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Identification of Aminoethylcysteine Ketimine Decarboxylated Dimer, a Natural Antioxidant, in Dietary Vegetables

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Aminoethylcysteine ketimine decarboxylated dimer (simply named dimer) is a natural sulfur-containing tricyclic compound detected, until now, in human urine, bovine cerebellum, and human plasma. Recently, the antioxidant properties of this compound have been demonstrated. In this investigation, the presence of aminoethylcysteine ketimine decarboxylated dimer was identified in garlic, spinach, tomato, asparagus, aubergine, onion, pepper, and courgette. Identification of this compound in dietary vegetables was performed using gas chromatography, high-performance liquid chromatography, and gas chromatography−mass spectrometry. Results from GC analysis range in the order of 10^{-4} μmol of dimer/g for all the tested vegetables. These results and the lack of a demonstrated biosynthetic pathway in humans might account for a dietary supply of this molecule.

KEYWORDS: Aminoethylcysteine ketimine decarboxylated dimer; dietary vegetables; antioxidant

INTRODUCTION

Aminoethylcysteine ketimine decarboxylated dimer is a natural, sulfur-containing, tricyclic member of a new family of sulfur-containing amino acids (*1*). Many of these ketimines and their derivatives have been found to be present in detectable amounts in mammalian tissues and fluids (*2*−*6*). Some of them were detected in the central nervous system, and for one of them the binding to bovine brain cortex membranes was demonstrated (*7*), suggesting a possible neurochemical role.

The product of spontaneous dimerization and decarboxylation of aminoethylcysteine ketimine was synthesized for the first time in 1961 (*8*), and, for many years, very little progress has been made on the understanding of its properties. Only recently the antioxidant properties of this compound have been demonstrated. Particularly interesting is the protective effect of the dimer on the copper-induced oxidation of low-density lipoprotein (*9*) exerted at concentrations comparable to those found in human plasma. Moreover, a scavenging activity on hydroxyl radicals, peroxynitrite, and its derivatives, comparable to α-tocopherol and more potent of ascorbic acid and glutathione has been described (*10*−*13*). However, even if the dimer has been detected in different biological samples such as human plasma and urine (*14*, *15*) and bovine cerebellum (*16*), an in vivo biochemical route leading to its synthesis has not yet been demonstrated. In a search for possible exogenous sources of this compound, some common vegetables daily present in mediterranean diet such as garlic, asparagus, aubergine, onion, pepper, tomato, courgette, and spinach were selected and checked for the presence of this molecule. Some of them were chosen on the basis of their high content in sulfur-containing amino acids (garlic, onion, asparagus, and spinach).

The gas chromatographic, gas chromatographic−mass spectrometric, and high-performance liquid chromatography detection of the dimer in all the above cited vegetables is now reported.

MATERIAL AND METHODS

Aminoethylcysteine ketimine decarboxylated dimer standard was prepared according to ref *12*. Fresh vegetables were obtained from local market.

Sample preparation: 10 g of very fresh vegetables (garlic, spinach, tomato, asparagus, aubergine, onion, pepper, and courgette) were mixed with 10 mL of water, then homogenized in a Waring blender apparatus at high speed, for 2 min, at room temperature. After centrifugation (20 min at 15000*g*) of the sample, the supernatant was taken and extracted three times with chloroform (1:4). The collected organic fraction was dried, and the residue was resuspended in 100 μL of methanol to be directly analyzed without derivatization by GC, GC-MS, and HPLC. The recovery of authentic dimer was tested by the standard addition method in each vegetable. Aliquots of the same sample were added

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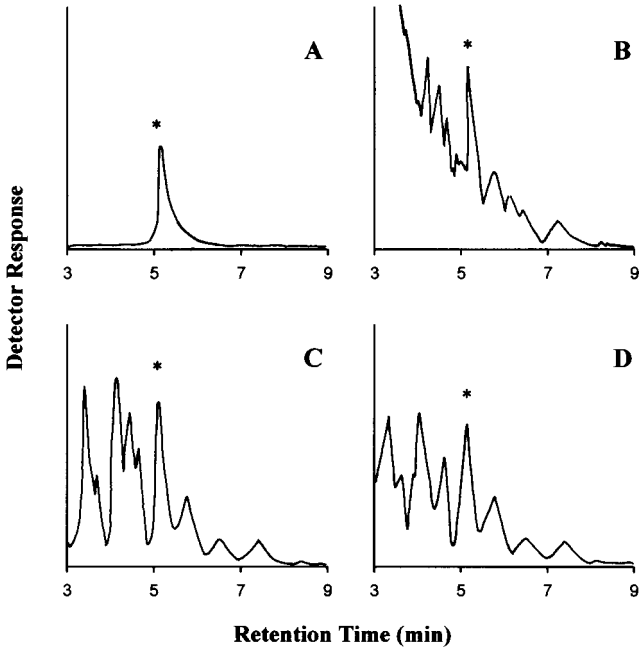

Figure 1. GC–FPD chromatograms of (A) standard aminoethylcysteine ketimine decarboxylated dimer; (B) garlic; (C) spinach; and (D) tomato extracts.

with three different quantities of this compound (500, 1000, and 1500 ng), and, after the extraction procedure, the residues were resuspended in 100 μL of methanol and analyzed by GC.

GC Analyses. Gas chromatographic analyses were performed on a Perkin-Elmer Sigma 300 chromatograph (Perkin-Elmer, Norwalk, Connecticut, U.S.A.) equipped with a flame photometric detector for sulfur-containing compounds. The Supelco glass column (180 cm × 2 mm i.d.) was packed with 3% OV-17 on Chromosorb W HP, 100−120 mesh (Supelco, Bellefonte, PA). Temperatures were 260 °C for the column, 270 °C for the injector, and 300 °C for the detector. Flow rates were 25 mL/min for N_2 carrier gas, 65 mL/min for H_2, and 110 mL/min for air.

GC-MS Analyses. Gas chromatographic−mass spectrometric analyses were performed on a Hewlett-Packard 5970A MSD system (Hewlett-Packard, Palo Alto, CA). Chromatographic separations were carried out on a 30 m × 0.25 mm i.d. fused-silica capillary column coated with cross-linked 5% phenyl-methyl siloxane, film thickness 0.25 μm, as stationary phase (Hewlett-Packard, Palo Alto, CA). Injection mode: splitless at a temperature of 260 °C. Column temperature program: 41 °C (3 min), then to 280 °C at a rate of 8 °C/min and held for 8 min. The carrier gas was helium at a constant flow of 2.5 mL/min, and the average linear velocity was 60 cm/s. The spectra were obtained in the electron impact mode at 70 eV ionization energy; ion source 280 °C; ion source vacuum 10^{-5} Torr. When the selected ion monitoring acquisition mode was used, the dwell time was 50 ms. Chromatograms and spectra were collected by a Hewlett-Packard 59970 Chem Station.

HPLC Analyses. High performance liquid chromatography analyses were carried out with a Waters chromatograph equipped with a model 626 pump, a 6005 controller and a model 996 photodiode array detector linked to a Millennium 2010 Data Station (Waters Corporation, Millford, MA).

The column, 250 × 4 mm i.d., was packed with ODS, 5 μm, (Hypersil, Bellefonte, PA). The mobile phases were A water, B acetonitrile−water (80:20 v/v). A linear gradient from 100% A to 100% B was developed in 30 min. The flow rate was 1 mL/min at room temperature, and absorbance was recorded at 308 nm.

RESULTS AND DISCUSSION

The results of the three analytical methods (GC, GC-MS, HPLC) used to test the presence of the dimer in plant food were positive in all tested vegetables (garlic, spinach, tomato, asparagus, aubergine, onion, pepper, and courgette).

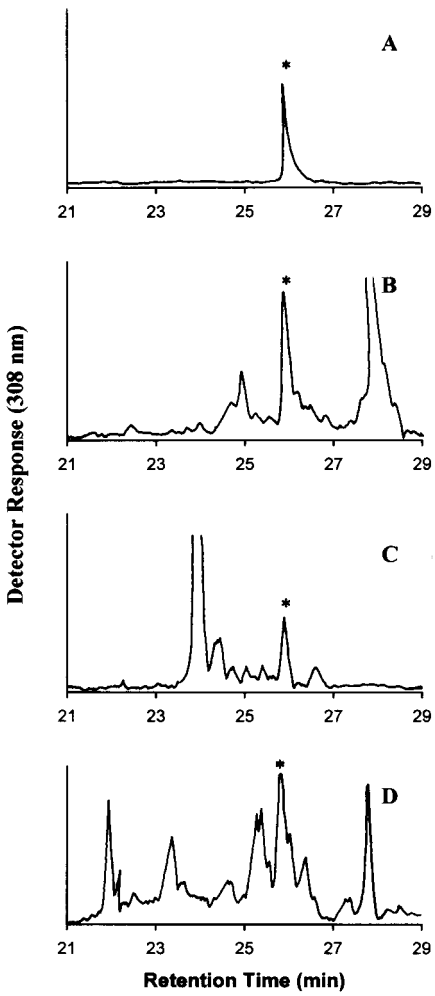

Figure 2. HPLC chromatograms of (A) standard aminoethylcysteine ketimine decarboxylated dimer; (B) garlic; (C) spinach; and (D) tomato extracts.

In Figure 1 are shown, as representative of all vegetables analyzed, the GC profiles of garlic (B), spinach (C), and tomato (D). In all of them, a peak with the same retention time of the dimer standard is present. The flame photometric detector selective for sulfur-containing compounds used for GC analyses and the coelution with the synthetic molecule are highly indicative of the presence of the dimer in all the above cited vegetables.

The same samples were also analyzed by HPLC and the corresponding chromatograms are shown in Figure 2. Moreover, the dimer peak when eluted from HPLC, collected, dried, solubilized in methanol, and submitted to GC analysis again showed the same chromatographic pattern as the authentic compound.

Identification of the dimer in all the vegetable extracts was further confirmed by GC-MS where the mass spectrometer was used both in the scan and in the selected ion monitoring mode. The selected ion chromatogram of the dimer standard is shown in Figure 3A, and the reconstructed ion chromatogram from spinach is shown in Figure 3B. Comparing this ion chromatogram with that obtained from authentic dimer, the same cluster of main ions (i.e., *m*/*z* 228, 200, and 154) is recognizable. Moreover, both in authentic dimer and in the extracts the cluster showed almost the same relative abundance. Because of the complexity of the extract chromatograms, in Figure 3 only part

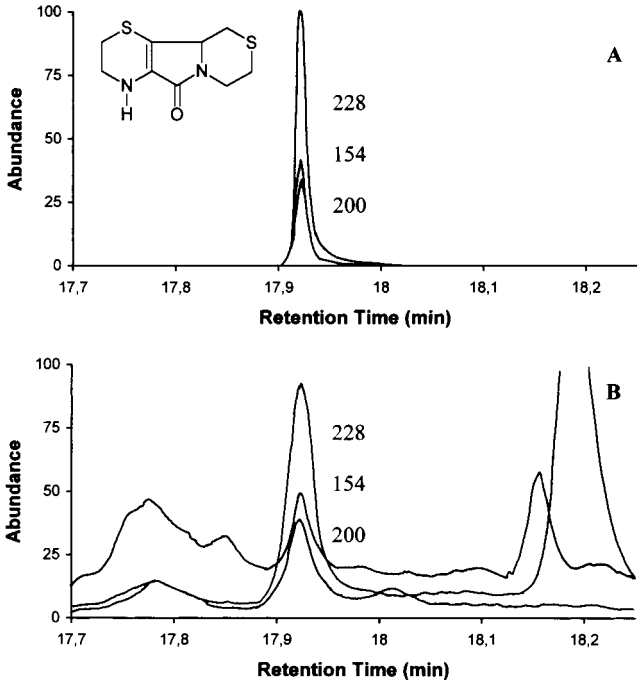

Figure 3. (A) Formula and selected ion chromatograms (*m*/*z* 228, *m*/*z* 200, *m*/*z* 154) of standard aminoethylcysteine ketimine decarboxylated dimer; (B) selected ion chromatograms of the dimer extracted from spinach.

Table 1. Content of Aminoethylcysteine Ketimine Decarboxylated Dimer in Vegetables Present in Mediterranean Diet

samples	amount of dimer (nmol/g)[a]
garlic	0.10 ± 0.02
spinach	0.18 ± 0.05
tomato	0.13 ± 0.03
asparagus	0.25 ± 0.04
aubergine	0.08 ± 0.02
onion	0.68 ± 0.15
pepper	0.43 ± 0.11
courgette	0.52 ± 0.13

[a] Each value is expressed as mean ± SD ($n = 3$).

of the entire chromatogram is shown. Even if an accurate quantitation of the dimer was not the aim of this work, however, an estimate of the endogenous content of this compound can be given.

The dimer content was calculated from the area of the GC peak as compared to a five point standard curve (slope: 1293 ± 14.07; *y* intercept: 7629.50 ± 176.95; $r^2 > 0.99$). The calibration curve was linear from 5 to 200 ng, and the lower limit of detection was 1 ng (absolute quantity). The recovery of authentic dimer was >97%. Results from GC analysis range in the order of 10^{-4} μmol of dimer/g for all the tested vegetables as shown in Table 1.

In this study, we have presented three independent methods of identification for the presence of the dimer in common plant food. Data obtained by GC-flame photometric detector selective for sulfur-containing compounds coupled with HPLC and GC-MS methods lead us to conclude that the dimer is present in garlic, spinach, tomato, asparagus, aubergine, onion, pepper, and courgette.

The finding of the dimer in common vegetable constituents of human diet, the presence of this molecule in human plasma at micromolar concentrations, and the lack of a demonstrated biosynthetic pathway in humans might account for a dietary supply of this molecule. Further studies are needed to investigate the absorption, the fate, and the correlation between diet and the dimer levels in human plasma.

ABBREVIATIONS USED

GC, gas-chromatography; FPD, flame photometric detector; HPLC, high performance liquid chromatography; GC-MS, gas chromatography−mass spectrometry.

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