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Reaction of pyridoxamine with malondialdehyde: Mechanism of inhibition of formation of advanced lipoxidation end-products

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Summary. Advanced glycation end products (AGEs) and advanced lipoxidation end products (ALEs) are implicated in many age-related chronic diseases and in protein aging. Recent studies suggest that pyridoxamine (PM) is an efficient AGEs/ALEs inhibitor in various biological systems. Because malondialdehyde (MDA) is an important intermediate in the formation of ALEs during lipid peroxidation, the purpose of this study is to determine whether PM can trap MDA directly and thereby prevent ALEs formation. PM reacted readily with MDA under physiological conditions. Within 6 h, a 1-pyridoxamino-propenal adduct derived from reaction of equimolar PM + MDA was detected. A 1-amino-3-iminopropene complex and a dihydropyridine-pyridinium complex were also identified after 7 d incubation. PM also greatly inhibited the lipofuscin-like fluorescence formation induced by MDA reaction with bovine serum albumin (BSA). Our results showed clearly that PM inhibited the formation of ALEs by trapping MDA directly under physiological condition, and provide insight into the mechanism of action of PM in protecting proteins against carbonyl stress.

Keywords: Advanced glycation end products (AGEs) – Advanced lipoxidation end products (ALEs) – Carbonyl stress – Pyridoxamine (PM) – Malondialdehyde (MDA)

Introduction

The Maillard reaction and advanced lipid peroxidation reactions, which can cause the biochemical modification of tissue proteins and lead to the formation of AGEs and ALEs, are associated with diabetic complications, atherosclerosis, uremia, neurodegenerative diseases and normal physiological aging (Brownlee, 1995; Colaco and Harrington, 1994; Thorpe and Baynes, 1996; Miyata et al., 1999; Baynes and Thorpe, 2000; Miyata et al., 2000). A variety of reactive carbonyl intermediates derived from Maillard and lipid peroxidation reactions act as intermediates in formation of AGEs and ALEs (Miyata et al., 2000; Yin, 1995). These toxic carbonyl compounds react with amino group of proteins resulting in various chemical modifications and crosslinks that alter the structure and function of protein. An effort to inhibit formation of AGEs/ALEs by carbonyl-trapping compounds has emerged recently as a therapeutic approach for inhibiting these age- and disease-dependent changes in biomolecules (Shapiro, 1998).

MDA, as one of the most important intermediates of lipid peroxidation, is a reactive unsaturated dicarbonyl that can readily bind to and crosslink biomacromolecules such as structural and functional proteins and nucleic acids (Esterbauer et al., 1991). The concentration of MDA in plasma is increased in most (if not all) acute and chronic diseases such as diabetes and Alzheimer's disease (Slatter et al., 2000; Markesberry, 1995). MDA can react with protein and form enamine and fluorescent pyridinium products: 1-amino-3-iminopropene, dihydropyridine, and dihydropyridine-pyridinium complex, implicating MDA in the formation of lipofuscin-like fluorophores (Esterbauer et al., 1991; Yin, 1996). MDA has also been shown to be mutagenic in bacteria, in mouse and human kidney cells, and is carcinogenic in rats (Niedernhofer et al., 2003). MDA reacts with DNA bases to produce adducts to deoxyguanosine, deoxyadenosine and deoxycytidine (Wang et al., 2004). In these studies, the formation of DNA–DNA cross-links by MDA was proposed to be one of the most serious forms of DNA damage.

PM was originally described as a post-Amadori inhibitor of AGEs formation (Khalifah et al., 1999; Metz et al.,

2003). Later it was also found to inhibit the formation of the ALEs during lipid peroxidation reactions (Onorato et al., 2000). Further research also proved that PM can trap reactive carbonyl intermediates of carbohydrate and lipid degradation including glyoxal, glycolaldehyde, methylglyoxal, 4-hydroxy-2-nonenal and 1,4-dicarbonyls (Voziyan et al., 2002; Nagaraj et al., 2002; Amarnath et al., 2004). In streptozotocin (STZ)-diabetic rats, PM also retards the development of diabetic complications including nephropathy and retinopathy (Degenhardt et al., 2002; Stitt et al., 2002). Lipids are now recognized an important source of chemical modification of tissue proteins in both hyperglycemic (diabetic rat) and normoglycemic (Zucker obese rat; Alderson et al., 2003) animals, and PM, as an AGEs/ALEs inhibitor, interferes strongly in lipid peroxidative damage to protein. PM also inhibits formation of N^{ε}-(carboxymethyl)lysine (CML) and N^{ε}-(carboxyethyl)lysine (CEL) in skin collagen, though CML and CEL may be derived from either glycoxidation or lipid peroxidation reactions (Degenhardt et al., 2002).

In this paper, we demonstrate that PM reacted readily with MDA under physiological conditions and protects protein against modification by MDA.

Materials and methods

Materials

$PM \cdot (HCl)_2$ was purchased from Sigma Chem. Co. (St. Louis, MO, USA). 1,1,3,3-tetramethoxypropane (TMP) was obtained from Fluka Chemie AG (Buchs, Switzerland). Bovine serum albumin (BSA) was from Amresco (Solon, USA) and other chemicals used were purchased from Sangon (Shanghai, China).

Preparation of MDA

MDA stock solution (10 mM) was prepared immediately before use by hydrolyzing 1,1,3,3-tetramethoxypropane (TMP), according to the method of Kikugawa (Kikugawa et al., 1980). Briefly, 0.084 ml (0.5 mM) TMP was mixed with 1.0 ml 1.0 N HCl, and shaken at 40°C for about 2.5 min. After the TMP was hydrolyzed, the pH was adjusted to 7.4 with 6 N NaOH, and the stock solution was finally diluted to 50 ml with 0.2 M sodium phosphate buffer (pH 7.4). The concentration of MDA in the stock solution was estimated by absorbance at 267 nm using $\varepsilon_{MDA} = 31500$.

Reaction of PM with MDA

PM (5.0 mM) was incubated with equimolar MDA (5.0 mM) in 0.2 M sodium phosphate buffer pH 7.4, at 37°C. Sodium azide (0.02%, w/w) was added to prevent bacterial growth. Samples were analyzed by HPLC or LC/MS.

HPLC analysis of the reaction mixture of MDA with PM

HPLC analysis was performed on a Reverse-phase Vydac C18 analytical column (250 mm × 4.6 mm, 10 um) using a Waters™ Alliance 2690 HPLC system with a model 996 photodiode array detector. The mobile phase consisted of solution A (0.1% trifluoroacetic acid in water) and solution B (acetonitrile, 100%). The flow rate was 0.7 ml/min and the elution gradient was as follows: 0–3 min, 0% B; 3–10 min, 20% B; 10–45 min, 50% B.

LC/MS analysis of the reaction products of PM and MDA

LC/MS experiments were carried on an LC/MS-2010 quadrupole mass spectrometer interfaced with an electrospray ionization (ESI) source (LC/MS-ESI). The positive electrospray ionization mode has been the choice of detection. Separation were done by a Thermo Hypersil-Keystone Hypurity C18 (150 mm × 2.1 mm, 5 um) analytical column in the following conditions: Solution A was 0.2% acetic acid in water and solution B was 100% methanol, the flow rate was 0.2 ml/min and the elution gradient was as follows: 0–2 min, 15% B; 2–6 min, 35% B; 6–10 min, 0% B. The wavelength of the SPD-M10Avp diode array detector was set in the range of 210–360 nm. The ESI/MS source was set as follows: the temperatures were maintained at 250°C, 250°C and 200°C for the probe, CDL, and block respectively. The voltages were set at 4.5 kV, −30 V, 25 V, 150 V, and 1.5 kV for the probe, CDL, Q-array 1, 2, 3 bias, Q-array radio frequency and detector respectively. The flow rate of nebulizer gas was 4.5 L/min. The ions used for selected ion monitoring were chosen by scanning in the positive ion mode from m/z = 50–700.

Effect of PM on the MDA modified proteins

BSA (10 mg/ml) was incubated with MDA (1.0 mM) in the absence or presence of different concentrations of PM (PM and MDA were added simultaneously to reaction system) in 0.2 M sodium phosphate buffer, pH 7.4 (containing 0.02% sodium azide) at 37°C for 24 hours. Modified protein was precipitated by adding an equal amount of 20% trichloroacetic acid (TCA) and centrifugation at 3000 × g for 3 min. In order to fully remove PM and fluorescent MDA polymer, the pellet was washed for 3 times with 20% TCA and collected after centrifugation. Finally, the precipitated protein was redissolved in 0.2 M sodium phosphate buffer pH 7.4, and the concentration was adjusted to 5.0 mg/ml. The sample was diluted 20-fold before the fluorescence was read at an excitation wavelength of 395 nm and an emission wavelength of 460 nm.

Results and discussion

HPLC analysis of the MDA + PM reaction

The standard absorbance spectrum of PM shows three peaks at neutral pH: 219, 251 and 325 nm. Under acidic conditions, a proton $[H]^+$ bonds to the nitrogen group of the pyridinium ring resulting in an absorption peak at 293 nm (Brealey and Kasha, 1955).

After PM (5.0 mM) was incubated with MDA (5.0 mM) in 0.2 M phosphate buffer pH 7.4, at 37°C for 6 h, the reaction mixture was analyzed at acidic pH by HPLC; products detected by absorbance at 293 nm. As shown in Fig. 1, the retention time of PM was at 6.3 min, while MDA has no absorption at 293 nm in this system. A new product (product 1) was observed in the reaction of PM and MDA, with retention time at 11.3 min (Fig. 1A). The UV-absorption spectrum showed that absorption maximum at 277 nm under acidic conditions (Fig. 2).

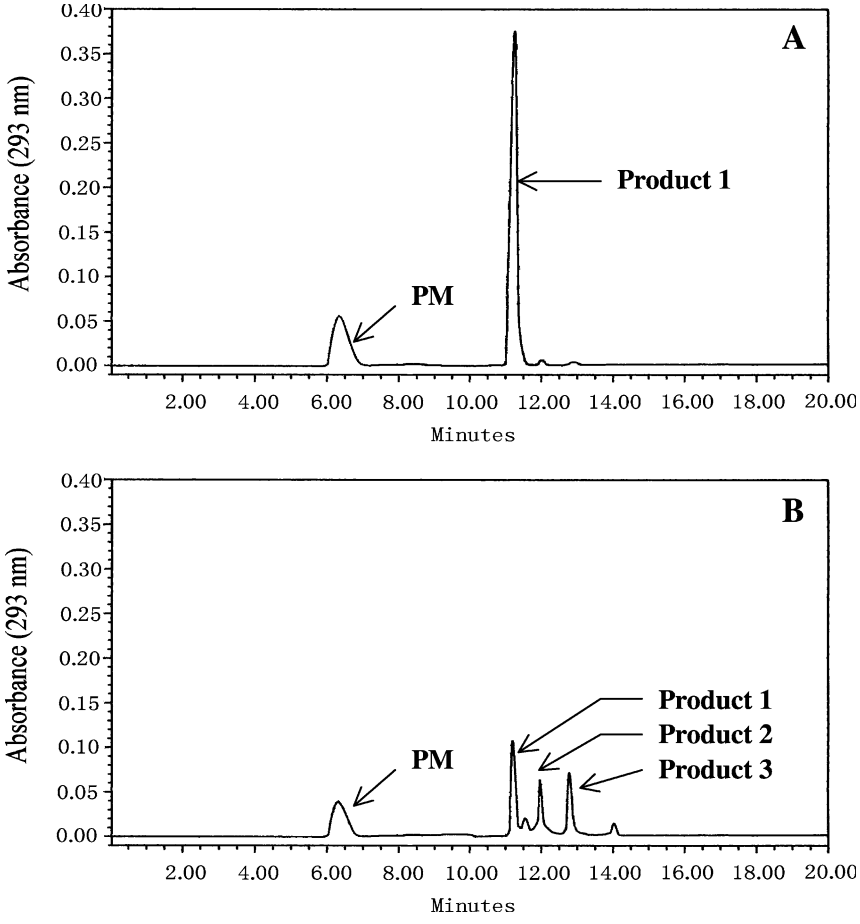

Fig. 1. HPLC analysis of the reaction between PM and MDA. PM (5.0 mM) was incubated with MDA (5.0 mM) in 0.2 M sodium phosphate buffer pH 7.4, at 37°C. **A** PM and MDA were incubated for 6 h. **B** PM and MDA were incubated for 7 d

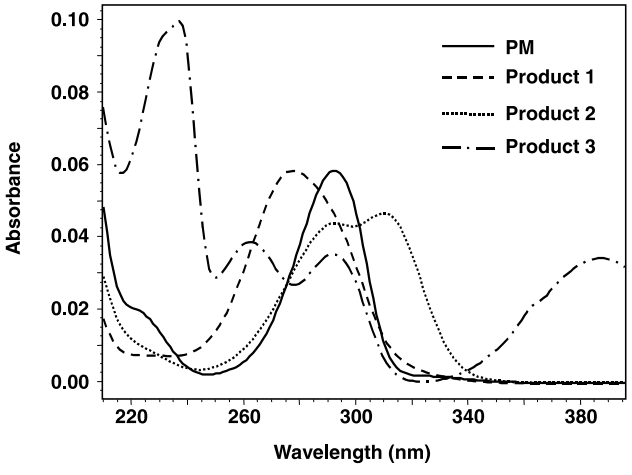

Fig. 2. UV-absorption spectrum of PM and PM + MDA products. The UV-absorption spectrum of PM and PM + MDA products were obtained during HPLC analysis using a model 996 photodiode array detector

When the incubation was continued for 7 days under the same conditions, two other products were observed (Fig. 1B), at retention times of 12.0 min (product 2) and 13.8 min (product 3), respectively. The UV-absorption spectrum of product 2 showed maxima at 293 and 310 nm (Fig. 2). The spectrum of product 3 was more complex, with UV-absorption maxima at 237, 262, 293 and 388 nm (Fig. 2).

The reaction kinetics of PM and MDA during 0 to 24 h were also analyzed by HPLC. As demonstrated in Fig. 3, the amount of PM decreased and the product 1 increased during the first 6 hours of incubation. The reaction proceeded with a half-time of approximately 45 min for PM consumption, and the yield of product 1 reached a plateau at about 6 h.

Identification of reaction products by LC/MS

LC/MS was employed to identify the reaction products. When the reaction mixture was incubated for up to 6 h, product 1, detected by a diode array detector (DAD) (Fig. 4A) corresponded to a product detected in the total ion current chromatogram (TIC) (Fig. 4B) at approximately 3.8 min. The mass spectrum of product 1

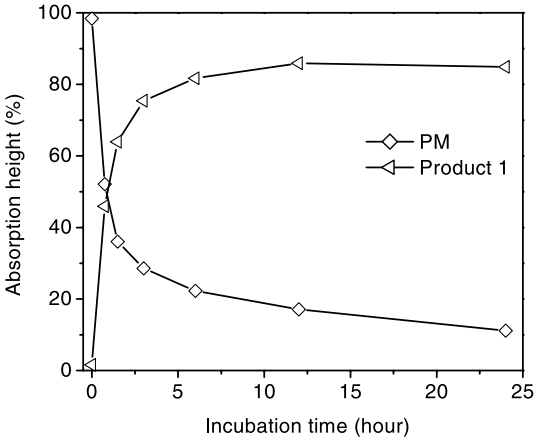

Fig. 3. Kinetics of decrease in PM and formation of product 1 (expressed as percentage, based on the absorbance of PM) during reaction of PM and MDA. Absorbance was measured at 293 nm

showed four main peaks: m/z 223 $[M_{P1}+H]^+$, m/z 245 $[M_{P1}+Na]^+$, m/z 261 $[M_{P1}+K]^+$ and m/z 467 $[2M_{P1}+Na]^+$, respectively (Fig. 4C).

When the reaction mixture was incubated for 7 days, two new products were identified in addition to m/z 233. The other two were m/z = 373 $[M_{P2}+H]^+$ and 517 $[M_{P3}{}^+]$ (Fig. 5).

It is well known that MDA can react with amino acids and proteins and form a series of products, including enamine, 1-amino-3-iminopropene and dihydropyridine derivatives (Esterbauer et al., 1991). It is also known that protein can be cross-linked with an MDA-derived dihydropyridine-pyridinium structure (Itakura et al., 1996). According to the molecular weight and considering that PM and amino acid possess the same primary amino group, which can react with MDA, products 1, 2, 3, are proposed to be the enamine adduct of MDA to PM, the 1-amino-3-iminopropene crosslink, and the dihydropyridine-pyridinium complex (Fig. 6). The proportions of PM:MDA in the product 1, 2, 3 were 1:1, 2:1, 2:4 respectively. Considering that the UV-absorption of enamine show maxima at about 271–280 nm in water (Kikugawa et al., 1981), 1-amino-3-iminopropene at about 300 nm (Itakura and Uchida,

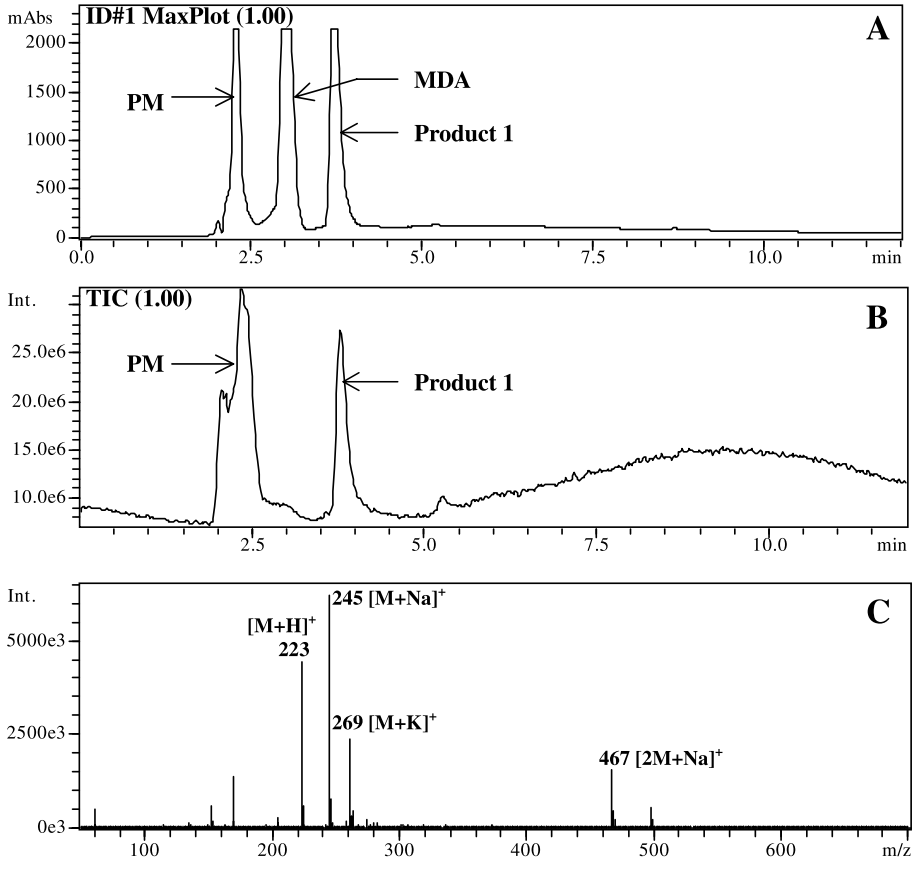

Fig. 4. LC/MS analysis of reaction mixture after 6 h incubation. **A** The diode array detector chromatogram; **B** the total ion chromatogram; **C** the mass spectrum corresponding to the total ion chromatogram at retention time 3.77 min

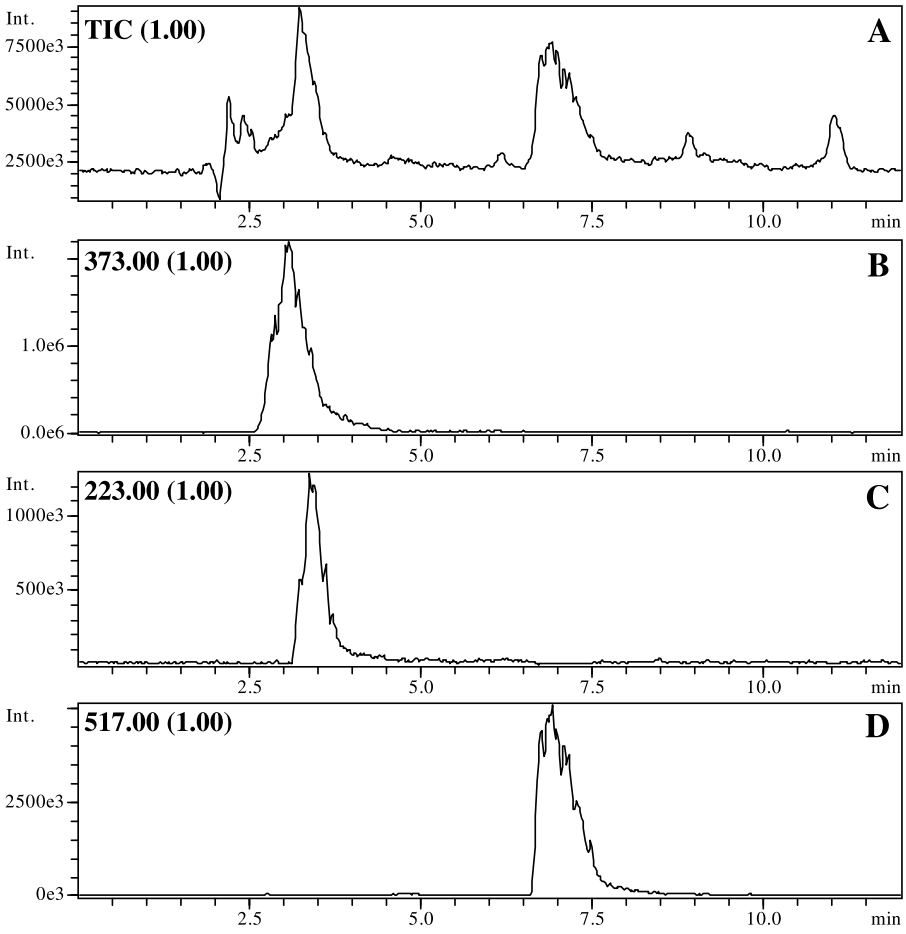

Fig. 5. LC/MS analysis of reaction mixture after 7 d incubation. **A** The total ion chromatogram; **B** m/z 373 selective ion chromatogram (product 2); **C** m/z 223 selective ion chromatogram (product 1); **D** m/z 517 selective ion chromatogram (product 3)

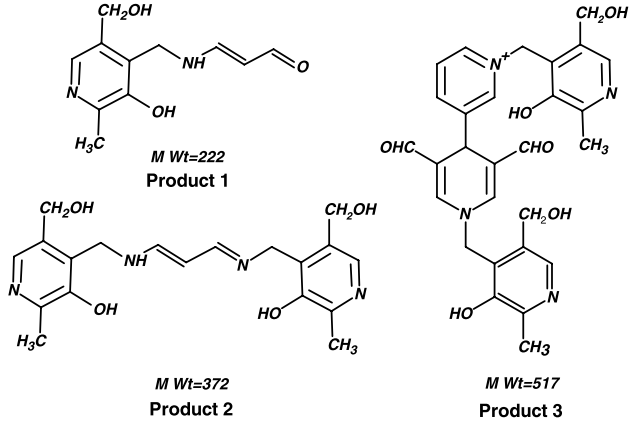

Fig. 6. Proposed product structures of PM + MDA reaction. Products 1, 2 and 3 are 1-pyridoxamino-propenal, the 1-amino-3-iminopropene crosslink, and dihydropyridine-pyridinium complex, respectively

2001), dihydropyridine and dihydropyridine-pyridinium derivative showing three absorption maxima at about 235, 260, and 398 nm (Itakura et al.; Kikugawa et al.), our products matched with the reported spectra very well.

Effect of PM on the MDA modification of protein

MDA was frequently reported to react with amino compounds forming fluorescent products such as dihydropyridine and dihydropyridine-pyridinium derivatives (Esterbauer et al., 1991; Itakura et al.; Chio and Tapple, 1969). Slatter et al. also showed that the dihydropyridine derivative is the major product of MDA and propylamine reaction under physiological conditions (Slatter et al., 1998). In our experimental conditions, MDA modified protein showed lipofuscin-like fluorescence at 395 ex/460 em which is in accordance with the fluorescence of dihydropyridine. As is seen in Fig. 7, when BSA (10 mg/ml) was incubated with MDA (1.0 mM) at 37°C for 24 h, the lipofuscin-like fluorescence increased by 25-fold compared with BSA control. Addition of different concentration of

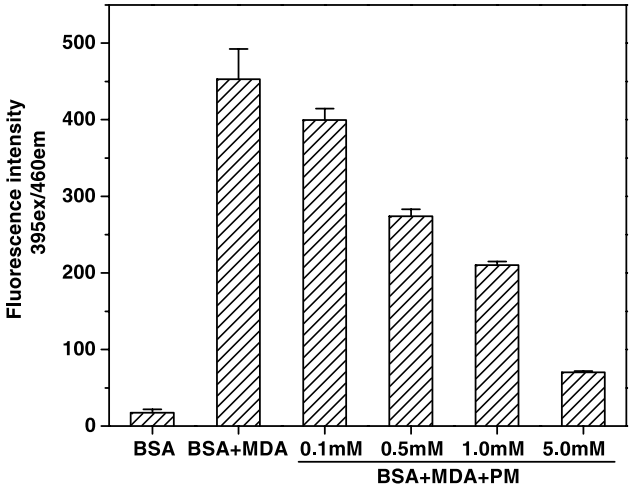

Fig. 7. Protective effect of PM on chemical modification of BSA by MDA. BSA (10 mg/ml) was incubated alone or with MDA (1.0 mM) in the absence or presence of different concentrations of PM (0.1 mM, 0.5 mM, 1.0 mM and 5.0 mM) in 0.2 M sodium phosphate buffer, pH 7.4, at 37°C for 24 hs. After protein was precipitated by trichloroacetic acid, and the concentration was adjusted to 0.25 mg/ml, the lipofuscin-like fluorescence was measured at ex = 395 nm, em = 460 nm. Data are means ± s.d. of triplicates

PM (0.1 mM, 0.5 mM, 1.0 mM, 5.0 mM) inhibited the formation of lipofuscin-like fluorescence (by 12%, 40%, 54%, and 85% respectively). Our results suggest that PM may inhibit AGEs/ALEs formation, in part, by reacting directly with MDA, preventing the formation of MDA adducts and crosslinks to protein.

Conclusion

Overall, PM is thought to inhibit the formation of AGEs/ALEs by a mechanism depending on carbonyl trapping. Because MDA is one of the most important dicarbonyl intermediates of lipid peroxidation, we have studied the reaction of PM with MDA and three products have been identified: a molecule of 1-pyridoxamino-propenal, a 1-amino-3-iminopropene crosslink and a dihydropyridine-pyridinium complex. Our data clearly demonstrate that PM inhibits the formation of ALEs by directly detoxifying MDA at physiological condition. Moreover, PM also greatly depressed the formation of lipofuscin-like fluorescence during reaction of MDA with BSA, suggesting a direct mechanism by which PM may inhibit lipid-dependent carbonyl stress in biological systems.

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