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OCCURRENCE OF TWO BRANCHED PENTA-AMINES IN LEGUME SEEDS HAS NOT BEEN CONFIRMED

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ABSTRACT

We detected N^4-bis(aminopropyl)spermidine [3(3)(3)4], a quaternary branched penta-amine and N^5-aminobutylhomospermine [4(4)44], a tertiary branched penta-amine by liquid chromatography and gas chromatography of the concentrated/purified polyamine fraction acid-extracted from the legume seeds of *Crotalaria spectabilis*, and other legume seeds of *Vicia sativa* (narrow-leaved vetch), *Vicia villosa* (hairy vetch) and *Phaseolus coccineus* (runner bean), respectively, and we have reported tentatively the natural occurrence of the two penta-amines in legume seeds. We validated and evaluated the analyses of the two penta-amines in our previous studies and showed here some unreproducible polyamine profiles among the seeds supplied from markets, which we cultivated and collected in fields. Contamination of the branched penta-amines during concentration/purification to obtain polyamine fraction before performing the two chromatographical analyses, was not excluded. Therefore, the natural occurrence of the two branched penta-amines in leguminous plant seeds has not been confirmed.

Keywords: Polyamines; Branched penta-amines; Legume seeds; HPLC; GC-MS; Crotalaria spectabilis

INTRODUCTION

More than 35 kinds of natural biogenic polyamines (linear diamines, triamines, tetra-amines, penta-amines and hexa-amines, and branched triamines, tetra-amines and penta-amines, etc.) can act as a controller for acid/base-buffering function and a free radical scavenger and can interact with various acidic biomolecules in cells. Furthermore, plant polyamines are important for carbon dioxide (CO_2) assimilation for photosynthesis in plants. Therefore, polyamines have various

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respective functions in the adaptation to environment for plant cell growth and the differentiation of cells to multicellular plant organs. We comprehensively have examined endogenous cellular polyamines of the various organs of 95 seed plant species (Hamana et al., 1991, 1992, 1996, 2017, 2019, 2025a; Matsuzaki et al., 1990; Otsuka et al., 2005) and reported finally an overview of polyamine distribution profiles in plants, in Hamana et al., 2025b. Leguminous plant seeds, especially edible beans, are important as an agricultural crop product. Leguminous plants such as *Crotalaria* and *Vicia* species are used as a green manure plant or soil improvement plant in the fields of agriculture and environmental science.

In our polyamine analyses of 73 kinds of legume seeds of 73 leguminous plants taxonomically belonging to the family Fabaceae in the kingdom Plantae, we detected N^4-bis(aminopropyl)spermidine [3(3)(3)4], a branched penta-amine, in the concentrated/purified polyamine fraction acid-extract from the mature seeds of a leguminous plant, *Crotalaria spectabilis,* supplied from a market by high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) in Hamana et al., 1996. In subsequent years, 3(3)(3)4 was not detected in the polyamine fraction acid-extracted from other stored (old) seeds by HPLC and high-performance gas chromatography-MS (HPGC-MS) in Otsuka et al., 2005.

The presence of N^5-aminobutylhomospermine [4(4)44], another branched penta-amine, and the absence of N^5-aminobutylhomospermidine [4(4)4] were found in HPLC, GC and GC-MS of the concentrated/purified polyamine fraction acid-extracts from the mature seeds, supplied from a market, of *Vicia sativa* in Haman et al., 1991 and *Vicia villosa* in Hamana et al., 1996 and *Phaseolus coccineus* in Hamana et al., 1992. On the other hand, the branched penta-amine 4(4)44 was not detected in the polyamine fraction from the immature seeds of *Vicia sativa* collected in the field (Hamana et al., 1991) and in the fresh mature seeds of *Vicia sativa,* supplied from a market and newly analyzed (Otsuka et al., 2005). When we have analyzed the polyamine fraction acid-extracted from the mature seeds of *Phaseolus coccineus* cultivated in the author's (Hamana) home garden, 4(4)44 was not detected by HPLC (data were not shown in our previous reports).

Several liner penta-amines and linear hexa-amines, in addition to some usual diamines and triamine, were detected and identified with high reproducibility in the concentrated/purified polyamine fractions (samples) from the 73 leguminous mature seeds cultivated under different environments. Therefore, detection of 3(3)(3)4 and 4(4)44 in legume seeds in our previous studies was validated and evaluated in the present report.

MATERIALS AND METHODS

In our previous polyamine analyses, immature, mature (fresh mature) and/or old (old mature) (stored old) (5 year's storage after harvest) legume seeds were supplied by Kaneko Seed Co. Ltd., Maebashi, Gunma, Japan, purchased from food markets in Gunma, Japan, harvested from the

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author's (Hamana) home garden, Tsumagoi, Gunma and/or collected in the fields at Shimokoide, Maebashi. Therefore, unfortunately, environmental conditions of seed production and seed storage of the 73 legume seeds were not consistent. Minor incorporation of polyamines from soil and leguminous root nodules during growth of the leguminous plants are possible. Mature *Crotalaria spectabilis* and *Crotalaria juncea* seeds were supplied by Kaneko Seed and stored for 1 year in our laboratory, as old seeds (Hamana et al., 1996; Otsuka et al., 2005). Old seeds (5 year's storage in Kaneko Seed) of *Vicia sativa* (Haman et al., 1991) and (fresh) mature seeds of *Vicia sativa* (Otsuka et al., 2005), mature seeds of *Vicia villosa* (Hamana et al., 1996) and mature sees of *Phaseolus coccineus* (Hamana et al., 1992) were supplied by Kaneko Seed. Immature seeds of *Vicia sativa* were collected in the fields (Otsuka et al., 2005).

Polyamine extraction from seeds and concentration/purification procedures in our previous studies are following. After washing with water and weighting, leguminous seeds (10-100g) were homogenized in the same weight of 10% (1.0M) perchloric acid (PCA) and were homogenized using a mixer (T-fal BL13) and an ultrasonic disintegrator (Branson sonifier SFX). After centrifugation at 10,000 ×g, the supernatant (5% PCA extract) was applied to a column (3cm I.D. × 1cm) of cation-exchange resin (-SO_3^-), DOWEX W50x8, and then polyamines were concentrated by elution with 6M HCl from the column. After evaporating at 90-95°C, for 10-30 min, dissolving in water, and then neutralizing and centrifuging, the supernatant was obtained as concentrated/purified polyamine fraction.

We have developed high-performance liquid chromatography (HPLC), gas chromatography using a packed column (without mass spectrometric apparatus) (GC) and gas chromatography-mass spectrometry using a packed column (equipped with mass spectrometric apparatus) (GC-MS), and high-performance gas chromatography-mass spectrometry using a capillary column (equipped with mass spectrometric apparatus) (HPGC-MS) (Niitsu et al., 1993; Hamana et al., 2016, 2025b). Before the three GC analyses, polyamine fractions (samples) were heptafluorobutyrylated by heating with CH_3CN and heptafluorobutylic anhydride (Niitsu et al., 1993). Retention chart by FID (Flame Ionization Detector) as the detector was obtained in GC. Retention chart by MS as the detector was obtained in GC-MS and HPGC-MS. We chemically synthesized the most of biogenic polyamines to use as authentic standards. In the three GC analysis, 3(3)(3)4 (detected in HPLC) in the original polyamine sample is detected as N^4-aminopropylspermidine [3(3)4] and N^4-aminopropylnorspermidine [3(3)3], and N^5-bis(aminobutyl)homospermidine [4(4)(4)4] (detected in HPLC) is detected as 4(4)4 (Niitsu et al., 1993; Hamana et al., 2016). When 4(4)4 is not detected, the existence of 4(4)(4)4 in the original polyamine sample can almost be excluded. However, retention peak identification of 4(4)4 was difficult in our GC methods. When 4(4)44 is detected, the existence of 4(4)(4)44 is possible in the original polyamine sample.

RESULTS AND DISCUSSION

The branched penta-amine 3(3)(3)4 was not found in other (stored, old) seed samples of *Crotalaria spectabilis* (Otsuka et al., 2005) nor the (fresh) mature seeds of *Crotalaria juncea* supplied by Kaneko Seed (Hamana et al., 1996) (Fig. 1) and other various plant organs including the 73 legume seeds in the 95 seed plant species analyzed in our previous studies. We analyzed the branched penta-amine in various bacteria and archaebacteria (archaea) (Hamana and Matsuzaki, 1992; Hamana and Hayashi, 2025) at the same time in our laboratory. Before HPLC and GC analyses, contamination of the branched penta-amine during concentration/purification to the polyamine fraction from the mature seeds of the *Crotalaria spectabilis* supplied by Kaneko Seed in Hamana et al., 1996 was not excluded. Although the column of DOWEX W50x8 was re-used after the recycle by washing with acetone, 2M NaOH, 6MHCl and water at room temperature, contamination of the residual branched penta-amine from the previous polyamine samples is not excluded. Minimize contamination risks through the use of fresh reagents, new columns. Therefore, the natural occurrence of 3(3)(3)4 in the seed of *Crotalaria spectabilis* was not confirmed by our previous studies.

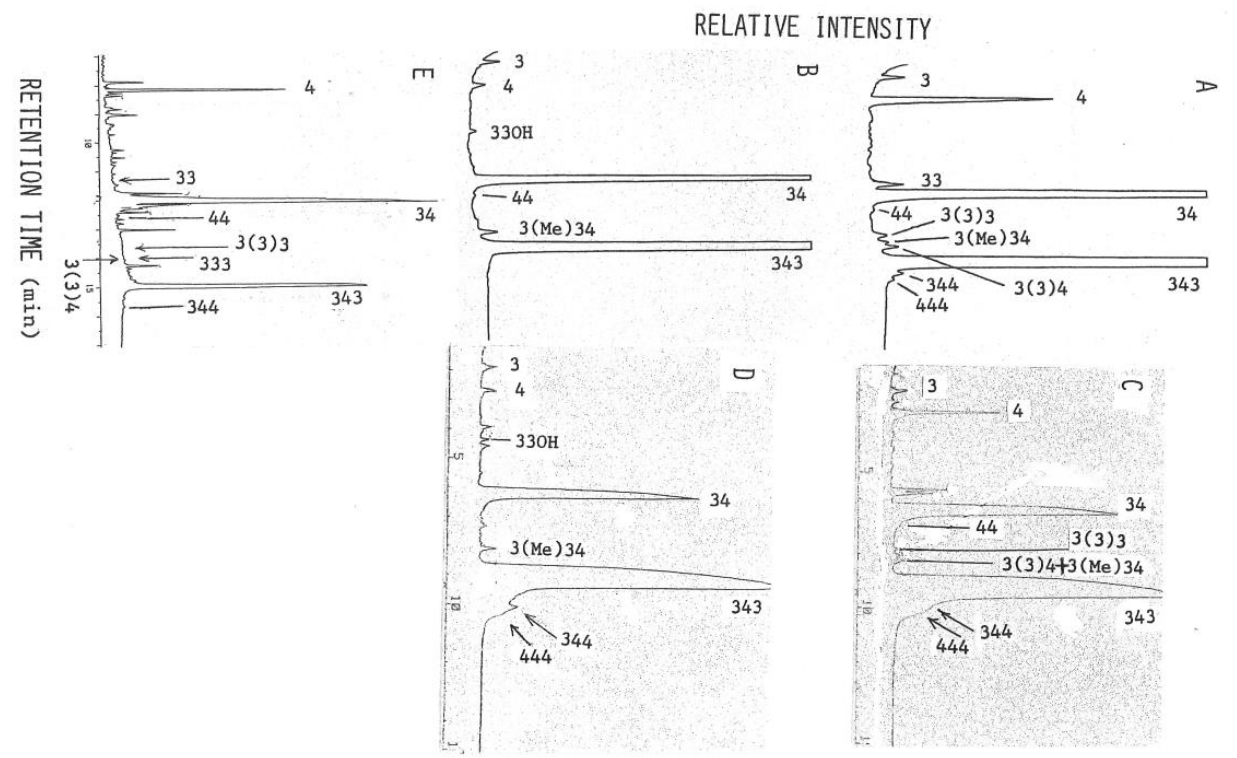

Fig. 1: GC retention charts of the concentrated/purified polyamine fraction acid-extracted from the mature seeds (supplied by Kameko Seed) of *Crotalaria spectabilis* (A and C) and *Crotalaria juncea* (B and D) (Hamana et al., 1996). A and B were obtained by using FID in GC, and C and D were obtained by using MS in GC-MS in Hamana et al., 1991 and are shown. HPGC retention chart of the old seeds (storage in our laboratory) of *Crotalaria*

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***spectabilis* (E) was obtained by using MS in HPGC-MS (Otsuka et al., 2005). Arrow (→) indicates retention position of authentic polyamine standards. Bar (ー) indicates the minor polyamine peaks identified. Numeric codes (number of methylene (CH_2) groups between amino (NH_2) or imino (NH) groups) are abbreviations for polyamines.**

We detected 4(4)44 in the polyamine concentrated/purified fraction acid-extract from the seeds of three leguminous plants *Vicia sativa*, *Vicia villosa* and *Phaseolus coccineus* supplied from the market by HPLC, GC and GC-MS, and reported the major occurrence of the branched penta-amine and its long polyamine derivatives in the former and the minor occurrence of the branched penta-amines in the last two (Fig 2). However, we have not obtained reproducible detection of 4(4)44 in the concentrated/purified polyamine fraction from the seeds of *Vicia sativa*, *Vicia villosa* and *Phaseolus coccineus* in our studies (Fig. 2). Although minor contamination of the branched penta-amine during concentration/purification of the last two polyamine fractions from the concentrated/purified polyamine fraction of the old seeds of *Vicia sativa* was possible. Major contamination of 4(4)44 into the concentrated/purified fraction of the old seeds of *Vicia sativa* was excluded because we have not chemically synthesized authentic 4(4)44 in our laboratory. The branched penta-amine was not detected in the immature seeds of *Vicia sativa* collected from the field and the fresh mature seeds of *V. sativa* supplied by Kaneko Seed (Otsuka et al., 2005). On the other hand, polyamine profiles of the mature seeds of *Glycine max* did not change during storage for 1 year in our laboratory after harvest (Otsuka et al., 2005). At present, fungicides and bactericides containing =NH and/or =N— such as Benomyl, Thiuram, Captan and Thiamethoxam are frequently used for the storage of legume seeds for seedling in agriculture but not used for edible beans in food products. We discussed with Kaneko Seed Co. about possible contamination of polyamine-related chemicals used during preservation of the old seeds of *Vicia sativa* in Hamana et al., 1991, however no information led us to believe there was a possibility of contamination. Contamination of chemicals related to 4(4)4 and 4(4)44 in the old seeds were not excluded. Experimental reproducibility by standardizing seed sources, storage conditions, and extraction procedures were need. Therefore, the natural occurrence of 4(4)44 in the three leguminous seeds was not confirmed in our studies. Biochemically synthesis of the unusual, branched polyamines in plants, as a biogenic polyamine, is unlikely. Furthermore, the branched polyamines have never been found in other organisms.

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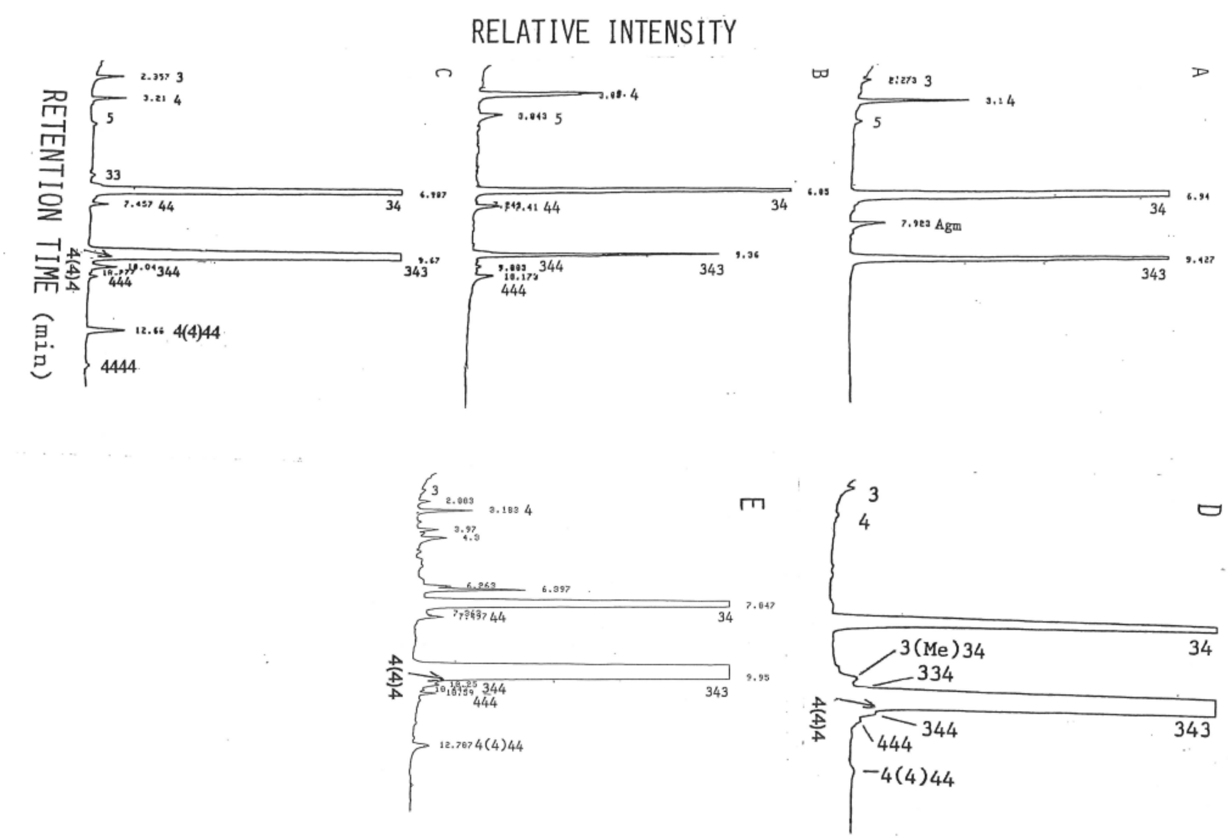

Fig. 2: GC retention charts (obtained using FID in GC) of the concentrated/purified polyamine fraction acid-extracted from the immature seeds (collected in field) (A) (Hamana et al., 1991; Otsuka et al., 2005), mature seeds (supplied by Kaneko seed) (B) (Otsuka et al., 2005) and old seeds (supplied by Kaneko Seed) (C) (Hamana et al., 1991) of *Vicia sativa,* and the mature seeds (supplied by Kaneko Seed) of *Vicia villosa* (D) (Hamana et al., 1996) and of *Phaseolus coccineus* (E) (Hamana et al., 1992). Generally, polyamine-overloaded GC can detect minor peaks, while minor peaks disappear in a major peak on overloaded GC chart. Arrow (→) indicates retention position of authentic polyamine standards. Bar (一) indicates the minor polyamine peaks identified. Abbreviation of 4444 in Otsuka et al., 2005 should be corrected to 4(4)44.

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