detector (Erma Inc., Tokyo, Japan), and a temperature-controlled column oven, all from Kontron Instruments (Milan, Italy). The chromatographic separations were performed on an ION-300 polymeric resin column (300 mm × 7.8 mm, Interaction-Laboratory, San Jose, CA) at 50 °C. A sulfuric acid solution (1 μM) was used as eluent at 0.4 mL/min. Glucose was recorded with a refractive index detector (RID-10A, Shimadzu, Tokyo, Japan) and quantified by the external standard method within the range of 0.01−0.8 g/100 mL.

Determination of Asparagine (Asn). Asn was analyzed in a plate reader by an automated procedure. A 20 μL aliquot of sample and 180 μL of 0.1 M borate buffer (pH 10) were placed per well in a 96-well microplate (Biogen Científica, Madrid, Spain). Sample was previously diluted in borate buffer (1/1000). The plate-reader automatically dispensed 50 μL of OPA solution. The microplate was shaken for 15 s, and the fluorescence was recorded at 360 and 460 nm excitation and emission wavelengths, respectively. A Synergy HT-multimode microplate reader with automatic reagent dispenser and temperature control from Biotek Instrumens (Winooski, VT) was used. Biotek Gen5 data analysis software was used. The OPA solution was daily prepared and

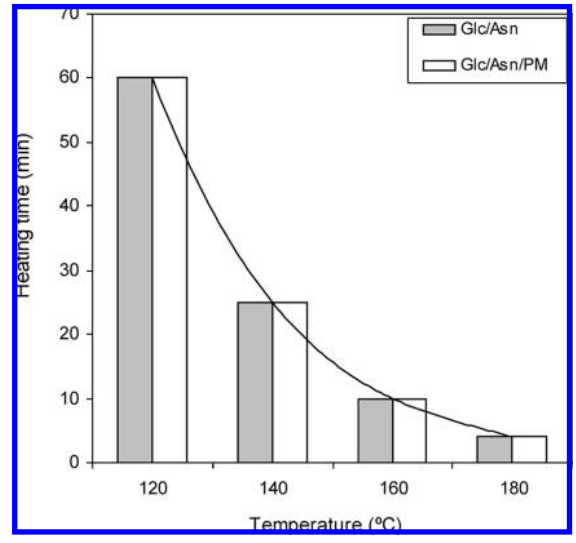

Figure 2. Time necessary (min) to reach the maximum level of acrylamide formation at each temperature investigated in the glucose/asparagine (Glc/Asn) and glucose/asparagine/pyridoxamine (Glc/Asn/PM) model systems.

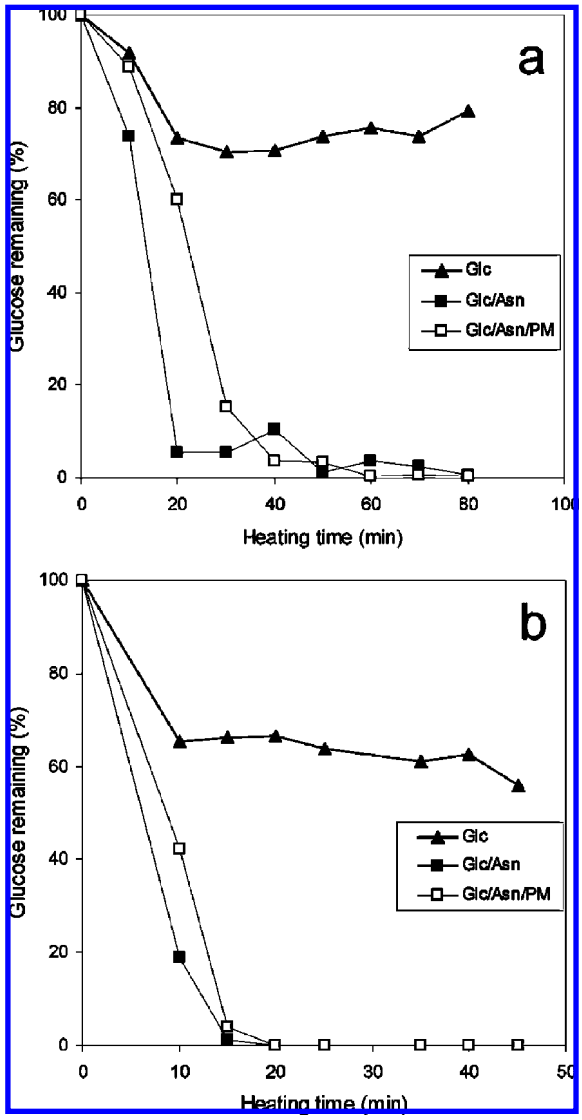

Figure 3. Reactivity of glucose (expressed as percentage of remaining glucose) in the glucose, glucose/asparagine, and glucose/asparagine/pyridoxamine systems at 120 and 140 °C. Residual standard deviation was below 5%.

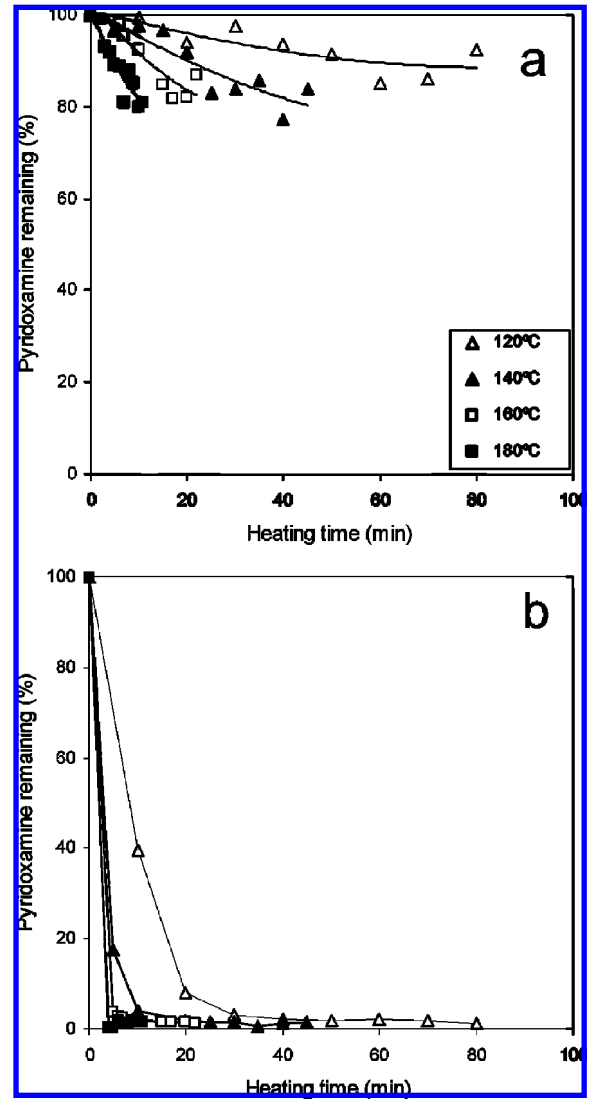

Figure 4. Reactivity of pyridoxamine (expressed as percentage of remaining pyridoxamine) in a pyridoxamine system (**a**) and in a glucose/asparagine/pyridoxamine system (**b**). Residual standard deviation was below 3%.

100-fold diluted with borate buffer. The OPA stock solution (stable for 1 week) was prepared 6 h before use and stored in the dark at 4 °C and contained 100 mg of *o*-phthaldehyde dissolved in 1.0 mL of methanol, 500 μL of β-mercaptoethanol, and 8.5 mL of 0.1 M borate buffer (pH 10). A blank using borate buffer instead of the sample and calibration solutions (10, 25, 50, 100, 200, and 300 mM asparagine) was used in each assay. Asparagine solutions were also diluted 1000-fold with borate buffer.

Analysis of Pyridoxamine. PM was quantified by a HPLC−fluorescence according to the method of Nagaraj et al. (*16*). Sample (10 μL) was injected into a Kromasil column (250 mm × 4 mm, 5 μm, Sugerlabor, Madrid, Spain). The samples were appropriately diluted with distilled water. The vitamin was eluted in a gradient with 5% heptafluorobutyric acid (phase A) and 100% acetonitrile (phase B) at 1 mL/min. The column was equilibrated in 95% phase A and 5% phase B. The elution program was as follows: time 0−1 min, 5% B; time 10 min, 20% B; time 11 min, 40% B; time 12 min, 40% B; time 20 min, 5% B; time 45 min, 0% B. Data acquisition was performed by acquiring chromatograms at an excitation wavelength of 290 nm and an emission wavelength of 395 nm. The quantification of pyridoxamine was performed using a calibration curve. Stock solution of PM was prepared at a concentration of 130 μg/mL.

Measurement of Browning. Two hundred microliters of reaction mixture was placed per well in a 96-well microplate (Biogen Científica). Browning was measured at a wavelength of 420 nm after appropriate dilution. The instrument for determining A_{420} was a microplate reader (BioTek Instruments).

Statistical Analysis. Data were analyzed using Microcal Origin, version 7.5 (Origin Laboratory Corp., Northampton, MA). Values are presented as means ± SD. Differences with $P < 0.05$ were considered to be statistically significant using the Tukey test.

RESULTS AND DISCUSSION

Effect of Pyridoxamine on the Formation of Acrylamide. Two model systems (Glc/Asn, Glc/Asn/PM) were designed for studying the fate of PM on the chemistry of AA formation. Systems were prepared under low-moisture conditions in closed glass tubes to resemble the processing of cereals under baking conditions and heated at 120−180 °C for different times. The amount of water present in the matrix has an important effect on the yield of AA, being dramatically reduced in both dry systems and with water content higher than 25%. Model systems of Asn and substances with carbonyl residues, both in dry and aqueous solutions, have been largely used to elucidate the pathways of acrylamide formation (see refs *2*, *5*, *and 13*).

Figure 1 illustrates AA formation in the presence of PM (Glc/Asn/PM) as compared with a control model system (Glc/

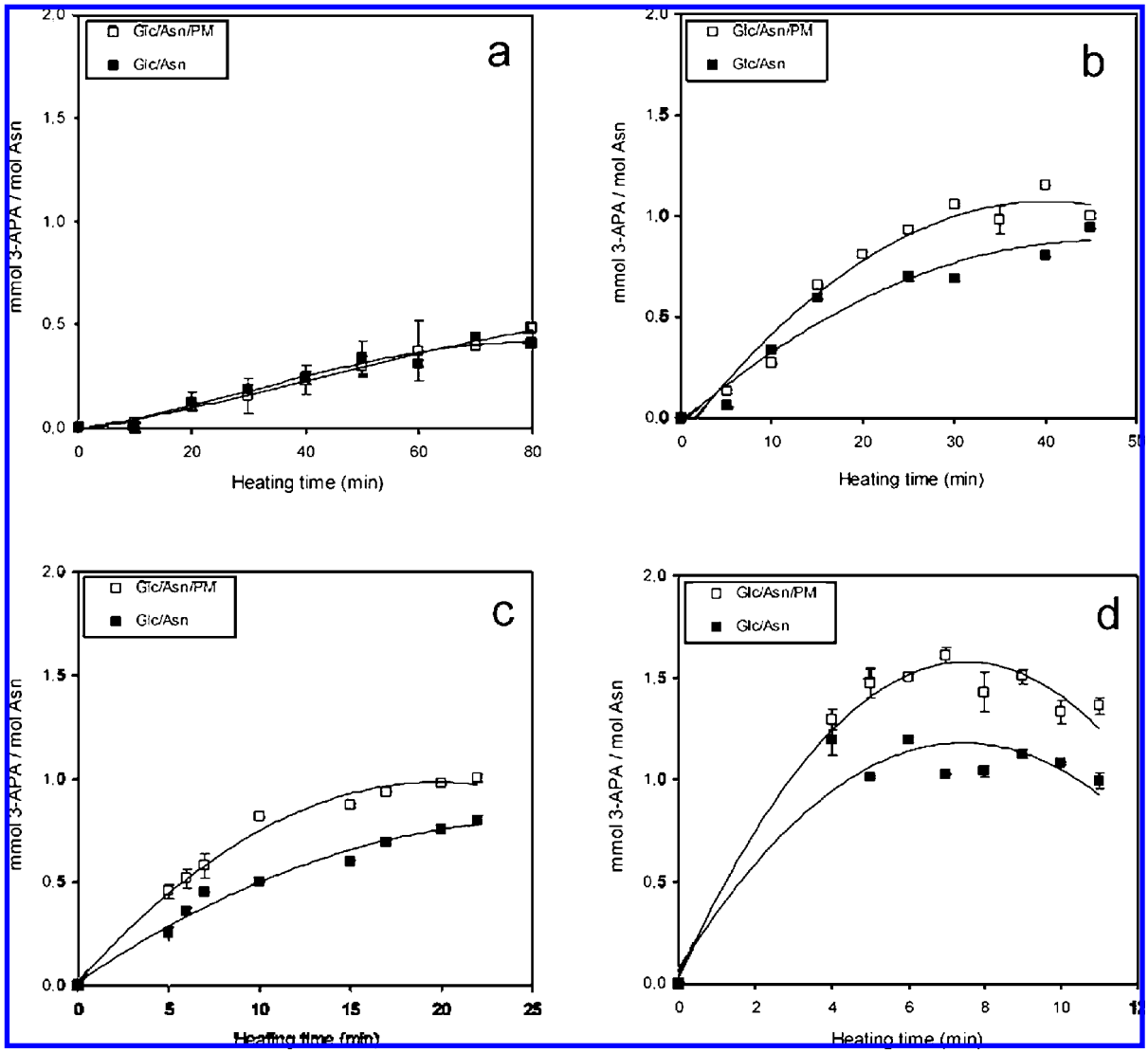

Figure 5. Formation of 3-aminopropanamide in glucose/asparagine model system (solid box; control) and glucose/asparagines/pyridoxamine model system at 120 °C (**a**), 140 °C (**b**), 160 °C (**c**), and 180 °C (**d**).

Asn) without the addition of PM at different temperatures and times. Classical kinetics of AA in the MR describe both the formation and elimination processes for whcih several different mathematical models have been drawn to elucidate the process and follow two consecutive first-order reactions (see refs *13 and 17*). Kinetic modeling of AA formation/elimination is not the aim of this paper. Results obtained for the Glc/Asn model system are in line with results in model systems (see ref *18*) and foods (*19*) reported in the literature, where prolonged heating times and high temperatures decreased the net formation of AA. AA has two reactive sites, the conjugated double bond and the amide group, and it has been proposed that losses of acrylamide are due to polymerization, evaporation, and Michael addition reactions with other food constituents (*5*). At 120 °C a plateau was observed after 60 min of heating where the formation and elimination of AA are in balance. Inhibition of AA by PM was observed at all temperatures but become relevant at temperatures >120 °C. As expected, the time to reach the maximum level of acrylamide was reduced by increasing temperature, being 60, 30, 10, and 4 min for 120, 140, 160, and 180 °C, respectively, but the time to reach the maximum level of AA for each temperature remained equal in both systems and was not affected by the addition of PM as depicted in **Figure 2**.

Becalski et al. (*17*) reported in dry systems that the amount of AA was reduced when the temperature was increased from 155 to 185 °C, at least when heating was longer than 10 min. It is clear that AA is an intermediary of the MR rather than an end-product, which implies that it is also subject to further degradation reactions as has been clearly shown by kinetic modeling (*13*). If percentages of AA reduction are compared at the time at which the highest AA was formed, inhibition rates of 25, 44, 51, and 51% were obtained at 120, 140, 160, and 180 °C, respectively. Inhibition of AA by PM was more effective at higher temperatures, at which up to 51% of reduction was obtained, which is promising for its further application during baking of cereal products.

In line with the literature, with increasing temperature, the reaction rate of both AA formation and elimination increased (*13, 20*), but it is worth noting that the curves of both Glc/Asn and Glc/Asn/PM systems showed the same rate of acrylamide formation/elimination. A correlation was observed between the time necessary to reach the maximum AA yields at each temperature, providing supportive evidence that was described before (**Figure 2**), and it was not affected by the presence of PM. In line with previous studies, time and temperature drive AA formation (*19, 21*), but PM did not induce new chemical pathways for AA and just a net reduction in the reaction rate was suggested.

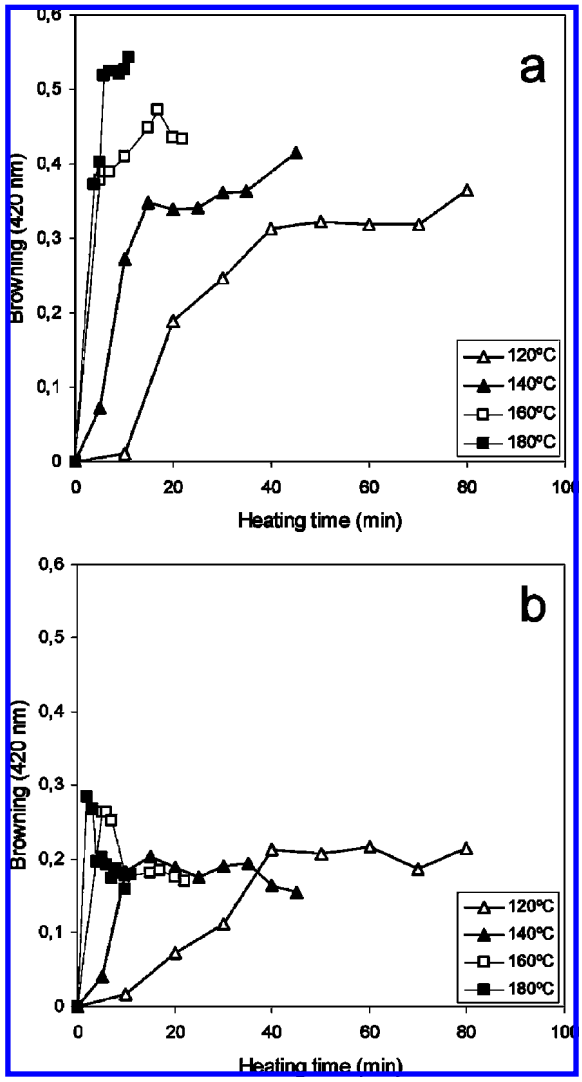

Figure 6. Browning (expressed as absorbance at 420 nm) in a glucose/asparagine system (**a**) and in a glucose/asparagine/pyridoxamine system (**b**). Residual standard deviation was below 7%.

Effects of PM on Glc, Asn, 3-APA, and Browning. For better understanding of whether the presence of PM in the reaction mixture influences the mechanism of reaction for AA formation, Glc and Asn were analyzed as reactants. As expected, consumption of Glc was increased with the presence of amino groups, and the reaction was rapidly catalyzed at high temperatures. **Figure 3** shows the glucose remaining (percent from initial glucose content) in Glc, Glc/Asn, and Glc/Asn/PM model systems. The rate of glucose decomposition was just slightly reduced at 120 °C (**Figure 3a**) and 140 °C (figures **3b**) by the addition of PM. At higher temperatures, the reaction took place so quickly that the potential effect of PM on Glc degradation could not be observed or quantified (data not shown). At physiological conditions it had been demonstrated that PM was able to trap hexoses and pentoses by forming a Schiff base and finally degrades into unknown compounds and pyridoxal (*22*), but this situation cannot be extrapolated to classical cooking temperatures.

In the case of degradation of Asn, an increase in the temperature led to higher losses as time increased in the Glc/Asn model, but Asn remained when heated alone (data not shown). These results are in agreement with previous papers (*13, 23*). In contrast, the degradation rate of Asn was not significantly affected by the addition of PM regardless of the temperature applied. Average values of 30, 21, 17, and 9% for remaining Asn were obtained at 120, 140, 160, and 180 °C, respectively. Moreover, in the Glc/Asn model system, Glc was consumed more rapidly than Asn, which is in line with results reported in the literature (see ref *13*). This fact can be explained by the regeneration of Asn from the initial condensation products such as the Amadori rearrangement product and the possible formation of diglucosylamine (*10, 24*). It was concluded that PM did not influence significantly the rates of glucose and asparagine consumption, and its effect on AA formation would take place in a more advanced stage of the reaction.

The thermal stability of PM was also evaluated in the presence of Glc and Asn, where thermal decomposition of PM is limited when heated alone (**Figure 4a**). Degradation was found to be up to 20% depending of the temperature and time, indicating that most of the PM remains reactive. However, degradation of PM is rapidly enhanced in the presence of Glc and Asn in the reaction media, being almost consumed after only a few minutes (**Figure 4b**) because PM is participating in the advanced stages of the MR. At 160 and 180 °C a complete loss of PM was observed after just 2 min of reaction. Then, PM reacted very efficiently in the Glc/Asn reaction system, where PL and PN are also detected as reaction products in the chromatogram profile as well as other minor unknown compounds.

Previous studies have demonstrated that 3-APA is a transient intermediate in AA formation during thermal treatment (*15, 25*). Furthermore, it may also be formed by enzymatic decarboxylation of Asn (*26*), via decarboxylation of the Schiff base (*4*), and through the Schiff base formed from Asn and α-hydroxycarbonyl (*27*) or reaction with pyruvic acid (*5*). This point was confirmed in the present study when the concentration of 3-APA in the reaction mixtures clearly showed an exponential increase followed by a decrease after prolonged time depending on the temperature (**Figure 5**). When the temperature was increased, 3-APA yields increased in both model systems. However, the presence of PM did not show an inhibitory action on 3-APA; in contrast, PM enhanced the net formation of 3-APA at 140, 160, and 180 °C (no significant effect was found at the lowest temperature). These data suggest that the action mechanism of PM could be taking place at this step, blocking the reaction of 3-APA through AA or blocking other routes of AA formation (such as degradation of the Amadori products or Schiff base and formation of reactive intermediary compounds) through the formation of 3-APA.

Browning is a classical feature of the extent of the MR in its advanced/final stages, and it has been directly related to AA formation in both model systems and foods (see ref *28*). **Figure 6** depicts the extent of browning in the control model system and with the addition of PM. In the Glc/Asn model (**Figure 6a**), browning increased rapidly with temperature until a maximum; after that, browning remains more stable with time. Results are in line with previous studies (*17*). Similar behavior was observed in the Glc/Asn/PM system (**Figure 6b**). Browning was also directly related with the temperature, where a maximum is obtained. At 120 and 140 °C, browning increased steadily, whereas at 160 and 180 °C the absorbance reached a peak and finally decreased followed by a plateau. However, browning was nearly 2-fold less in the presence of PM. These findings indicate that PM strongly reduces the advanced and final stages of the MR and, subsequently, PM action on AA is connected to its inhibitory effect on the activity of the MR and the formation of intermediary reactive compounds as reported for in vivo studies.

Dose−Response and Structure-Related Effect of PM on AA Formation. The dose−response of different vitamins on AA

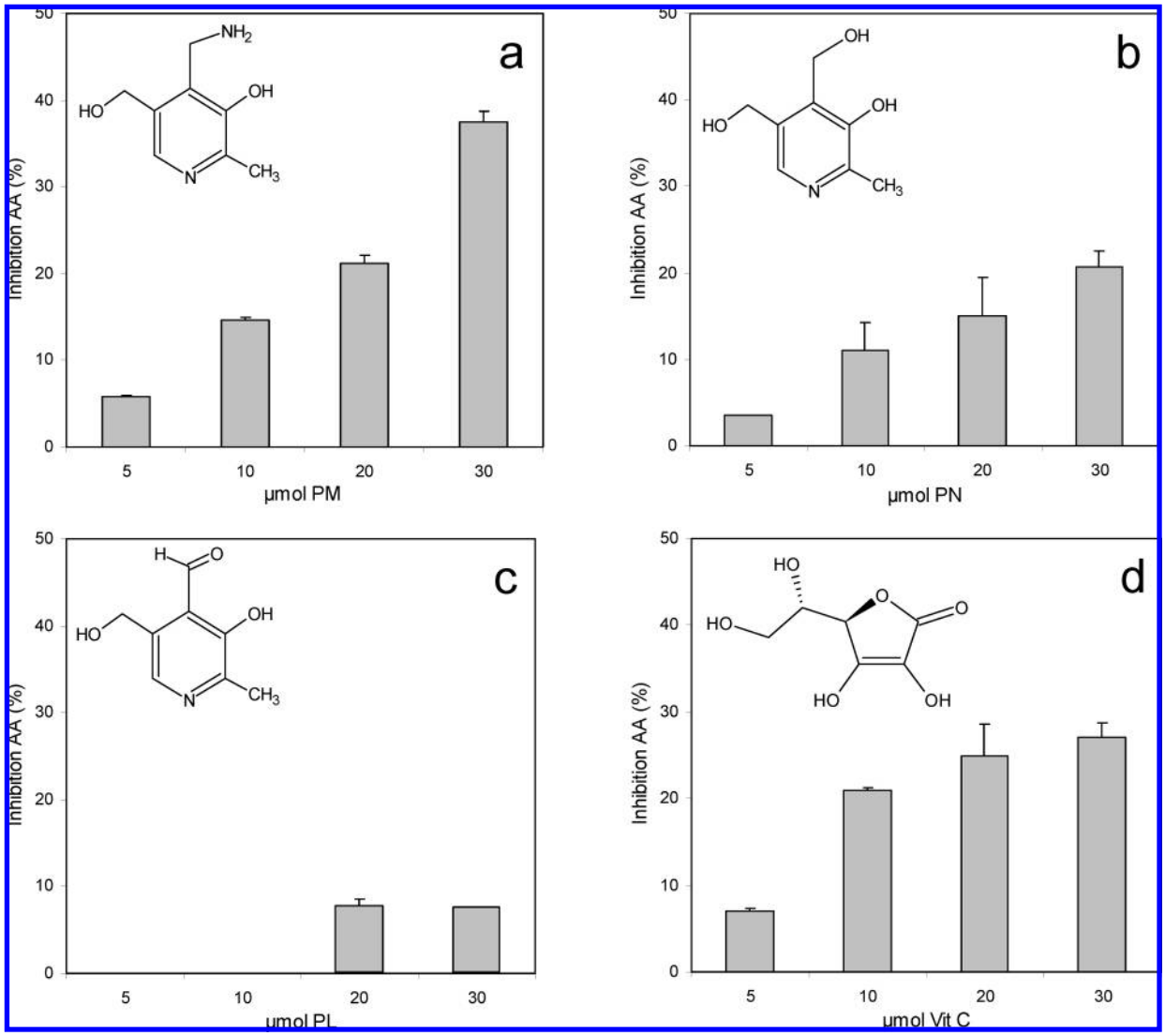

Figure 7. Dose−response effect of PM on the acrylamide inhibition (percentage from control) in a glucose/asparagine model system with added pyridoxamine (**a**), pyridoxine (**b**), pyridoxal (**c**), and ascorbic acid (**d**).

formation was investigated. For this purpose, three B_6 derivates (PM, PN, PL) and vitamin C at different concentrations (5, 10, 20, and 30 μmol) were added to the Glc/Asn model system and heated at 140 °C for 30 min. Results are depicted in **Figure 7** and are expressed as percentage of AA inhibition as compared with the control system without the addition of vitamin. All of the compounds showed inhibition activities to some extent, except for pyridoxal at concentrations of 5 and 10 μmol, which did not show significant differences from control. However, a dose-dependent response for PM, PN, and vitamin C was observed. At the highest concentration tested (30 μmol), PM, PN, PL, and vitamin C reduced the formation of AA by 38, 21, 8, and 27%, respectively. Among the vitamers examined, PM provided the highest inhibition rates followed by vitamin C and pyridoxine. Pyridoxal (the alcohol form of vitamin B_6) appeared to inhibit acrylamide formation only very slightly. On the other hand, vitamin C showed a moderate inhibition, confirming the protective action against acrylamide found in previous paper, mainly owing to lowering of the pH (*19*, *29*). It is observed that the dose−response for vitamin C is not linear and a degree of saturation is observed at 30 μmol. However, the activity of PM increased linearly with time, showing a remarkable dose−response.

It is interesting to compare the different responses observed between the inhibition rates for the three forms of vitamin B_6. This finding could be attributed to a structure-related effect (**Figure 7**). It has been demonstrated that in the case of PL the inhibition involves competitive Schiff base condensation of the aldehyde group with protein amino groups at glycation sites (*30*). In this respect, the inhibition exerted by PM necessarily follows a different pattern, as it lacks an aldehyde group as described below. In contrast, some authors have reported that PN is not an AGE inhibitor (*9*). The structural features of PM are critical in the catalysis of transamination reactions, where important residues are the phenolic hydroxyl group and the aminomethyl group at positions 3 and 4 of the pyridinium ring, respectively. PM was effective in blocking carbonyl compounds; the amino group reacts with the carbonyl moiety of α-keto acid, which is the key point of its usefulness for AA mitigation. Then, PM can act through nucleophilic reactions with carbonyl intermediates in the MR, autoxidation of carbohydrates, or lipid peroxidation.

It has been frequently reported that PM is a potent inhibitor of the formation of AGEs (*9*, *10*). This discovery stimulated our interest in this B_6 vitamer as a possible inhibitor of AA because the formation of this compound is closely linked to the MR. AA formation involves the condensation between carbonyl groups of reducing sugars with amino groups to form *N*-glycoconjugate, which is in equilibrium with the Schiff base (*3*). Recently, it has been described that the Schiff base

mentioned is the first step in the formation of AA (*4*, *5*). Our results suggest that PM did not affect this early step of the MR because it did not greatly influence the decomposition of the reactants at 140, 160, and 180 °C. The effect of PM in the degradation rates of Glc and Asn was residual.

Previous studies indicated that α-hydroxycarbonyls are much more efficient than α-dicarbonyls in converting asparagine into AA (*5*), but opposing evidence has also been reported recently. Yuan et al. (*31*) showed that almost 80% of AA was formed through participation of α-dicarbonyls such as methylglyoxal. Similar results were obtained by Zyzak et al. (*4*) studying AA formation in a semidry food model system, showing that a variety of carbonyl sources (glucose, ribose, glyceraldehyde, glyoxal) could generate AA from Asn under heating. The reactivity of carbonyl is higher when the sugar chain is shorter; glyoxal becomes 3-fold more reactive than glucose on a molar basis. Then, discrepancies could be due to the water content in the reaction medium used when the reaction is limited in dried systems at pyrolysis conditions. Amrein et al. (*32*) also concluded that methylglyoxal and glyoxal play important roles in AA formation in model systems. Therefore, the inhibition mechanism of PM might be related with these compounds because it is known that this form of vitamin B_6 is capable of reacting with the reactive dicarbonyls intermediates of the MR, especially glyoxal, methylglyoxal, glycolaldehyde, or 3-deoxyglucosones (*10*, *16*), forming stable adducts (*33*) that would inhibit the conversion to the next steps of the reaction. The action mechanism is attributable to its structural features as mentioned before; PM possesses a nucleophilic amino group (see **Figure 7a**) that has the capacity to scavenge toxic carbonyl compounds, inactivating these key intermediates on the AA and thereby preventing progression to its formation. Nagaraj et al. (*16*) concluded that the inhibitory effect of PM is mediated by the formation of a methylglyoxal−pyridoxamine dimer in biological systems. In our investigation, we were not able to identify the formation of dimers between PM and dicarbonyls or PM degradation products, but this aspect is still under investigation due to the complexity of chromatographic profiles in the Glc/Asn/PM system.

The strong decrease in the degree of browning by the addition of PM found in the reaction mixture also confirmed the inhibitory action of PM on AA formation as the color is directly associated with its formation through the MR (*28*, *34*). It is important to stress that this suppression of the browning confirms the hypothesis of the capacity of PM to trap these colored compounds and precursors formed during the degradation of sugars in the MR. Nevertheless, the inhibitory effect is limited not only to scavenging of dicarbonyl compounds but also to blocking the pathway of formation of acrylamide via 3-APA. There was an increase of 3-APA formation likely because of no conversion to AA, confirming the studies reported by other authors where 3-APA takes part in acrylamide formation (*25*, *26*). In addition, 3-APA is converted into AA more efficiently in the presence of aldehydes (*35*). Consequently, PM reacts with aldehydes and therefore competes with 3-APA for available carbonyl groups.

PM suppressed AA formation even at the lowest concentration investigated. PM is more effective as compared with the other B_6 vitamers, pyridoxal and pyridoxine; even inhibition was considerably higher than vitamin C at the same concentration studied. On the basis of the data obtained in the present work, a working hypothesis was settled on, whereby the PM mechanism of action to reduce AA formation is mediated through scavenging dicarbonyls. Dicarbonyl compounds such as glyoxal and methylglyoxal are established as important intermediates of the MR (a) from retro-aldol fragmentation of Schiff base, (b) from autoxidation of sugars, (c) from oxidative degradation of the Amadori product, and (d) and as part of the end-products of lipid peroxidation. At this point, the formation of conjugated intermediary compounds from the reaction of PM and advanced products of the MR is not confirmed. Theefore, a forthcoming investigation is focused on the determination of methylglyoxal or glyoxal pyridoxamine dimers and adducts of two molecules of PM and two molecules of MG or glyoxal. On the other hand, application of PM as a mitigating agent for acrylamide formation is also potentially relevant to foods rich in lipids where advanced products of oxidation have been recently implied in the formation of acrylamide.

ACKNOWLEDGMENT

We thank D. Gómez for technical assistance.

LITERATURE CITED

(1) FAO/WHO. Joint FAO/WHO Expert Committee on Food Additives: Summary and conclusions report from sixty-fourth meeting, Rome, 8−17 Feb 2005; available at ftp://ftp.fao.org/es/esn/jecfa/jecfa64_summary.pdf.

(2) Friedman, M.; Levin, C. E. Review of methods for the reduction of dietary content and toxicity of acrylamide. *J. Agric. Food Chem.* **2008**, *56*, 6113–6140.

(3) Mottram, D. S.; Bronislaw, L.; Wedzicha, A. T. D. Acrylamide is formed in the Maillard reaction. *Nature* **2002**, *419*, 448–449.

(4) Zyzak, D. V.; Sanders, R. A.; Stojanovic, M.; Tallmadge, D. H.; Eberhart, B. L.; Ewald, D. K.; Gruber, D. C.; Morsch, T. R.; Strothers, M. A.; Rizzi, G. P.; Villagran, M. D. Acrylamide formation mechanism in heated foods. *J. Agric. Food Chem.* **2003**, *51*, 4782–4787.

(5) Stadler, R. H.; Roberts, F.; Riediker, S.; Varga, N.; Davidek, T.; Devaud, S.; Goldmann, T.; Hau, J.; Blank, I. In-depth mechanistic study on the formation of acrylamide and other vinylogous compounds by the Maillard reaction. *J. Agric. Food Chem.* **2004**, *52*, 5550–5558.

(6) Leklem, J. E. Vitamin B6. In *Modern Nutrition in Health and Disease*, 9th ed.; Shils M. E., Olson J. A., Shike M., Ross, A. C., Eds.; Williams and Wilkins: Baltimore, MD, 1999; pp 413−421.

(7) Reddy, V. P.; Beyaz, A. Inhibitors of the Maillard reaction and AGE breakers as therapeutics for multiple diseases. *Drug Discov. Today* **2006**, *11*, 646–654.

(8) Alderson, N. L.; Chachich, M. E.; Youssef, N. N.; Beattie, R. J.; Thorpe, S. R.; Baynes, J. W. The AGE inhibitor pyridoxamine inhibits lipemia and development of renal and vascular disease in Zucker obese rats. *Kidney Int.* **2003**, *63*, 2123–2133.

(9) Booth, A. A.; Khalifah, R. G.; Hudson, B. G. Thiamine pyrophosphate and pyridoxamine inhibit the formation of antigenic advanced glycation end-products: comparison with aminoguanidine. *Biochem. Biophys. Res. Commun.* **1996**, *220*, 113–119.

(10) Voziyan, P. A.; Metz, T. O.; Baynes, J. W.; Hudson, B. G. A post-amadori inhibitor pyridoxamine also inhibits chemical modification of proteins by scavenging carbonyl intermediates of carbohydrate and lipid degradation. *J. Biol. Chem.* **2002**, *277*, 3397–3403.

(11) Onorato, J. M.; Jenkins, A. J.; Thorpe, S. R.; Baynes, J. W. Pyridoxamine, an inhibitor of advanced glycation reactions, also inhibits advanced lipoxidation reactions. Mechanism of action of pyridoxamine. *J. Biol. Chem.* **2000**, *275*, 21177–21184.

(12) Loeb, A. L.; Anderson, R. J. Antagonism of acrylamide neurotoxicity by supplementation with vitamin B6. *Neurotoxicology* **1981**, *2*, 625–633.

(13) Knol, J. J.; van Loon, W. A. M.; Linssen, J. P. H.; Ruck, A.-L.; van Boekel, M.J. S.; Voragen, A. G. J. Toward a kinetic model for acrylamide formation in a glucose−-asparagine reaction system. *J. Agric. Food Chem.* **2005**, *53*, 6133–6139.

(14) Rufián-Henares, J. A.; Arribas-Lorenzo, G.; Morales, F. J. Acrylamide content of selected Spanish foods: survey of biscuits and bread derivatives. *Food Addit. Contam*. **2007**, *24*, 343–350.
(15) Bagdonaite, K.; Viklund, G.; Skog, K.; Murkovic, M. Analysis of 3-aminopropionamide: a potential precursor of acrylamide. *J. Biochem. Biophys. Methods* **2006**, *69*, 215–221.
(16) Nagaraj, R. H.; Sarkar, P.; Mally, A.; Biemel, K. M.; Lederer, M. O.; Padayatti, P. S. Effect of pyridoxamine on chemical modification of proteins by carbonyls in diabetic rats: characterization of a major product from the reaction of pyridoxamine and methylglyoxal. *Arch. Biochem. Biophys*. **2002**, *402*, 110–119.
(17) Becalski, A.; Lau, B. P.-Y.; Lewis, D.; Seaman, S. W. Acrylamide in foods: occurrence, sources, and modeling. *J. Agric. Food Chem*. **2003**, *51*, 802–808.
(18) Ehling, S.; Shibamoto, T. Correlation of acrylamide generation in thermally processed model system of asparagine and glucose with color formation, amounts of pyrazines formed, and antioxidative properties of extracts. *J. Agric. Food Chem*. **2005**, *53*, 4813–4819.
(19) Rydberg, P.; Eriksson, S.; Tareke, E.; Karlsson, P.; Ehrenberg, L.; Törnqvist, M. Investigations of factors that influence the acrylamide content of heated foodstuffs. *J. Agric. Food Chem*. **2003**, *51*, 7012–7018.
(20) Vleeschouwer, K.; Van der Plancken, I.; Van Loey, A.; Hendrickx, M. Impact of pH on the kinetics of acrylamide formation/elimination reactions in model systems. *J. Agric. Food Chem*. **2006**, *54*, 7847–7855.
(21) Bråthen, E.; Knutsen, S. H. Effect of temperature and time on the formation of acrylamide in starch-based and cereal model systems, flat breads and bread. *Food Chem*. **2005**, *92*, 693–700.
(22) Adrover, M.; Volanova, B.; Muñoz, F.; Donoso, J. Inhibition of glycosylation processes: the reaction between pyridoxamine and glucose. *Chem. Biodivers*. **2005**, *2*, 964–975.
(23) Vleeschouwera, K.; Planckena, I. V.; Loey, A. V.; Hendrickx, M. E. The kinetics of acrylamide formation/elimination in asparagine−glucose systems at different initial reactant concentrations and ratios. *Food Chem*. **2008**, *111*, 719–729.
(24) Martins, S. I. F. S.; Van Boekel, M. A. J. S. A kinetic model for the glucose/glycine Maillard reaction pathways. *Food Chem*. **2005**, *90*, 257–269.
(25) Granvogl, M.; Schieberle, P. Thermally generated 3-aminopropionamide as a transient intermediate in the formation of acrylamide. *J. Agric. Food Chem*. **2006**, *54*, 5933–5938.
(26) Granvogl, M.; Jezussek, M.; Koehler, P.; Schieberle, P. Quantitation of 3-aminopropionamide in potatoes—a minor but potent precursor in acrylamide formation. *J. Agric. Food Chem*. **2004**, *52*, 4751–4757.
(27) Schieberle, P.; Köhler, P.; Granvogl, M. News aspects on the formation and analysis of acrylamide. Chemistry and Safety of Acrylamide in Food. *Adv. Exp. Med. Biol*. **2005**, *561*, 205–227.
(28) Gökmen, V.; Açar, Ö.Ç.; Arribas-Lorenzo, G.; Morales, F. J. Investigating the correlation between acrylamide content and browning ratio of model cookies. *J. Food Eng*. **2008**, *87*, 380–385.
(29) Levine, R. A.; Smith, R. E. Sources of variability of acrylamide levels in a cracker model. *J. Agric. Food Chem*. **2005**, *53*, 4410–4416.
(30) Khatami, M.; Suldan, Z.; David, I.; Li, W.; Rockey, J. H. Inhibitory effects of pyridoxal phosphate, ascorbate and aminoguanidine on nonenzymatic glycosylation. *Life Sci*. **1988**, *43*, 1725–1731.
(31) Yuan, Y.; Zhao, G.-H.; Hu, X.-S.; Wu, J.-H.; Liu, J.; Chen, F. High correlation of methylglyoxal with acrylamide formation in glucose/asparagine Maillard reaction model. *Eur. Food Res. Technol*. **2008**, *226*, 1301–1307.
(32) Amrein, T. B.; Andres, L.; Giuseppe, G.; Manzardo, G.; Amado, R. Investigations on the promoting effect of ammonium hydrocarbonate on the formation of acrylamide in model system. *J. Agric. Food Chem*. **2006**, *54*, 10253–10261.
(33) Voziyan, P. A.; Khalifah, R. G.; Thibaudeau, C.; Yildiz, A.; Jacob, J.; Serianni, A. S.; Hudson, B. G. Modification of proteins in vitro by physiological levels of glucose. *J. Biol. Chem*. **2003**, *278*, 46616–46624.
(34) Mustafa, A.; Andersson, R.; Rosen, H.; Kamal-Eldin, A.; Aman, P. Factors influencing acrylamide content and color in rye crisp bread. *J. Agric. Food Chem*. **2005**, *53*, 5985–5989.
(35) Perez-Locas, C.; Yaylayan, V. A. Further insight into thermally and pH-induced generation of acrylamide from glucose/asparagine model systems. *J. Agric. Food Chem*. **2008**, *56*, 6069–6074.

Received for review September 15, 2008. Revised manuscript received December 2, 2008. Accepted December 6, 2008. This work was carried out within the framework of COST 927 thermally processed foods: possible health implication. Research has been partly fund by Consejeria Educación y Ciencia (CAM) under project ANALISYC Program, S-505/AGR-0312. G.A.-L. was the recipient of a grant from Conserjería de Educación de la Comunidad de Madrid.

JF802870T