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Azetidine-2-carboxylic acid in garden beets (*Beta vulgaris*)

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Abstract

Azetidine-2-carboxylic acid (L-Aze) is a toxic and teratogenic non-protein amino acid. In many species, including man, L-Aze is misincorporated into protein in place of proline, altering collagen, keratin, hemoglobin, and protein folding. In animal models of teratogenesis, it causes a wide range of malformations. The role of L-Aze in human disease has been unexplored, probably because the compound has not been associated with foods consumed by humans. Herein we report the presence of L-Aze in the garden or table beet (*Beta vulgaris*).

Keywords: Azetidine-2-carboxylic acid; Beets; *Beta vulgaris*; Garden beet; Table beet; Teratogen; Toxin; Autoimmunity

1. Introduction

Non-protein amino acids, found principally in plants, higher fungi, and marine algae, do not participate in protein synthesis, but serve as poisons that may deter predators and as reservoirs for nitrogen storage (Rosenthal and Bell, 1979; Wink, 1997). Their consumption can cause a broad spectrum of human diseases including acute toxicity, neurodegeneration, and autoimmune disorders (Rubenstein, 2000; Bell, 2003). Azetidine-2-carboxylic acid **2** (L-Aze) is a non-protein amino acid homologue of proline **1** (Fig. 1), previously identified in Liliaceae and in the sugar beet (Fowden, 1956, 1972; Fowden and Richmond, 1963). Sugar beets are processed for the extraction of sucrose, and their by-products are often deployed as livestock feed, as are fodder beets.

We are unaware of efforts to determine whether L-Aze **2** is present in garden or table beets or related foods consumed by humans. Therefore, we have conducted liquid chromatographic–mass spectrometry (LC–MS) studies. The initial investigations were carried out in a commercial laboratory SurroMed, Inc., Menlo Park, CA in August 2003. To validate and extend these observations, we performed a second set of experiments in the Stanford University Mass Spectrometry Laboratory in May 2005.

2. Results and discussion

The first investigations were done on a number of different kinds of vegetables, including garden beets of one variety, purchased at a grocery store located on the San

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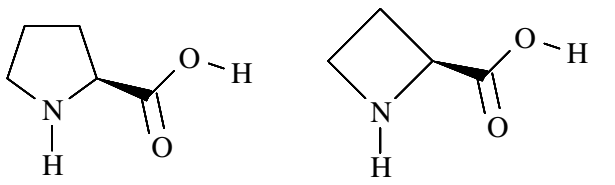

Fig. 1. Chemical structures of L-proline and its homologue L-azetidine-2-carboxylic acid, the difference being a five- vs. four-member ring.

Francisco Peninsula. The primary source of the vegetables was not determined.

L-Aze **2** was found to be present in the root of garden beets, the plant part ordinarily consumed by humans. The ratio of the free amino acids L-Aze **2** to L-proline **1** was determined to be from 5% to 20%, the result depending on the amino acid extraction method (see Section 4). Mass chromatograms from the beet extract for L-proline **1** and L-Aze **2** are displayed in Fig. 2. A strong-cation exchange cartridge was used for extraction. In contrast, L-Aze **2** was not found in extracts of the leaves of garden beets (*Beta vulgaris*), the leaves of spinach (*Spinacia oleracea*), the leaves of Swiss chard (*Beta vulgaris flavescens*) carrot roots (*Daucus carota*), or potato roots (*Solanum tuberosum*), as displayed in Fig. 3. Note that this study did not specifically determine the stereochemistry of these amino acids; future work with chiral chromatography and/or NMR spectroscopy is recommended.

The second set of experiments was done on four different varieties of garden beets purchased at three different locations on the San Francisco Peninsula (Fig. 4). The beets were grown in four different regions of California: the northern San Joaquin Valley, the central San Joaquin Valley, the Imperial Valley, and the Salinas Valley. In these investigations, L-Aze **2** was found in all four varieties of garden beets. The observed ratio of L-Aze **2** to L-proline **1** ranged from 1% to 5% in the four different varieties of garden beets.

These studies confirm that substantial amounts of L-Aze **2** are present in garden beets grown in four different regions and harvested approximately two years apart. The findings were made using two different liquid chromatography–mass spectrometry methods in two different laboratories.

Fowden reported in 1956 that L-Aze **2** was present in sugar beets in a concentration about 2% that of proline

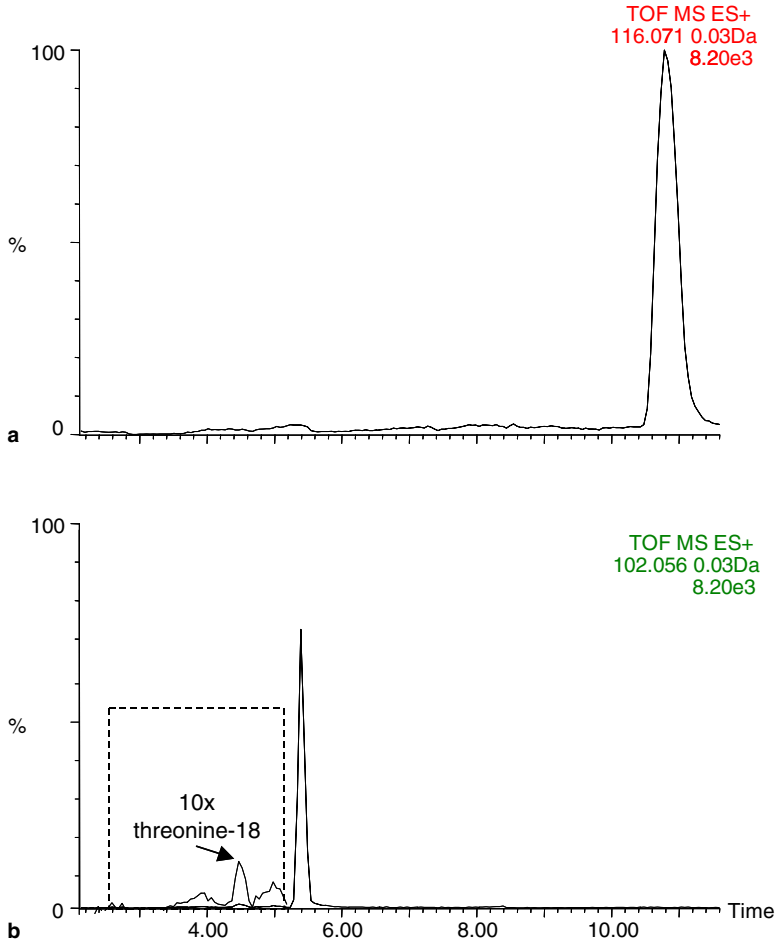

Fig. 2. Selected mass chromatograms (intensity of the characteristic mass vs. chromatographic elution time) from garden beet extract: (a) proline **1**; (b) azetidine-2-carboxylic acid **2** (with a resolved water-loss signal from threonine).

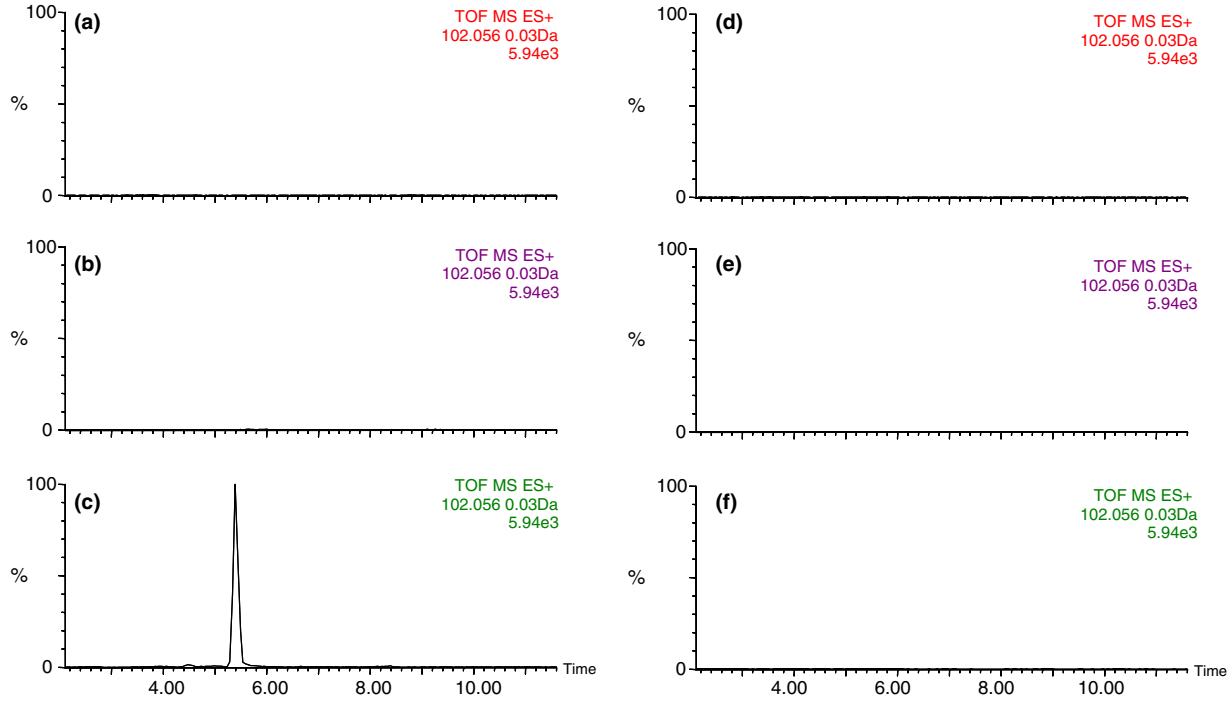

Fig. 3. Mass chromatograms of azetidine-2-carboxylic acid **2** mass chromatograms from: (a) Swiss chard extract; (b) beet leaves extract; (c) beet root (bulb); (d) carrot root; (e) spinach leaves; (f) potato root.

1, an amount that corresponds with these findings. Although the absolute concentration of L-Aze **2** was not determined in this study, relating the relative amounts of the amino acids L-Aze **2** and L-proline **1** is of utility, noting a prior determination that a one-cup serving of garden beets (246 grams) contains an average 49.2 mg of proline **1** (United States Dept. of Agriculture, National Nutrient Data Base, Standard Reference, Release 17, June 2004).

These data show that garden beets are a source of significant amounts of L-Aze **2**, a compound known to cause toxic and teratogenic effects in animal models. To the extent that L-Aze **2** makes its way from sugar beets or fodder beets into the livestock and their dairy products, as well as into environmental run-off systems, such foods may be a secondary source of L-Aze **2**.

The misincorporation of L-Aze **2** in place of proline **1** changes the folding of proteins and alters the structure of collagen, keratin, and hemoglobin (Tsai et al., 1990; Lubec et al., 1985; Tan et al., 1983; Trasko et al., 1976; Baum et al., 1975; Trotter et al., 2002). In animal models of teratogenesis, it causes malformation of the inner ear, lung, teeth, palate, vertebra, long bones, neural crest, and other structures (Van de Water and Galinovic-Schwartz, 1986; Adamson and King, 1987; Moullect et al., 1975; Joneja, 1981; Badamante and Hall, 1980). Exposure to L-Aze **2** during critical periods of embryogenesis could result in transient impairment of the expression of signalling proteins such as those involved in brain development (Sur and Rubenstein, 2005).

L-Aze **2** that has been previously ingested and misincorporated into proteins may be released after protein breakdown and thus may re-enter protein synthesis endogenously as has been shown in the pathogenesis of disorders associated with β-*N*-methylamino-L-alanine (Murch et al., 2004; Cox et al., 2005). Although L-Aze **2** causes congenital malformations and severe biochemical abnormalities in many species, its role in the pathogenesis of human congenital and acquired diseases, including autoimmune disorders, is not established and requires further investigation.

Data currently available are unsuitable for use in determining dose–response curves or toxicity thresholds in humans. For instance, in experiments conducted on abnormal collagen production in mouse embryo otic explants, the investigators used nutrient media containing L-Aze **2** at concentrations of 150 and 300 μg/ml (Berggren et al., 1997). Such data cannot be extrapolated into meaningful information regarding the pathogenesis of chronic neurodegenerative and autoimmune diseases, especially those with latency periods often spanning decades.

Epidemiologically useful information would require large-scale, carefully designed studies done on defined populations, conducted over long intervals. Our purpose is to call attention to the fact that a widely eaten food, indeed a staple for many millions of people in various parts of the world, contains significant amounts of a potentially noxious non-protein amino acid that readily misincorporates in proteins.

Garden beets and sugar beets belong to the goosefoot family (Chenopodiaceae). The other members of this family are spinach and Swiss chard. L-Aze **2** was not found in the leaves of any of these three plants (Fig. 3). Further studies are needed to determine whether L-Aze **2** is present

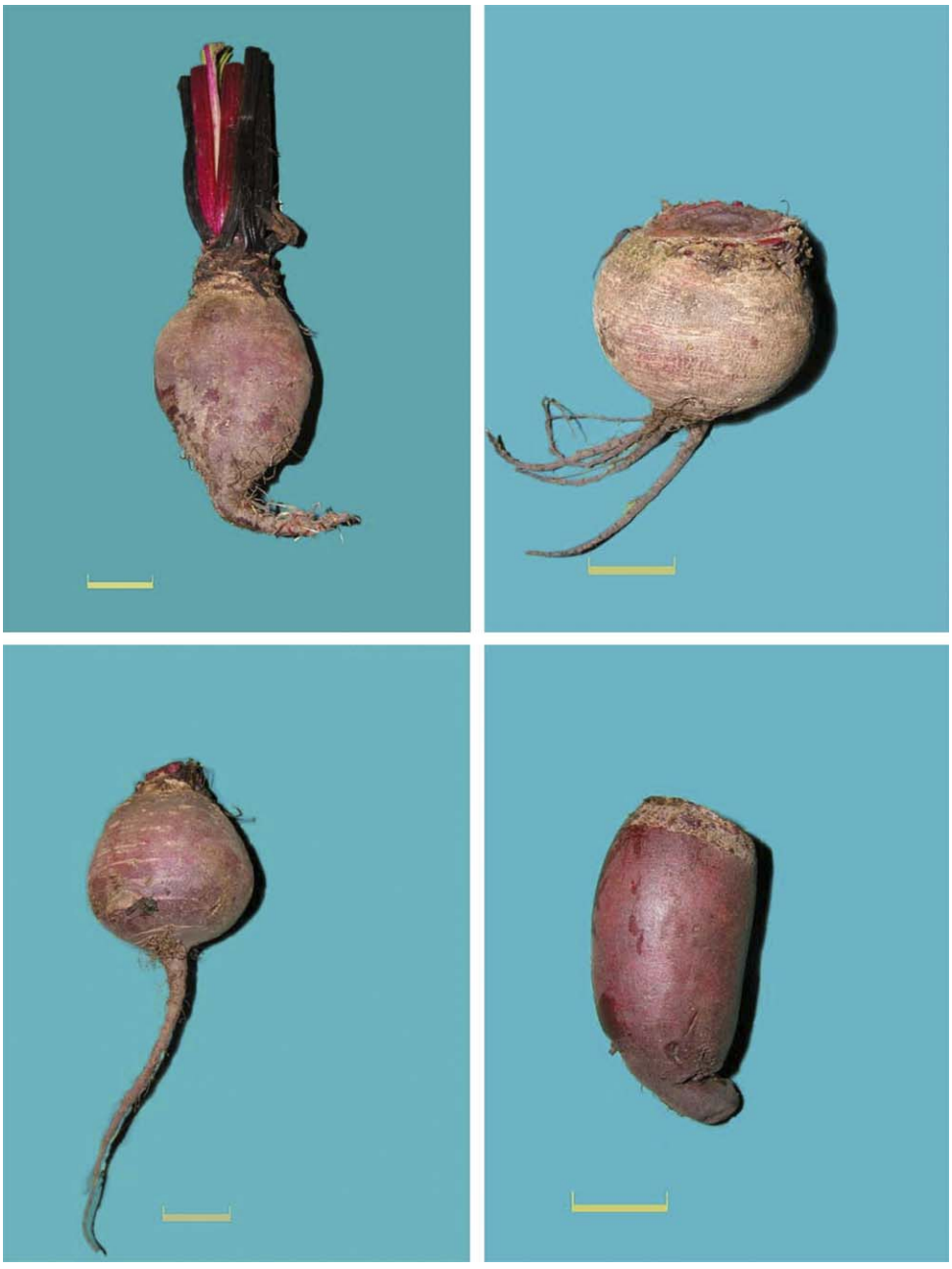

Fig. 4. Photograph showing the four different varieties of garden beets grown in four different regions of California used in the second set of experiments. The length of the scale bars is 2.5 cm. The photograph shows the distinctive morphologies of each variety.

in structural parts of spinach and Swiss chard that correspond with the fleshy roots of beets. Whether the non-protein amino acid is synthesized de novo within the root system of beets or arises from associated microorganisms is unclear (Fowden, 1956).

3. Concluding remarks

We present data indicating that the non-protein amino acid, azetidine-2-carboxylic acid **2**, is present in the roots of garden beets.

4. Experimental

4.1. Initial studies

4.1.1. Amino acid extraction

Six beet roots of the same variety were separated from the stems and leaves and washed under tap water to remove soil. After the roots were dry, they were diced into fine strips and then boiled in 300 ml of HPLC water for 2 h. The extract was allowed to cool, then filtered and transferred in 5 ml aliquots to 40 tubes and vacuum dried. Thereafter the amino acids were extracted using a strong-cation

exchange (SCX) cartridge (AccuBond SPE cartridge from Agilent), and in a second experiment, using a mixed-mode cation exchange cartridge (Oasis MCX cartridge, Waters Corp. Milford, MA).

For the cation-exchange purification procedure, each tube of the dried beet extract was reconstituted with 0.1% formic acid (1 ml). The cation exchange cartridge was washed with MeOH (1 ml and then HPLC-grade H_2O (2 ml) before the sample was loaded. The cartridge plus sample was then washed with 0.1% HCO_2H (2 ml) and the desired amino-acid-containing fraction eluted with diluted NH_4OH (2 ml, pH 11.5). The sample was pooled and divided into 20 tubes and vacuum dried again. Similar extraction was independently performed for the beet leaves, the Swiss chard leaves, the spinach leaves, carrots (roots) and red potatoes.

4.1.2. Liquid chromatography–mass spectrometry

After cation-exchange extraction, one tube of the dried sample was reconstituted with 200 μl of 0.3% heptafluorobutyric acid (HFBA) and centrifuged to remove particulates. The supernatant was further acidified with 2 μl of concentrated HFBA to reach a final pH of 2–3. Ten microliter of the sample were injected into the LC–MS system which consisted of an Agilent capillary 1100 HPLC system (Palo Alto, CA) coupled on-line to an electrospray ionization, time-of-flight mass spectrometer, model LCT, from Waters Corp. (Milford, MA). A reverse-phase LC column (Phenomenex, Synergi Hydro-RP, Torrance, CA) was used for separating the amino-acid-containing mixture. Buffer A was 0.3% HFBA in HPLC-grade H_2O; buffer B was 0.3% HFBA in CH_3CN. The separation was performed with a linear gradient from 0% to 25% B in 65 min with a flow rate of 15 μl/min.

To test the LC–MS system for this application, samples of L-azetidine-2-carboxylic acid **2** (Aldrich, A0760), L-proline **1** (Fluka, 81709), and L-threonine (Fluka, 89179) were used without further purification. Attention was paid to the exact mass coincidence of L-Aze **2** and the minor water-loss channel of threonine associated with its ionization; these two compounds were found to be well-separated chromatographically.

4.2. Second set of experiments

4.2.1. Amino acid extraction

Each of the four varieties of beets was processed and analyzed independently.

The beet roots were separated from their stems and leaves, and washed under tap water. They were then air dried, weighed, and approximately 400 g (3–6 beets) were cut into fine strips, approximately $3 \times 3 \times 20$ mm each, and re-weighed. Less than 4 g were lost in the chopping process. The beet solids were boiled in HPLC grade H_2O (750 ml, EM Sciences, WX0004-6). After cooling, the aqueous phase was gravity filtered. The liquid filtrate was dried under vacuum for further use. Whether boiling affects the concentration of L-Aze **2** has not been studied.

Aliquots of the dried beet extract (approximately 1% of the total volume) were reconstituted with 0.1% HCO_2H (J.T. Baker, 0129-01) to a total volume of 1 ml. Strong cation exchange (SCX) cartridges (AccuBond, Agilent, 188-1550) were conditioned with MeOH (1 ml, Burdick and Jackson) and HPLC-grade H_2O (2 ml). Aliquots (1 ml) of the sample were loaded and then the cartridges were washed with 0.1% HCO_2H (2 ml). Compounds of interest were eluted with diluted NH_4OH (2 ml, Fisher HPLC grade, A639-500, pH 11.5). The collected samples were dried under vacuum.

Authentic standards of L-azetidine-2-carboxylic acid **2** (Aldrich, A0760). L-proline **1** (Fluka, 81709), and L-threonine (Fluka, 89179) were used for method development and spiking experiments.

4.2.2. Liquid chromatography–mass spectrometry

The dried samples from the SCX extraction were weighed, then reconstituted in H_2O (0.5 ml). Aliquots (200 μl) were taken from each sample. In order to match the sample buffer to the HPLC initial conditions, three volumes of CH_3CN (Burdick and Jackson) were added to each sample aliquot, resulting in a composition of 75% CH_3CN. The samples were then centrifuged to separate the precipitate. The supernatant (800 μl) was recovered and dried to reduce volume. The samples were reconstituted in 200 μl of sample-loading buffer (2.6 mM ammonium acetate, Fisher HPLC grade), in CH_3CN–H_2O (6:4). Five microliter of sample were injected for LC–MS/MS analysis. HPLC was performed on an Agilent 1100 system at a flow rate of 300 μl/min. A 6-min isocratic elution from a 2.1×100 mm hydrophobic interaction chromatography column (5 μm particle size, 100 Å pore size, PolyHYDROXYETHYL A) was performed using 2.6 mM ammonium acetate in CH_3CN–H_2O (6:4).

The HPLC was directly coupled to a Micromass Quattro Premier triple quadrupole mass spectrometer with electrospray ionization. The mass spectrometer was operated in multiple reaction monitoring mode, using the transitions 101.9 to 55.8, 73.8 and 83.3 m/z for L-Aze **2**, and 115.8 to 69.8 and 98.1 m/z for proline **1** (major fragment ions are underlined). Threonine was determined not to be interfering under these LC–MS conditions.

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