Phytochemistry, Vol 24, No. 8, pp. 1653–1655, 1985
Printed in Great Britain

0031–9422/85 $3 00 + 0.00

DETERMINATION OF METHYLPUTRESCINE OXIDASE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

FRIEDHELM FETH, VICTOR WRAY and KARL G. WAGNER

Gesellschaft für Biotechnologische Forschung, D-3300 Braunschweig, West Germany

(*Revised received* 10 *December* 1984)

Key Word Index—Methylputrescine oxidase; HPLC.

Abstract—*N*-Methyl-Δ^1-pyrrolinium chloride, the product of the title enzyme, was synthesized by methylation of aminobutyraldehyde diethylacetal followed by acidic cleavage. After purification to homogeneity, it was characterized by NMR and UV spectroscopy. The compound had an absorption maximum at 210 nm; previous data indicating a maximum at 267 nm were shown to arise from an impurity. An HPLC method for the assay of *N*-methylputrescine oxidase from plant material was developed based on the separation of *N*-methyl-Δ^1-pyrrolinium chloride on a cation exchange column and direct detection at 210 nm. The enzyme activity was measured in the protein fraction extracted from plant roots and treated by gel filtration on disposable PD 10 columns. A K_m value of 1.9 mM was determined for methylputrescine and the enzyme from tobacco roots. The enzyme activities from *N. tabacum* and *Datura stramonium* were compared.

INTRODUCTION

N-Methyl-Δ^1-pyrrolinium chloride is a key metabolite in the biosynthetic pathway leading to the tropane alkaloids and nicotine [1–3]. It is obtained from *N*-methylputrescine by oxidation. *N*-Methylputrescine oxidase has been demonstrated in the roots of tobacco, *Datura stramonium* and *Atropa belladonna* plants [4, 5]; and has been purified about 150 fold from tobacco roots [6]. Furthermore its activity was found to be enhanced after the decapitation of tobacco plants [4].

For the study of the regulation of nicotine biosynthesis in tobacco roots and tissue culture we established enzyme assays of important key enzymes. In order to avoid the necessity of synthesizing and applying radioactive substrates, the enzyme assays were based on the use of HPLC [7, 8]. This offers the further advantage of a more direct detection of the enzymic product than with the former assay [6]. For *N*-methylputrescine oxidase a new method has been developed for the synthesis of its substrate *N*-methylputrescine [8]. In the present work the chemical characterization of the enzymic product, *N*-methyl-Δ^1-pyrrolinium chloride, an enzymatic assay based on the determination of this product by HPLC and data on the activity of *N*-methylputrescine oxidase in the roots of different origin are presented.

RESULTS AND DISCUSSION

Chemical properties of N-*methyl-*Δ^1*-pyrrolinium chloride*

N-Methyl-Δ^1-pyrrolinium chloride was prepared via γ-formylaminobutyraldehyde diethylacetal by reduction with $LiAlH_4$ and acidic cleavage of the acetal group [9, 10]. For detection by HPLC we relied on a report [11] which described this compound with an absorption maximum at 267 nm and a rather high molar extinction coefficient (ε2240). Figure 1A shows an HPLC analysis of the compound detected at 267 nm. However, due to difficulties during the establishment of the HPLC based enzymatic assay, the HPLC analysis was performed more thoroughly. Figure 1B shows the elution profile obtained at lower wavelength and clearly indicates two compounds, one with a strong absorption at the lower wavelength which is not detected at 267 nm and a second with a low absorption at 220 nm and a strong absorption at 267 nm. The UV-absorption spectrum of the preparation determined in 1 M HCl revealed two maxima at 263.5 nm and 210 nm, respectively. An impurity could also be detected in the NMR spectra.

Therefore we decided to further purify this compound with the help of the procedure described by Mizusaki *et al.* [10] using Dowex ion-exchanger. The authors applied this method to purify *N*-methyl-Δ^1-pyrrolinium chloride from tobacco root extracts. The purification procedure eliminated the compound absorbing at 267 nm in our HPLC profile (Fig. 1A). The UV-absorption spectrum was consistent with the results obtained by HPLC analysis and showed only a maximum at 210 nm; a molar extinction coefficient of about 900 was determined. Furthermore the purified compound could be unequivocally assigned to *N*-methyl-Δ^1-pyrrolinium chloride from the NMR data (Table 1). Hence the physical data reported [11] must have been derived from an impurity.

HPLC enzyme activity assay

After homogenization the plant material was extracted by the procedure including a purification step on prepacked disposable PD 10 gel filtration columns [7]. The enzyme assay was performed in the presence of 8.3 mM methylputrescine. *N*-Methyl-Δ^1-pyrrolinium chloride was detected without derivatization, by its UV-absorption at 210 nm and eluted on a HPLC cation exchange column at pH 3.5 (Fig. 1C).

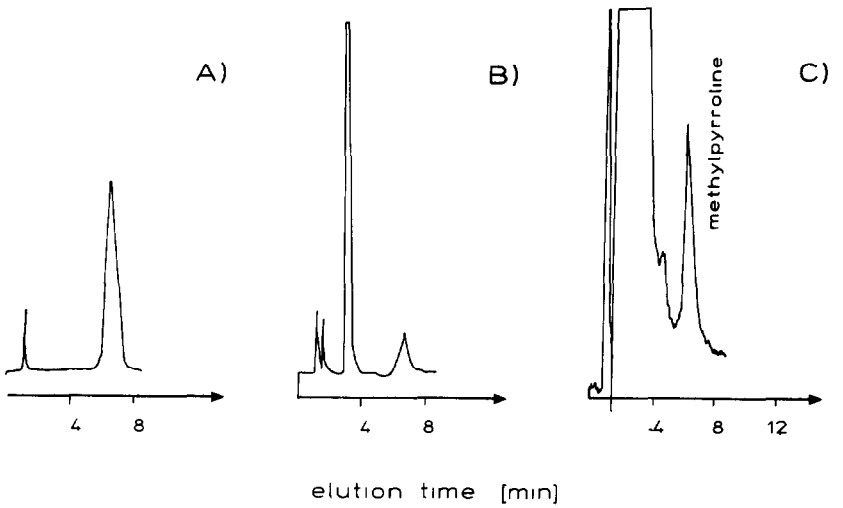

Fig. 1. HPLC analysis of *N*-methyl-Δ^1-pyrrolinium chloride. (A) and (B), Analysis of the compound prepared as described in the Experimental section, without the purification on Dowex 50 WX8: detection at 267 nm (A) and 220 nm (B). (C) Determination of the enzymatic product, detection at 210 nm. Analyses were performed on a Nucleosil 10 SA column, 50 μl sample injection; flow rate 1.5 ml/min. The isocratic mobile phase was 0.25 M $(NH_4)_2HPO_4$ of pH 3.5 with 20% acetonitrile for (A) and (B) and 0.3 M $(NH_4)_2HPO_4$ of pH 3.5 with 5% acetonitrile for (C).

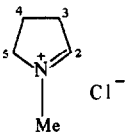

Table 1 NMR data of *N*-methyl-Δ^1-pyrrolinum chloride in D_2O*

	^1H			^{13}C		
Position	δ	J	Multiplicity†	δ	J	Multiplicity
2	8.585	—	*br s*	182.47	(^{14}N–C) 12.8	*d*
3	3.147	(3–4) 8.0	*br t*	36.79	—	*t*
4	2.292	—	*p*	20.54	—	*t*
5	4.115	(4–5) 8 0	*t*	61.39	—	*br t*
Me	3.578	—	*br s*	41.41	(^{14}N–C) 4.9	*q*

*^{1}H and ^{13}C NMR spectra were recorded, at ambient temperature, on a Bruker AM-300 NMR spectrometer locked to the deuterium resonance of the solvent at 300.1 and 75 5 MHz, respectively.

†The abbreviations used to indicate the multiplicities of the ^{1}H and SFORD ^{13}C signals are as follows; *br*, broad; *s*, singlet; *d*, doublet; *t*, triplet; *q*, quartet; *p*, pentet.

Properties of the enzyme

The enzyme is stable in the treated extract and at 4° its activity is not reduced after 24 hr. The kinetics of the enzymatic reaction under our assay conditions revealed a linear increase in the product concentration for about one hour. With the enzyme extracted from the roots of *N. tabacum* var. Samsun a K_m value of 1.9 mM was determined for methylputrescine. This value is higher by a factor of about four than that (0.45 mM) reported for the enzyme extracted from the roots of *N. tabacum* L. var. Bright Yellow [6].

Enzyme activities in the roots of different origin

Following the procedure described in the Experimental section the enzyme activities were determined in the roots of individual plants of *N. tabacum* and *D. stramonium* and also in the root material grown in a root organ culture of

D. stramonium. Usually 1.5 g of fresh roots were extracted and treated by gel filtration which resulted in an enzyme fraction containing about 2 mg of protein. The specific activities determined with the roots of *N. tabacum* var. Samsun varied from 1.7 to 6.3 nmol/min/mg, yielding a mean value of 3.3 nmol/min/mg for eight plants. The deviations were much smaller and in the expected range of ± 20% when two parallel extractions from the root of the same plant were performed, indicating that the variance originates in the physiology of the individual plants. However, the variance found with the eight individual plants for the enzyme methylputrescine oxidase was significantly smaller than that found for putrescine methyltransferase [8], which varied by a factor of ten.

Mizusaki *et al.* [4] reported a specific activity for methylputrescine oxidase from the Bright Yellow variety of *N. tabacum* of only 0.27 nmol/min/mg which is significantly smaller than that found with the variety Samsun. However, these authors reported an increase in the specific activity to 0.72 nmol/min/mg after decapitation of the plants. With the variety Samsun we could not detect such an increase in the specific activity. On the other hand with the high values found for *N. tabacum* Samsun one could speculate that this enzyme is not rate limiting for the synthesis of *N*-methyl-Δ^1-pyrrolinium chloride but rather the preceding enzyme, putrescine methyltransferase, which is regulated. In this respect it is interesting to note that unlike Mizusaki *et al.* [4] we could determine methylputrescine oxidase activity in a callus culture of *N. tabacum* var. Samsun [F. Feth, unpublished results], whereas the activity of putrescine methyltransferase could not be detected.

Mizusaki *et al.* [4] reported specific activities for methylputrescine oxidase of 0.11 and 0.17 nmol/min/mg for enzymes extracted from the roots of *D. stramonium* and *Atropa belladonna*, respectively. We found a specific activity of 0.26 nmol/min/mg in the roots of *D. stramonium*. The specific activity extracted from the root material obtained from the *D. stramonium* organ culture was on average 0.60 (four determinations) nmol/min/mg indicating a broad variance.

EXPERIMENTAL

Materials. γ-Aminobutyraldehydediethylacetal was purchased from Merck AG. *Nicotiana tabacum* var. Samsun and *Datura stramonium* plants were grown in a greenhouse with natural light conditions. Most of the young tobacco plants were kindly provided by Dr. W. Huth from the Institut für Viruskrankheiten, Biologische Bundesanstalt Braunschweig.

Chemical synthesis of N-*methyl*-Δ^1-*pyrrolinium chloride* γ-Methyl-aminobutyraldehydediethylacetal was prepared from γ-aminobutyraldehydediethylacetal via formylation with ethylformate (yield 35%) and reduction with $LiAlH_4$ (yield 68%) as described in ref. [10]. For the cleavage of the acetal group 300 μl (1.7 mmol) of the distilled product were incubated with 1.5 ml 2 M HCl at 60° for 30 min; after evaporation, 110 mg (1.3 mmol) *N*-methyl-Δ^1-pyrrolinium chloride were obtained (76% yield). This product was purified by chromatography on Dowex 50 WX8 by modifying the procedure used in ref. [10] to purify *N*-methyl-Δ^1-pyrrolinium chloride from tobacco root extract. The above product (110 mg) was applied onto a 20 ml Dowex column, washed with 90 ml 1 M HCl and eluted with 70 ml 2 M HCl. After evaporation of the pooled fractions 60 mg of purified product was obtained.

Enzyme extraction was performed at 0–4° with buffer A (100 mM K-Pi of pH 7.5, 5 mM EDTA, 10 mM mercaptoethanol, 0.5% Na ascorbate, 2% polyethyleneglycol 400) as described previously [7, 8]. After homogenization and centrifugation, the supernatant was applied onto a Sephadex G 25 disposable PD 10 column and eluted by buffer B (50 mM K-Pi of pH 8, 1 mM EDTA and 5 mM mercaptoethanol). Protein concentration was determined according to ref. [12].

Enzyme assay was performed in buffer B at 30° and started by the addition of 20 μl 50 mM methylputrescine to 100 μl enzyme soln. For the determination of the K_m values the incubation time was 30 min, otherwise 45 min. The reaction was stopped by the addition of 10 μl 1 M HCl and the samples were frozen for determination of the product by HPLC.

HPLC chromatography. N-Methyl-Δ^1-pyrrolinium chloride was separated on a 10 μm cation exchange column Nucleosil 10 SA (SO_3Na) 4 × 250 mm from Macherey & Nagel. The HPLC system consisted of a LDC Constametric II pump, Rheodyne probe injector and Kontron Uvikon 730 LC. The isocratic mobile phase was 0.3 M $(NH_4)_2HPO_4$ of pH 3.5, 5% acetonitrile. The column was run at 1.5 ml/min and the detection was at 210 nm.

Acknowledgements—We are grateful to Drs. H.-A. Arfmann and G. Höfle for helpful discussions.

REFERENCES

1. Bush, L. P. and Saunders, J. L. (1977) *Tob. Chem. Symp. Am. Chem. Soc. New Orleans*, pp. 389.
2. Leete, E. (1979) *Planta Med.* **36**, 97.
3. Waller, G. R. and Dermer, O. C. (1981) *Biochem. Plants* **7**, 317.
4. Mizusaki, S., Tanabe, Y., Noguchi, M. and Tamaki, E. (1973) *Plant Cell Physiol.* **14**, 103.
5. Yoshida, D. (1973) *Bull. Hatamo Tobacco Exp. Stn.* **73**, 239.
6. Mizusaki, S., Tanabe, Y., Noguchi, M. and Tamaki, E. (1972) *Phytochemistry* **11**, 2757.
7. Wagner, R. and Wagner, K. G. (1984) *Phytochemistry* **23**, 1881.
8. Feth, F., Arfmann, H.-A., Wray, V. and Wagner, K. G. (1985) *Phytochemistry* **24**, 921.
9. Hasse, K. and Schührer, K. (1962) *Biochem. Z.* **336**, 20
10. Mizusaki, S., Kisaki, T. and Tamaki, E. (1968) *Plant Physiol.* **43**, 93.
11. Leete, E. (1967) *J. Am. Chem. Soc.* **89**, 7081.
12. Bradford, M. M. (1976) *Analyt. Biochem.* **72**, 248.