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Identification and Content of 1-Malonylaminocyclopropanecarboxylic Acid in Germinating Cocklebur Seeds

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A major conjugate of 1-aminocyclopropanecarboxylic acid in germinating cocklebur (*Xanthium pennsylvanicum* Wallr.) seeds was isolated and identified as 1-malonylaminocyclopropanecarboxylic acid (MACC). The change in MACC content during the germination period of this seed also was examined.

Key words: 1-Aminocyclopropanecarboxylic acid — Cocklebur — 1-Malonylaminocyclopropanecarboxylic acid — Seed germination — *Xanthium pennsylvanicum*.

Ethylene has a regulatory role in the germination of cocklebur seeds and is produced from its immediate precursor, ACC, in these seeds as in other plant tissues (Satoh and Esashi 1980, 1982, 1983a). Recently, ACC has been found in both its free and conjugated forms in several plant tissues, including peanut seeds. A major ACC conjugate has been identified as MACC (Amrhein et al. 1981, 1982, Hoffman et al. 1982, 1983a, b). We found that ACC also occurs in conjugated form in addition to the free form in soaked axial (Satoh and Esashi 1983a) and cotyledonary (Satoh and Esashi 1983b) tissues of cocklebur seeds. More recently, we found that the isomers of 1-amino-2-ethylcyclopropanecarboxylic acid, analogs of ACC, are malonylated in cocklebur seed tissues. This suggests that there is malonylation of endogenous ACC in these tissues (Satoh and Esashi 1984). No ACC conjugate in cocklebur seed tissues, however, has yet been identified. We here report the isolation of a major ACC conjugate from soaked cocklebur seed tissues and its identification as MACC. Changes in its content during germination are discussed.

Materials and Methods

Isolation and identification of a major ACC conjugate—Axial segments 3-mm long (580 g dry wt) were excised from after-ripened lower cocklebur (*Xanthium pennsylvanicum* Wallr.) seeds then incubated on a water substratum at 23°C for 2 days in a dark room. The incubated segments were rinsed with 10 volumes of water then blotted dry on filter paper and weighed (1.8 kg fr wt), after which they were homogenized for 5 min at 20,000 rpm in 6 liters of aqueous ethanol at a final concentration of 50% (v/v) in a PHYSCOTRON NS-600 (NITI-ON, Chiba, Japan). The homogenate was left overnight at 4°C then centrifuged at 10,000 × *g* for 15 min. The pellet formed was washed with 1 liter of 50% ethanol and similarly centrifuged. The supernatants were combined and reduced to a 400-ml aqueous solution in an evaporator at 40°C, then partitioned against 400 ml of chloroform.

Abbreviations: ACC, 1-aminocyclopropanecarboxylic acid; MACC, 1-malonylaminocyclopropanecarboxylic acid.

In the following purification steps, the ACC conjugate was assayed as an ethylene-forming substance after acid hydrolysis. A portion of the combined eluates from the chromatography columns and the water extracts from the scraped zones of the TLC plates was hydrolyzed with HCl to obtain ACC, after which it was assayed for ethylene according to the method of Lizada and Yang (1979).

The aqueous solution was applied to a column of Dowex-50W (H^+) (bed volume 260 ml). The passed-through fraction was loaded onto a column of Dowex-1 ($HCOO^-$) (bed volume 260 ml), and after washing this column with 9 liters of water, the adsorbed organic acids were eluted with 750 ml of 40% (v/v) formic acid. The eluate was dried by concentration, then the residue was taken up in 40 ml of water and centrifuged to remove insoluble material.

The clear supernatant produced was condensed to about 2 ml and chromatographed on silicagel coated plates (PLC plates Silicagel 60, Merk Co.) with solvent I (1-butanol–acetic acid–water, 60 : 15 : 25, v/v). Eight plates were used to separate the whole sample. Development was carried out for 18 cm and repeated twice with an air-drying interval. The separated ACC conjugate was applied to the same type of 8 plates and developed 3 times with solvent II (1-propanol–conc. NH_4OH, 7 : 3, v/v).

The sample produced after the second silicagel TLC was purified first by chromatography on cellulose coated plates (Avicel SF, Asahi Kasei Co.) with solvent I then by HPLC. HPLC was done with a Shodex Ionpack KC-811 column (8 mm i.d. × 30 cm) eluted at 40°C with 0.1% phosphoric acid at a flow rate of 1 ml·min^{-1} (Satoh and Esashi 1984). The purity of the isolated ACC conjugate was checked by cellulose TLC with solvents I, II and III (ethyl acetate–formic acid–water, 50 : 15 : 10, v/v).

A portion of the isolated ACC conjugate was hydrolyzed in 6 M HCl at 100°C for 2 h, then the hydrolyzate was separated on a Dowex-50W (H^+) column into the passed-through and retained fractions. The former fraction was analyzed for possible malonic acid by HPLC. The amount of malonic acid present was determined by comparing its peak area with that of the standard curve obtained with known amounts of authentic malonic acid. The latter fraction was eluted with 2 M NH_4OH and, after the evaporation of NH_4OH, it was tested for ACC by cellulose TLC with solvents I, II and IV (phenol–water, 3 : 1, v/v). The amount of ACC present was estimated by the ACC-determination method. The isolated ACC conjugate and its hydrolyzate were put through reactions with ninhydrin and fluorescamine (Weigele et al. 1972) to assay their primary amines. The isolated ACC conjugate was methylated with diazomethane and analyzed by GC-MS with a Jeol JMS-01SG-2 instrument equipped with an OV-1 GC column (1 m × 3 mm i.d.) operating at 30 eV.

Ethylene production and contents of ACC and MACC during the germination period—Twenty lower cocklebur seeds were sown in a 9-cm Petri dish on two layers of filter paper wet with 9 ml water at 23°C in a dark room. At 0 (dry seeds), 24 and 48 h after the start of imbibition, the seeds were checked for germination and weighed, after which they were transferred to 0.5 ml of 50 mM sodium phosphate, pH 6.8 in a 30-ml glass vial. The vial was sealed with a rubber stopper and left for 2 h at 23°C in the dark. A 1-ml gas sample then was taken from the sealed vial with a syringe and assayed for ethylene by gas chromatography (Satoh and Esashi 1980). Ethylene production rates were expressed on the basis of the fresh weights measured at the beginning of the 2-h enclosures. Immediately after the determination of ethylene, the seeds were cut into their axial and cotyledonary parts with a razor blade, and the seed coat fragments discarded. The seed parts were weighed and assayed for their contents of ACC and MACC as described previously (Satoh and Esashi 1983b). Experiments were run in triplicate.

Synthesis of MACC—A 1.67 g portion of ethyl malonyl chloride (Aldrich Chemical Co., once distilled) and 5 ml of 4 M NaOH were slowly added over 1.5 h under stirring to an ice-cold solution of 1.01 g of ACC in 6 ml of 2 M NaOH. After an addition of 1 ml of 4 M NaOH, the

reaction mixture was left for 4 h at room temperature. The mixture then was partitioned against 10 ml of ether. The aqueous phase was passed through an Amberlite IR-120 (H^+) column (bed volume 50 ml) which was washed with water until the effluent became neutral. The effluent and washings were combined and evaporated to an oily residue under a vacuum at 40°C. The residue was kept in a refrigerator for about 1 day and gave MACC crystals (mp 60–75°C).

MACC was recrystallized from acetonitrile: yield 0.95 g (50.6%); mp 139–140°C (decomposition). Elementary analysis: calculated for $C_7H_9O_5N$ (187.5): C, 44.92%; H, 4.85%; N, 7.48%. Found: C, 44.69%; H, 4.78%; N, 7.53%. ^{1}HNMR (90 MHz): δ [D_2O, sodium 3-(trimethylsilyl)tetradeuteriopropionate=0.00] 1.28 and 1.59 (multiplet centers of the AA'BB' system of the cyclopropane ring, 4H), 3.42 (s, 2H, methylene proton in the malonyl moiety). IR: ν_{max}^{KBr} cm^{-1}: 3,260 (secondary amide, stretching), 3,100–2,900 (carboxylic OH, stretching), 1,740 (amide carbonyl, stretching), 1,705 (carbonyl, stretching), 1,612 (carboxylic ion, stretching), 1,548 (secondary amide, deformation), 1,442 ($-CH_2-CH_2-$, stretching). Synthetic MACC was methylated with diazomethane and analyzed by GC-MS as described above.

Results and Discussion

In a preliminary experiment, an ACC conjugate capable of evolving ethylene after HCl-hydrolysis was found in both the aqueous ethanol-soluble and -insoluble fractions of 24-h soaked axial segments of cocklebur seeds. As judged by the amounts of ethylene evolved, the amount of ACC conjugate in the soluble fraction was 10 times that in the insoluble one. The crude ACC conjugate in the soluble fraction was chromatographed as one compound with respective Rfs of 0.73, 0.25 and 0.70 in cellulose TLC when developed with solvents I, II and III. Therefore, subsequent identification was made only for the ACC conjugate in the soluble fraction.

A large scale preparation for identification yielded approximately 8 μmoles of the ACC conjugate (in ACC equivalents) from 1.8 kg fresh weight of 2-day soaked axial segments. The Rf values of the isolated conjugate in cellulose TLC coincided with those of the crude conjugate. The conjugate appeared as one peak at Rt 8.49 min on HPLC. The Rt value of the isolated conjugate was identical to that of synthetic MACC.

The HCl-hydrolyzate of the conjugate was separated into the passed-through and retained fractions by cation exchange column chromatography, and these respective fractions were analyzed by HPLC and TLC. The HPLC of the passed-through fraction showed only one organic acid with an Rt 7.83 min; it was identical to the value for authentic malonic acid. In contrast, the cellulose TLC of the retained fraction gave an amino acid capable of producing ethylene; its respective Rf values were 0.43, 0.48 and 0.55 with solvents I, II and IV. These Rf values coincided with those of authentic ACC (Satoh and Esashi 1983b). The ratio of the amount of malonic acid to that of ACC in the hydrolyzate was 1.07. The ACC conjugate did not react with ninhydrin or fluorescamine, whereas one of its hydrolysis products, ACC, gave positive reactions. This is evidence that the conjugate is an N-substituted derivative of ACC.

All these results suggested that the ACC conjugate was MACC. This was confirmed by GC-MS analysis of the methylated ACC conjugate, which showed an m/z spectrum identical to that obtained for the synthetic MACC dimethyl ester: m/z (relative intensity); 215 (M^+, 5), 184 (16), 183 (80), 156 (20), 155 (24), 152 (3), 142 (8), 127 (10), 124 (8), 116 (11), 115 (28), 114 (32), 101 (44), 100 (36), 96 (5), 84 (6), 83 (100), 82 (6), 74 (17), 59 (22), 57 (9), 56 (25), 55 (39), 54 (14). The spectrum was almost the same to the spectra reported previously (Amrhein et al. 1981, Hoffman et al. 1982). Thus, the major ACC conjugate in soaked cocklebur axial tissue is MACC, the same as in other plant tissues (Amrhein et al. 1981, 1982, Hoffman et al. 1982, 1983a, b). MACC also was detected as a major ACC conjugate in the aqueous ethanol

Table 1 Changes in the MACC content of axial and cotyledonary tissues of cocklebur seeds during germination in relation to ACC content and ethylene production

Imbibition period (h)	Germination (%)	Fresh weight ($mg \cdot seed^{-1}$)	Ethylene ($nmol \cdot g^{-1} \cdot h^{-1}$)	ACC ($nmol \cdot g^{-1}$)		MACC ($nmol \cdot g^{-1}$)	
				Axis	Cotyledon	Axis	Cotyledon
0 (Dry seeds)	0	68.8±0.7	Nd	Nd	Nd	2.96±0.06	3.48±0.04
24	0	99.7±0.7	Nd	0.046±0.019	Nd	2.22±0.35	2.19±0.07
48	75±13	107.6±1.2	0.091±0.030	1.34 ±0.18	0.150±0.025	6.03±0.69	2.55±0.12

Data given are the means of triplicate determinations ±SE. Nd, not detected.

extract of soaked cotyledonary tissue of cocklebur seeds. Therefore, the major ACC conjugate of germinating cocklebur seeds is MACC.

Changes in the MACC content of cocklebur seeds during germination in relation to changes in ethylene production and ACC content are shown in Table 1. In the experiment reported here, however, no ethylene production was detected at 24 h in contrast to previously reported results (Katoh and Esashi 1975) because ethylene was assayed after a modified short accumulation period of 2 h. Cocklebur seeds showed about 75% germination 48 h after the start of imbibition. At this time, the MACC content of the axial (but not the cotyledonary) tissue markedly increased and was accompanied by ethylene production by the seeds and by an increase in the ACC content in the cotyledonary and, particularly, axial tissues. During the first 24 h of imbibition, the MACC contents in both tissues decreased slightly, but this was a superficial decrease resulting from the increasing fresh weight of the seeds due to their water uptake. Our findings agree with the previously postulated view that MACC is only a secondary product of ACC and does not liberate free ACC for ethylene production in plant tissues (Amrhein et al. 1982, Hoffman et al. 1983a, b, Satoh and Esashi 1983a). Interestingly, dry cocklebur seeds contain abundant MACC. Recently, Hoffman et al. (1983b) reported that the content of MACC is a good indicator of the stress history in water-deficit wheat leaves. Similarly, we are now investigating what changes in the MACC content of cocklebur seeds take place during seed development and maturation.

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