ENZYMATIC SYNTHESIS OF 5-^{3}H-INDOLE-3-ACETIC ACID AND 5-^{3}H-INDOLE-3-ACETYL-MYO-INOSITOL FROM 5-^{3}H-L-TRYPTOPHAN

Lech Michalczuk and John R. Chisnell
Department of Botany and Plant Pathology
Michigan State University
East Lansing, Michigan 48824
U.S.A.

SUMMARY

Labeled l-tryptophan is converted to indole-3-acetamide and then to indole-3-acetic acid by enzymes from Pseudomonas savastanoi. Labeled indole-3-acetic acid can be converted to indole-3-acetyl-1-O-β-D-glucose and to indole-3-acetyl-myo-inositol by enzymes from kernels of Zea mays sweet corn.

Key Words: l-tryptophan, indole-3-acetamide, indole-3-acetic acid, indole-3-acetyl-1-O-β-D-glucose, indole-3-acetyl-myo-inositol.

INTRODUCTION

High specific activity ^{3}H-indole-3-acetic acid (IAA) is commercially available only by custom synthesis. However, 5-^{3}H-l-tryptophan can be converted to 5-^{3}H-indole-3-acetic acid in 70% yield, and at one-tenth the cost, by enzymes from Pseudomonas savastanoi by the following reaction sequence (1,2):

0362-4803/82/010121-08$01.00

Received September 22, 1980
Revised May 4, 1981

a) l-tryptophan + O_2 ⟶ indole-3-acetamide + CO_2 + H_2O

b) indole-3-acetamide + $2H_2O$ ⟶ indole-3-acetic acid + NH_4OH

Labeled IAA may then be converted to indole-3-acetyl-myo-inositol by enzymes from kernels of Zea mays by the reaction sequence (3):

c) indole-3-acetic acid + UDPG ⟷ indole-3-acetyl-1-O-β-D-glucose

d) indole-3-acetyl-1-O-β-D-glucose + myo-inositol ⟶ indole-3-acetyl-myo-inositol + glucose

The enzyme catalyzing reaction a has been characterized as tryptophan 2-monooxygenase (EC 1.13.12.3) (1, 4); the enzymes catalyzing reactions b, c and d have been characterized only as to the products formed (1, 3).

A chemical synthesis of ^{14}C-indole-3-acetyl-myo-inositol employing ^{14}C-IAA-imidazole as the acylation reagent and myo-inositol as the alcohol acceptor has been published (5) as has an enzymatic synthesis of ^{14}C-IAA using a similar Pseudomonas savastanoi enzyme preparation (2). However, the enzymatic syntheses here described are adapted to a high specific activity, microscale synthesis of ^{3}H-IAA-myo-inositol and ^{3}H-IAA. ^{3}H-IAA has been used to study transport (6) and metabolism (cf. 7) of IAA in plants and in a radioimmunoassay for IAA (8). IAA-myo-inositol is a major ester of IAA in Zea (9), and the labeled compound has been used to study turnover of the ester (10) and its transport (11).

EXPERIMENTAL

Materials:

5-^{3}H-l-tryptophan, specific activity 29 Ci/mmol (±10%), was obtained from Research Products International Corp. (a product of CEA, France) and used without isotopic dilution or prior purification. It was 75% radiochemically pure as determined on thin layer chromatograms (Silica Gel 60 tlc plates, E. Merck, Darmstadt) developed with Solvent A (methylethylketone-ethylacetate-ethanol-water [3:5:1:1]). [2-ring-^{14}C]indole-3-butyric acid (^{14}C-IBA), specific activity 508 μCi/mmole, was synthesized by Dr. J.D. Cohen (12). High performance

liquid chromatography (HPLC) was performed on a Whatman PXS 10/25 ODS reverse phase column (25 cm x 0.46 cm) with a Whatman CO:PELL ODS precolumn (7 cm x 0.2 cm) (Whatman Inc., Clifton, NJ). Tetrahydrofuran was redistilled over potassium metal under an N_2 atmosphere immediately prior to use. Gas-liquid chromatography was performed on a Varian 2740 gas chromatograph equipped with both a Varian TSD nitrogen specific thermionic detector and an FID with N_2 as carrier gas. Two 6 ft x 2 mm ID glass columns packed with 3% OV17 on 100/120 Gas Chrom Q (Applied Science, State College, PA) were used, one with the nitrogen specific detector for determination of the relative quantities of compound and one in conjunction with an FID for peak collection to determine radioactivity. Scintillation counting was performed with a Beckman LS7000 scintillation counter. SP Sephadex C-25 was from Pharmacia Inc., Piscataway, NJ; UDPG, indole-3-acetic acid and myo-inositol were products of Sigma Chemical Co., St. Louis, MO, and used without purification.

Enzyme preparation:

Pseudomonas savastanoi was grown as described by Kosuge et al (1), harvested by centrifugation and stored as a cell paste in liquid nitrogen. All enzyme preparative steps were performed at 0-3°. Approximately 10 g of frozen cells were thawed and suspended in 50 ml of glass distilled water, resedimented, and this procedure repeated three times. The pellet from the third centrifugation was suspended in 50 ml of 0.01 M potassium phosphate buffer, pH 7.6, containing 5 mM mercaptoethanol. The cell suspension was sonically disrupted (Branson Model 125, Branson Instruments, Inc., Danbury, Conn.) for five 30 sec pulses with 30 sec intervals between each pulse, and the resultant homogenate was centrifuged at 20,000 x g for 15 min. To the supernatant fluid (50 ml total volume), 15.6 g of solid $(NH_4)_2SO_4$ (enzyme grade, Mann Research Lab., Inc., New York, NY) was added, the resultant precipitate sedimented by centrifugation and then dissolved in 5 ml of 0.05 M Tris-HCl, pH 7.6, containing 5 mM mercaptoethanol. This solution was desalted on a 30 ml bed volume Sephadex G-50 column eluted with the buffer used to dissolve the $(NH_4)_2SO_4$

pellet. The desalted enzyme (10 ml total volume) was divided into 1 ml aliquots and stored at -70° for further use. Under these conditions both decarboxylating and hydroxylating enzymes were stable for at least 2 weeks.

An enzyme preparation from Zea, catalyzing reactions c and d, was prepared by the method of Kopcewicz et al (13). Stage I enzymes (the 10,000 x g supernatant of the crude homogenate) were made 85% saturated with respect to $(NH_4)_2SO_4$ by the addition of the solid salt. The precipitated proteins were suspended in 0.01 M Tris-HCl, pH 7.1, buffer and desalted on a Sephadex G-50 column to yield Stage II enzymes (3). This preparation, in 5.0 ml aliquots stored at -70°, was stable for at least 10 weeks.

Product characterization:

Indole-3-acetic acid: The time course for the conversion of 5-^{3}H-l-tryptophan to labeled indole-3-acetamide and IAA was followed by thin layer chromatography of aliquots of the enzyme reaction mixture. With Solvent A on Silica Gel 60, authentic l-tryptophan, indole-3-acetamide and IAA are at R_f 0.13, 0.75 and 0.87 respectively. The putative IAA was at R_f 0.87. The R_f of the putative IAA in another solvent system, chloroform-methanol-water (85:14:1), on Silica Gel 60 was also identical to that of authentic IAA (R_f 0.28). The GC retention time of the methyl ester, prepared using diazomethane (14), was identical to that of the methyl ester of authentic IAA. The identity of IAA was further established by its conversion to IAA-myo-inositol.

The radiochemical purity of ^{3}H-IAA was determined by thin layer chromatography in two solvent systems (Solvent A and chloroform-methanol-water [85:14:1]) on Silica Gel 60.

The specific activity of the ^{3}H-IAA was determined by a modification of the method of Cohen and Schulze (12). Approximately 2 nmole ^{14}C-IBA was added to 1 nmole biosynthetic ^{3}H-IAA. Methylation of the mixture and gas chromatography using the nitrogen specific detector (12) showed the mole ratio of IBA to IAA to be 0.78. Chromatography of a second aliquot of the mixed methyl esters with collection of the radioactivity at the retention times of IAA and

and IBA showed the ratio of the radioactivities of IAA to IBA to be 70,800. As can be deduced from the equations of Cohen and Schulze (12), the specific activity of the IAA in the mixture can be calculated by the following equation:

$$\left(\frac{\text{dpm IAA}}{\text{dpm IBA}}\right)\left(\frac{\text{moles IBA}}{\text{moles IAA}}\right)(\text{specific activity IBA}) = \text{specific activity IAA}$$

The specific activity of the ^{3}H-IAA was determined to be 28 Ci/mmole.

Indole-3-acetyl-myo-inositol: The putative mixed isomeric IAA-myo-inositols (15) were eluted from a 0.9 x 17 cm HPLC column of sulfonated styrene-divinylbenzene copolymer (Beckman PA-28) (5,16) with retention times identical to those of authentic IAA-myo-inositols. They had the same R_f values on thin layer chromatograms (Silica Gel 60 developed in Solvent A) as did authentic IAA-myo-inositols (R_fs 0.28 and 0.32). Upon ammonolysis in 15% NH_4OH for 45 min at 45°, the methyl ester prepared from the sample had a GC retention time identical to that of the methyl ester of authentic IAA. Ammonolysis of IAA esters yields the free acid and the amide. The amide was not volatile in the GC system used and was not detected. In a previous paper (3), GC-MS analysis of the trimethylsilyl derivatives of the reaction products of Zea enzyme Stage II and unlabeled authentic IAA showed mass spectral fragmentation patterns identical to those of authentic hexakis trimethylsilyl-IAA-myo-inositol.

The radiochemical purity of ^{3}H-IAA-myo-inositol was determined by thin layer chromatography on Silica Gel 60 developed in Solvent A.

The specific activity of the ^{3}H-IAA-myo-inositol was determined after purification to 94% radiochemical purity by first chromatographing it over an SP-sephadex column (1 ml bed volume) eluted with 2-propanol-H_2O (1:1) and then chromatographing it on a reverse phase HPLC column eluted with tetrahydrofuran-H_2O (18:82). The ester was ammonolyzed to free IAA, and the procedure described above was followed exactly. Approximately 3 nmole ^{14}C-IBA was added to 0.5 nmole ^{3}H-IAA from ammonolyzed biosynthetic ^{3}H-IAA-myo-inositol. The mole ratio of IBA to IAA was 2.13 and the ratio of radioactivity in IAA to IBA was 24,500. The specific activity of ^{3}H-IAA-myo-inositol was calculated to be 27 Ci/mmole.

Enzymatic Conversions:

5-^{3}H-indole-3-acetic acid: The 5-^{3}H-l-tryptophan (1.5 mCi, 0.05 µmol) in 1.5 ml of water was incubated with 1.0 ml of the Pseudomonas enzyme preparation in 0.05 M TrisHCl, pH 7.6, buffer for 2 hr at 37°. The reaction proceeded to 90% completion as determined by thin layer chromatography and was terminated by the addition of an equal volume of 2-propanol. Denatured proteins were removed by centrifugation and the supernatant fluid applied to a 0.6 x 20 cm DEAE-Sephadex-acetate column. The column was developed with 300 ml of a linear gradient of 1:1 2-propanol-water to 1:1 2-propanol-0.04 M potassium phosphate (pH 3.5). Elution was at 0.04 M phosphate (140 to 154 ml). Tubes containing more than 5% of the total eluted radioactivity were combined. The IAA was extracted into ether (4 x 14 ml), the ether dried over Na_2SO_4 and evaporated to near dryness, and the IAA taken up in 5 ml of acetonitrile. The yield was 68%, the radiochemical purity was 91%, and the specific activity was 28 Ci/mmole.

5-^{3}H-indole-3-acetyl-myo-inositol: The 5-^{3}H-l-tryptophan (1.5 mCi, 0.05 µmol) in 1.5 ml water was incubated with 1.0 ml of the Pseudomonas enzyme preparation and 1.5 ml of the Zea enzyme preparation Stage II, together with 5 µmol of UDPG and 5 µmol of myo-inositol in a total volume of 4 ml. The reaction was stopped by adding an equal volume of 2-propanol; the product was freed of anionic materials and purified on the PA-28 HPLC column developed with 1:1 2-propanol-water (3). The resultant product was stored in 1:1 2-propanol-water at -20° in sealed ampules. The yield was 40% based on l-tryptophan, the radiochemical purity was 70%, and the specific activity was 27 Ci/mmole.

Incubation of the tryptophan with both enzyme preparations simultaneously provided better yields of IAA-myo-inositol than did converting tryptophan to IAA and later esterifying the IAA to myo-inositol.

DISCUSSION

Our results indicate that high specific activity ^{3}H-tryptophan can be enzymatically converted to ^{3}H-IAA and ^{3}H-IAA-myo-inositol with little or no dilution of the specific activity. The determined specific activities of the products are within 10% of that of the manufacturer's stated specific activity of 29 Ci/mmole for the ^{3}H-l-tryptophan substrate. The manufacturer's accuracy in the specific activity determination was ±10%.

The modification of the specific activity determination method of Cohen and Schulze used here is a simplification of their calculations. This simplification eliminates the requirement for knowing the amount of internal standard added to the sample (in this case ^{14}C-IBA); only the specific activity need be known. It also eliminates the requirement that the isotopically labeled internal standard be chemically related, and requires only that the standard contain nitrogen and that the relative nitrogen specific detector response to the two compounds be determined. Lastly, by reducing the number of calculations the accuracy of the method is improved.

ACKNOWLEDGEMENTS

We thank Professor T. Kosuge for cultures of Pseudomonas savastanoi, and Dr. J. Cohen for suggesting this enzymatic synthesis and providing the ^{14}C-IBA. We also thank Miss M. La Haine for her expert assistance in manuscript preparation. This work was supported by the Metabolic Biology Section of the National Science Foundation grants PCM 76-12356 and PCM 79-04637 to Professor R. S. Bandurski and is journal article 9643 from the Michigan Agricultural Experiment Station.

REFERENCES

1. Kosuge T., Heskett M.G. and Wilson E.E. - J. Biol. Chem. 241: 3738 (1966)
2. Hutzinger O. and Kosuge T. - In "Biochemistry and Physiology of Plant Growth Substances", Wightman, F. and Setterfield, G. editors, The Runge Press LTD., Ottawa, Canada, (1968)
3. Michalczuk L. and Bandurski R.S. - Biochem. Biophys. Res. Comm. 93: 588 (1980)
4. Comai, L. and Kosuge, T - J. Bacteriol. 143: 950 (1980)
5. Nowacki J., Cohen J.D. and Bandurski R.S. - J. Labelled Compd. Radiopharm. 15: 325 (1978)
6. Gardner G., Shaw S. and Wilkins M.B. - Planta 121: 237 (1974)
7. Davies P.J. - Physiol. Plant. 28: 95 (1973)
8. Pengelly W. and Meins F. - Planta 136: 173 (1977)
9. Ueda M. and Bandurski R.S. - Phytochem. 13: 243 (1974)
10. Epstein E., Cohen J.D. and Bandurski R.S. - Plant Physiol. 65: 415 (1980)
11. Nowacki J. and Bandurski R.S. - Plant Physiol. 65: 422 (1980)
12. Cohen, J.D. and Schulze, A. - Anal. Biochem. - In Press.
13. Kopcewicz J., Ehmann A. and Bandurski R.S. - Plant Physiol. 54: 846 (1974)
14. Schlenk, H. and Gellerman, J.L. - Anal. Chem. 32: 1412 (1960)
15. Ehmann A. and Bandurski R.S. - Carbohydr. Res. 36: 1 (1974)
16. Cohen J.D. and Bandurski R.S. - Plant Physiol. 59(S): 10 (1977)