Phytochemistry, 1980, Vol. 19, pp. 2371–2374. © Pergamon Press Ltd. Printed in England. 0031-9422/80/1101-2371 $02.00/0

DETERMINATION OF GLYCINE BETAINE BY PYROLYSIS–GAS CHROMATOGRAPHY IN CEREALS AND GRASSES*

WILLIAM D. HITZ and ANDREW D. HANSON

MSU–DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, U.S.A.

(*Revised received* 29 *February* 1980)

Key Word Index—Gramineae; pyrolysis–GC; water-stress; betaines; quaternary ammonium compounds; glycine betaine.

Abstract—A procedure for determining glycine betaine levels in plants involving pyrolysis–gas chromatography is described. It has been successfully applied to the analysis of 22 species of grasses.

INTRODUCTION

Betaines occur widely in plants [1–5]. Glycine betaine is of particular interest because it has recently been shown to accumulate in response to salt and water stress [6–8]. This accumulation may be of adaptive significance [9].

Methods used for glycine betaine estimation in plants include (a) spectrophotometric assays as the reineckate [4] or periodate salt [10], (b) TLC or electrophoresis combined with scanning densitometry [7], (c) isotope dilution/micro-Kjeldahl determination following electrophoresis [6]. Since none of these combine speed with sensitivity and specificity, we developed a pyrolysis–GC assay which, coupled with an ion-exchange purification, allows determination of nmol quantities of *N,N,N*-trimethylbetaines. This procedure has been used to survey the betaine content of 22 species of cereals and grasses, before and after water stress.

RESULTS AND DISCUSSION

Ion exchange purification

Betaines are not retained on either strong anion or weak cation exchange resins [11]. The presence of a permanent positive charge on the quaternary ammonium group, in combination with a carboxyl group of relatively low pK_a, gives betaines unique charge properties which allow their isolation, as a class, by a three-resin ion-exchange system. First, an aqueous extract at neutral pH is passed through columns of a strong anion exchange resin (quaternary ammonium type, OH^- form) and a weak cation exchange resin (carboxylic acid type, H^+ form). This step removes (a) on the quaternary ammonium resin, all anions other than OH^- and all zwitterions other than betaines, (b) on the carboxylic acid resin, all cations (Table 1). The two resins can be used in series or in the form of a mixed bed. Secondly, the effluent from these two resins—which contains uncharged compounds as well as betaines—is passed through a strong cation exchange resin (sulfonic acid type, H^+ form). This resin suppresses the ionization of their carboxyl groups and therefore binds the betaines; after washing to remove neutral compounds, the betaines can be eluted with ammonia.

Pyrolysis

Pyrolytic dealkylation and deamination of the ammonium group are well-known reactions of quaternary ammonium compounds (QACs) [12]. In the

Table 1. Retention of various nitrogenous compounds by a mixed-bed column of Bio-Rex 70 and AG-1 ion exchange resins

Compound	Amount applied to column (μmol)	Analytical method	% Retained on column
Ethanolamine	1.0	scintillation counting	99
Betaine aldehyde	1.0	scintillation counting	99
Choline	1.0	scintillation counting	100
Phosphorylcholine	1.0	scintillation counting	100
Hordenine	1.0	UV absorbance	95
Serine	1.0	scintillation counting	100
Amino acid mixture*	2.7	ninhydrin assay	100
Glycine betaine	1.0	scintillation counting	0
β-Alanine betaine†	1.0	pyrolysis–GC	0
Nicotinic acid betaine (trigonelline)	1.0	UV absorbance	0
Proline betaine‡ (stachydrine)	1.0	scintillation counting	2

*Ninhydrin-positive compounds in an aqueous extract from barley shoots.

†Obtained from F. Larher.

‡Purified from alfalfa leaves exposed overnight in darkness to tracer [^{14}C]-formate.

*Michigan Agric. Expt. Stn. Journal Article No. 9203.

case of the halide form of choline esters, pyrolytic demethylation is the main reaction and the volatile dimethylamino derivatives which are produced may be separated by GC and determined in pmol quantities [13]. In contrast, low temperature pyrolysis of the OH^- form of glycine betaine favors deamination, giving trimethylamine (TMA) as the major product, which may be determined by GC [14].

We have found the TMA yield to be reproducible and directly proportional to the amount of glycine betaine pyrolysed over the range 20–150 nmol. Fig. 2 shows typical results, with an absolute yield of TMA over this range of 42–51% of theoretical. It is probable that side reactions (such as dealkylation) limit the absolute yield and that the products of these side reactions are not sufficiently volatile to be detected by the GC conditions employed. Although the absolute yield of TMA declined when less than 20 nmol of glycine betaine was pyrolysed, it remained reproducible; the assay can thus be extended to a lower limit of 2 nmol by the use of a separate standard curve (Fig. 2, inset). Pyrolysis probes were found to vary in the absolute TMA yields they gave (within the range 38–47% for 50 nmol samples of glycine betaine), so a standard curve must be prepared for each probe. Curves remain operative throughout the life of the probes (an average of 300 analyses). Halide salts of glycine betaine do not give TMA yields equivalent to the free base upon pyrolysis. Since betaines are recovered from the ion exchange procedure in the OH^- form, this form must be used for the preparation of standard curves.

A comparatively low pyrolysis temperature (350°) was selected because higher temperatures did not increase the TMA yield, and the low temperature increased the specificity of the assay for the trimethylammonium function. For example, mono- and dimethyl betaines, such as trigonelline and stachydrine, released only traces of volatile fragments and yielded no TMA upon pyrolysis at 350°. Further, admixtures of contaminants (e.g. a 50-fold molar excess of glutamic acid) did not affect the TMA yield of glycine betaine pyrolysed at 350°.

Choline–Cl, phosphorylcholine–HCl and β-alanine betaine–HCl yielded TMA upon pyrolysis (42, 17, and 74% of theoretical TMA yield, respectively), demonstrating that trimethyl QACs in general are detected by the pyrolysis–GC assay. This is not a difficulty, provided that the ion exchange procedure described above is used to exclude compounds other than betaines from the pyrolysed sample. While the identity of the betaines present in a plant extract must be verified independently (e.g. by TLC), the general form of the pyrogram is characteristic of the compound pyrolysed. Representative programs for glycine betaine and β-alanine betaine are given in Fig. 1.

Recovery of glycine betaine from barley leaf extracts

When methanol–chloroform–water extracts of either turgid or wilted barley leaves were given an addendum of 22 nmol of exogenous [methyl-^{14}C]-glycine betaine (1.4 nCi/nmol), the recovery of ^{14}C in the ion exchange fraction prepared for pyrolysis averaged 78.4% (s.e. = 5.9%). When 0.3–1.2 μmol of authentic glycine betaine was added to extract samples, each containing 0.7 μmol of endogenous glycine betaine and an addendum of [^{14}C]-betaine, the recoveries of the added glycine betaine by pyrolysis–GC were the same as the radiochemical recoveries from the column procedure. This demonstrates

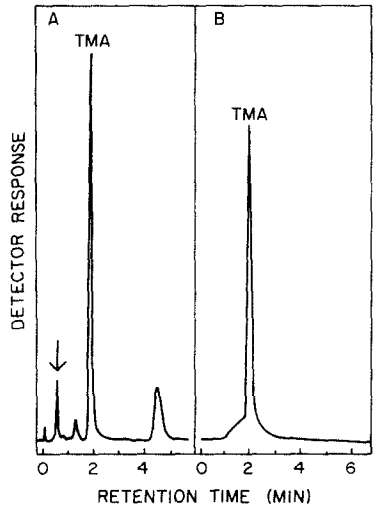

Fig. 1. Pyrograms of authentic betaines. (A) Glycine betaine, free base, 100 nmol. (B) β-Alanine betaine HCl, 50 nmol. Column was 4% Carbowax 20M with 0.8% KOH on Carbopack B. Pyrograms of glycine betaine isolated from barley leaves were identical to (A) except that the peak indicated by the arrow increased about 3-fold.

that there were no contaminants in the purified extracts which interfered with the pyrolysis reaction.

Betaine in the betaine-containing ion-exchange fractions of a series of turgid and wilted barley leaves was determined by both the pyrolysis–GC method (Y) and by a standard periodate method [10] (X). Agreement between methods was very good ($Y = 1.006\,X + 0.093$, $r = 0.96$), but the pyrolysis–GC method was up to 40 times more sensitive.

Betaine accumulation during water stress

Betaine was measured before and after a period of water stress in shoots of 22 species from the family Gramineae (Fig. 3). The main betaine present was taken to be glycine betaine since pyrograms from all species closely resembled those of authentic glycine betaine (Fig. 1A).

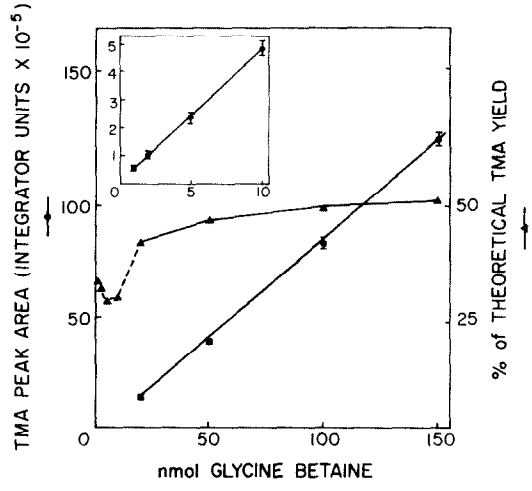

Fig. 2. Representative standard curves for determination of glycine betaine free base in the ranges 20–150 nmol and 1–10 nmol (inset). TMA yield as per cent of theoretical is also shown.

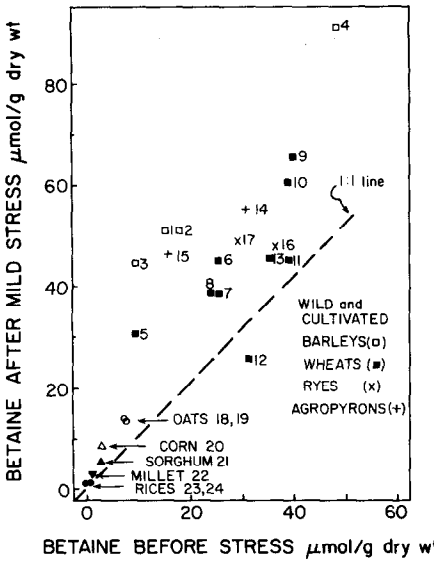

Fig. 3. Betaine contents of the shoots of some cereals and grasses before and after mild stress. Heights of points above the 1:1 line represent stress-induced betaine accumulation. Numbers next to points designate genotypes as follows: 1. *Hordeum bogdani* Wilensky; 2. *H. spontaneum* Koch; 3. *H. vulgare* L. cv Proctor; 4. *H. jubatum* L.; 5. *Triticum aestivum* L. cv Mexipak; 6. *T. aestivum* L. cv Genesee; 7. *T. dicoccoides* Koern; 8. *T. dicoccum* Schrank; 9. *T. tauschii* (Coss.) Schmalh; 10 *T. araraticum* Takubz., 11. *T. umbellulatum* (Zhuk.) Bowden; 12. *T. monococcum* L.; 13. *T. boeoticum* Boiss.; 14. *Agropyron desertorum* Fisch.; 15. *A. intermedium* (Host) Beauv.; 16. *Secale vavilovi* Grossheim; 17. *S. cereale* L. cv Rosen; 18. *Avena sativa* L. cv Froker; 19. *A. sterilis* L.; 20. *Zea mays* L. cv Great Lakes 333; 21. *Sorghum bicolor* Moench cv Northrup King 6636; 22. *Pennisetum americanum* (L.) Leeks cv Tift 23B; 23. *Oryza sativa* L. cv IR-120 (lowland); 24. *O. sativa* L. cv IRAT-13 (upland).

Further, TLC separations have shown glycine betaine to be the principal betaine in several grasses [9].

The following generalizations can be made from the results summarized in Fig. 3. (a) Glycine betaine was probably present before stress in all species, but the content showed much interspecific variation. (b) Most genotypes (all those falling above the 1:1 line in Fig. 3) accumulated betaine during stress. Also, the glycine betaine content before stress was a fair indication of the extent of accumulation after stress. (c) As in other families [7], betaine contents before and after stress were clearly related to taxonomic affinity. Within the Gramineae, all species which accumulated more than 20 μmol of glycine betaine per g dry wt were from the tribe Hordeae [15].

It has been suggested that glycine betaine functions as a compatible, cytoplasmic osmoticum during salt-stress or water-stress [8]. While this may be true of some species of Gramineae it seems evident that the accumulation of glycine betaine is not of universal importance in this family. For example, it occurs only to a very limited extent in millet, sorghum, and upland rice—all crops adapted to growth in drought-prone environments.

EXPERIMENTAL

Plant material. Barley (*Hordeum vulgare* L. cv Proctor) for use in glycine betaine recovery checks was grown and subjected to H_2O stress as described previously [16]. Seeds for the species survey of Fig. 3 were planted (6–10 seeds per pot) in 10 cm clay pots containing a moist sandy loam, and were stratified for 1 week at 4°. Plants were then grown in the greenhouse during March 1979. At the 3-leaf stage, plants were thinned to 5 per pot and a H_2O stress treatment was given by withholding irrigation for 4–6 days. For each species, stress was continued for *ca* 1 day after visible wilting occurred.

Extraction procedure. For glycine betaine recovery checks, second leaves of barley plants were extracted with MeOH–$CHCl_3$–H_2O as described in ref. [16]. For the species survey, whole shoots were freeze-dried, weighed and then extracted with 5 ml H_2O at 100° for 30 min, followed by overnight steeping at 4°.

Ion exchange separation. Resins were from Bio-Rad. The resins Bio-Rex 70 (200–400 mesh, H^+ form) and AG-1 (200–400 mesh, OH^- form) were routinely mixed in a 1:2 (v/v) ratio; this gives *ca* equal anion and cation exchange capacities per unit vol. of mixture. Aq. extract (1–5 ml) was applied to a column (1–1.5 ml packed vol.) of the mixed resins. The effluent from this column was passed directly onto a column of AG-50W (200–400 mesh, H^+ form, 1–1.5 ml). The extract was washed through the column of mixed resins with 4 ml H_2O. The AG-50W column was then washed with 4 ml H_2O, and bound betaines eluted with 4 ml 4N NH_4OH. The column eluate was dried under N_2 at *ca* 70° and redissolved in H_2O (usually 100 μl) before pyrolysis.

Pyrolysis–GC. Pyrolysis was on a ribbon probe of a Chemical Data Systems 100 series Pyrolyzer (Chemical Data Systems, Oxford, PA 19363) installed on a Varian 3700 GC equipped with a FID. Volatile pyrolysis products were separated on a 100 × 0.5 cm glass column packed either with 4% Carbowax 20 M containing 0.8% KOH on Carbopak B or with 20% SP-2100 on 80/100 mesh Gaschrom Q. Carrier gas (N_2) flow rate was 30 ml/min; column temp. was 75° for the Carbowax column and 85° for the SP-2100 column. Pyrolysis conditions were as follows: interface chamber temp. 200°; final pyrolysis ribbon temp. 350° for 10 sec; maximum heating rate (*ca.* 75° per msec). Samples (10 μl) were normally loaded on the ribbon probe in 2 × 5 μl applications with a 20 sec drying pulse at 100° after each application. Samples of β-alanine betaine were dried at room temp. The pyrolysis probe was placed in the interface chamber and fired as soon as the carrier gas pres. returned to normal. R_ts were measured from the time of probe firing. Any residues remaining on the probe after pyrolysis were removed by firing at 950° for 5 sec in air before the next sample application. The major peak obtained upon pyrolysis of glycine betaine (R_t *ca.* 1.9 min, Fig. 1A) was identified as TMA by comparison with authentic TMA on the two columns described above.

For calculation of theoretical TMA yield from pyrolysis, the absolute amount of TMA was determined by reference to standardized aq. solns of TMA, of which 1 μl samples were injected into the pyrolysis unit interface chamber. TMA in the standard solns was determined spectrophotometrically as the picrate salt [17].

Acknowledgements—Research supported by U.S. Department of Energy under Contract No. EY-76-C-02-1338. We thank Dr. F. Larher for the generous gift of β-alanine betaine, Dr. N. E. Good for valuable advice on ion-exchange purifications of betaines and C. E. Nelsen for technical assistance.

REFERENCES

1. Sethi, J. K. and Carew, D. P. (1974) *Phytochemistry* **13**, 321.
2. Brecht, C. A. L., Lousberg, R. J. J. C., Kuppers, F. J. E. M. and Salemink, C. A. (1973) *Phytochemistry* **12**, 2457.

3. Okogun, J. I., Spiff, A. J. and Ekong, D. E. (1976) *Phytochemistry* **15,** 836.
4. Cromwell, B. T. and Rennie, S. D. (1953) *Biochem J.* **55,** 189.
5. Larher, F. and Hamelin, J. (1975) *Phytochemistry* **14,** 205.
6. Hanson, A. D. and Nelsen, C. E. (1978) *Plant Physiol.* **62,** 305.
7. Storey, R. and Wyn Jones, R. G. (1977) *Phytochemistry* **16,** 447.
8. Wyn Jones, R. G. and Storey, R. (1978) *Aust. J. Plant Physiol.* **5,** 817.
9. Wyn Jones, R. G., Storey, R., Leigh, R. A., Ahmad, N. and Pollard, R. A. (1977) in *Regulation of Cell Membrane Activities in Plants,* (Marrè, E. and Ciferri, O., eds.) pp. 121–136. Elsevier, Amsterdam.
10. Wall, J. S., Christianson, D. D., Dimler, R. J. and Senti, F. R. (1960) *Analyt. Chem.* **32,** 872.
11. Carruthers, A., Oldfield, J. F. T. and Teague, H. J. (1960) *Analyst* **85,** 272.
12. Brewster, J. H. and Eliel, E. L., (1953) *Org. React.* **7,** 142.
13. Szilagyi, P. I. A., Schmidt, D. E. and Green, J. P. (1968) *Analyt. Chem.* **40,** 2009.
14. Prey, V., Braunsteiner, W. and Andres, H., (1974) *Z. Zuckerind.* **24,** 242.
15. Fernad, M. L. (1970) *Gray's Manual of Botany,* 8th edn., pp. 132–145. Van Nostrand Reinhold, New York.
16. Hanson, A. D., Nelsen, C. E. and Everson, E. H. (1977) *Crop Sci.* **17,** 720.
17. Murray, C. K. and Gibson, D. M. (1972) *J. Food Technol.* **7,** 720.