Determination of Methanol Using Alcohol Oxidase and Its Application to Methyl Ester Content of Pectins

Jerome A. Klavons* and Raymond D. Bennett

A method for the determination of methanol using alcohol oxidase is presented. The procedure offers the advantage over other photometric procedures that use potassium permanganate as the oxidant in that a subsequent reduction step is eliminated. The sensitivity of the assay is 1–20 μg/mL of methanol. The procedure is rapid, selective, and versatile. The methyl ester content on a sample of pectin was determined from the methanol liberated upon its hydrolysis.

A method often used to determine methanol in relation to pectin methyl ester content (Fishman et al., 1984) is via the oxidation of methanol to formaldehyde with potassium permanganate, followed by condensation with 2,4-pentanedione to yield the colored product 3,5-diacetyl-1,4-dihydro-2,6-dimethylpyridine (Wood and Siddiqui, 1971). The permanganate oxidation method requires the reduction of unreacted permanganate with sodium arsenite. In this laboratory, inconsistent results have been obtained by using this method, presumably due to the arsenite reduction step, which has been reported to be a complex process (Belcher and Nutten, 1960). Wood and Siddiqui reported that trace elements of permanganate interfere considerably in the assay (Wood and Siddiqui, 1971). The second step, involving condensation of formaldehyde with 2,4-pentanedione, provided accurate and reproducible results. Thus, it seemed that the method could be improved by developing a better procedure for oxidizing methanol to formaldehyde.

We present a modification of the permanganate oxidation procedure (Wood and Siddiqui, 1971) for the determination of methanol. The use of alcohol oxidase eliminates the reduction step with sodium arsenite and offers a twofold increase in sensitivity (1–20 μg/mL). An assay using this new method can be carried out in less than 1 h, compared to 2 h for the permanganate oxidation method. The specificity of alcohol oxidase for lower primary alcohols and the negligible or nonexistent production of chromophore from the condensation of 2,4-pentanedione with subsequent lower primary aldehydes other than formaldehyde provide a direct and specific procedure for the determination of methanol.

Alcohol oxidase from *Pichia pastoris* (alcohol: oxygen oxidoreductase, EC 1.1.3.13) was found to be a rapid and efficient catalyst for the oxidation of methanol to formaldehyde. It was found to be equally efficient in phosphate buffers ranging from 0.01 to 0.25 M. This provided great flexibility in the design of assay systems for the detection of methanol.

The ability to increase the ionic strength of the enzyme incubation enabled us to use this method to determine the methanol that was liberated upon hydrolysis of pectin and thus to measure the degree of methylation of the pectin. Pectic ester hydrolysis was accomplished in 0.5 M KOH and the hydrolysate titrated with dilute phosphoric acid to pH 7.5, the optimum pH of alcohol oxidase.

EXPERIMENTAL SECTION

Apparatus. A Cary Model 14 recording spectrophotometer was used to measure the absorbance of all samples.

Reagents. Alcohol oxidase from *P. pastoris* (alcohol: oxygen oxidoreductase, EC 1.1.3.13) was obtained from Sigma Chemical Co., St. Louis, MO. 2,4-Pentanedione, 99+%, Gold Label was obtained from Aldrich Chemical Co., Milwaukee, WI, and was distilled prior to use. Citrus pectin was obtained from Nutritional Biochemicals Corp., Cleveland, OH, with a molecular weight range of 150 000–300 000. All other reagents were analytical grade and of the highest purity obtainable.

Solutions: (A) diluted alcohol oxidase, approximately 1 unit of alcohol oxidase from *P. pastoris* (0.858 μL of current lot number) diluted to 1 mL with distilled water; (B) 0.02 M 2,4-pentanedione (freshly distilled) in 2.0 M ammonium acetate and 0.05 M acetic acid.

Procedure. Aliquots (1000 μL) of methanol standards or unknowns (1–20 μg) in potassium phosphte buffer in the range 0.01–0.25 M, pH 7.5, were placed in 16 × 126 mm Pyrex screw-cap culture tubes. Solution A, diluted alcohol oxidase (1000 μL) was added to each tube. The tubes were gently mixed and incubated at 25 °C for 15 min. Solution B (2000 μL) was added to each tube and mixed well by vortexing. The tubes were capped, placed in a water bath at 58–60 °C for 15 min, and cooled to room temperature. The absorbances were measured at 412 nm against a blank containing phosphate buffer of the appropriate concentration, pH 7.5, and dilute alcohol oxidase (A).

Determination of the Degree of Esterification of Pectin. A stock solution of pectin was prepared at a concentration of 1000 μg/mL of distilled water. Pectin methyl esters were hydrolyzed as follows: 25 mL of 1.0 N potassium hydroxide was added to aliquots of the stock pectin solution to give 50 mL of pectin solutions ranging in concentration from 50 to 400 μg of pectin/mL of 0.5 N potassium hydroxide. The solutions were incubated at room temperature for at least 30 min. The pectin hydrolysates were neutralized with dilute phosphoric acid to pH 7.5, using a pH meter, and then diluted to twice their original volumes with distilled water to give solutions ranging from 25 to 200 μg of pectin (hydrolyzed). Aliquots (1 mL) of the hydrolyzed pectin samples were then analyzed for methanol, as above. The pectin hydrolysates were analyzed for uronic acid via the carbazole procedure of McComb and McCready (1952).

RESULTS AND DISCUSSION

Aliquots of methanol ranging in concentration from 1 to 20 μg/mL in phosphate buffers, pH 7.5, ranging from 0.01 to 0.25 M were assayed. Methanol aliquots prepared in 0.5 M potassium hydroxide, neutralized to pH 7.5 with phosphoric acid, and then diluted to twice the original volume (of 0.5 M KOH) were also assayed. The results are shown in Table I. The calculated F value from an

Fruit and Vegetable Chemistry Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Pasadena, California 91106.

Table I. Effect of Phosphate Buffer Concentration on Methanol Determination (Final Absorbances at 412 nm)

phosphate buffer concn, M	methanol, μg/mL				
	1	5	10	15	20
0.01		0.253 ± 0.002 (n = 4)	0.503 ± 0.005 (n = 4)	0.737 ± 0.005 (n = 4)	0.945 ± 0.011 (n = 4)
0.025	0.049 ± 0.002 (n = 5)	0.249 ± 0.002 (n = 4)	0.493 ± 0.002 (n = 4)	0.728 ± 0.003 (n = 4)	0.927 ± 0.014 (n = 4)
0.05		0.234 ± 0.005 (n = 4)	0.478 ± 0.003 (n = 4)	0.726 ± 0.010 (n = 4)	0.950 ± 0.005 (n = 4)
0.25		0.256 ± 0.005 (n = 4)	0.517 ± 0.005 (n = 4)	0.714 ± 0.005 (n = 4)	0.947 ± 0.014 (n = 4)
0.5 M KOH[a]		0.255 ± 0.012 (n = 5)	0.516 ± 0.021 (n = 5)	0.772 ± 0.022 (n = 4)	0.984 ± 0.011 (n = 4)

[a] Neutralized with phosphoric acid to pH 7.5, volume doubled (as for degree of methylation of pectin) (see text).

Table II. Concentration of Methanol and Degree of Esterification of Pectin Solutions

pectin, μg/mL	methanol, μg/mL	% methyln of uronic acids
25	1.65 ± 0.01 (n = 5)	43.2
50	3.51 ± 0.08 (n = 5)	46.1
100	7.30 ± 0.04 (n = 5)	47.9
150	11.16 ± 0.11 (n = 5)	48.8
200	14.67 ± 0.05 (n = 5)	48.2
		mean: 46.8 ± 2.8

analysis of variance was less than 1 and showed that buffer concentration was not significant, over the range of phosphate buffers used, in the determination of methanol. A least-squares analysis was performed on all 87 data points. The equation of the straight line calibration curve was ABS_{412} = 0.04713[methanol] + 0.01914; r^2 = 0.9954.

Determination of the Degree of Esterification of Pectin. The pectin used was found to have a uronic acid content of 95.4%. Aliquots of pectin ranging in concentration from 25 to 200 μg/mL (final concentration) were analyzed for methanol. The percent esterification is expressed as the percent of the uronic acid present as methyl ester. The results are shown in Table II.

Phosphate Buffer, pH, and Concentration. pH 7.5 was used, as this the optimum pH of alcohol oxidase as indicated by the supplier. The effect of buffer concentration in the range 0.01–0.25 M was studied and was found not to be significant via an analysis of variance. This provides great versatility in the design of procedures to determine methanol from other sources.

Effect of Enzyme Concentration. Portions of 2, 1, and $^1/_2$ unit of alcohol oxidase per tube each gave similar results. At $^1/_4$ unit the final absorbance showed a 30% decrease based on a 15-min incubation. At $^1/_8$ unit the final absorbance showed a 58% decrease. A concentration of approximately 1 unit of alcohol oxidase per tube was chosen as an appropriate amount of enzyme to be used. This amounted to less than 1 μL of original enzyme (as supplied by Sigma Chemical Co.), of the current lot number.

Effect of Incubation Time. Incubation times of 15 and 30 min gave similar results. Incubation for 60 min led to a decrease in the final absorbance of 13% based on the use of 1 unit of alcohol oxidase per tube. Incubation for 120 min led to a decrease of 27% in the final absorbance. The alcohol oxidase, as supplied, was a crude enzyme preparation that likely contained other enzymes capable of further oxidizing formaldehyde (to formic acid). It was suspected that prolonged incubation leads to further oxidation. The reagent 2,4-pentanedione did not give a color with formic acid, and thus a decrease in final absorbance was observed. Therefore, the timing of the enzyme reaction is not critical provided that it does not approach 1 h.

Effect of Possible Interfering Substances. The effect of possible interfering substances, particularly that of other lower primary alcohols, was examined. The results are shown in Table III. The relatively high specificities of both the enzyme and the 2,4-pentanedione reaction

Table III. Effect of Possible Interfering Substances

substance	amt, μg	final abs at 412 nm
ethanol	20	+0.006
ethanol	100	−0.006
ethanol	1000	−0.018
ethanol	2000	+0.009
1-propanol	100	−0.007
1-propanol	1000	−0.011
1-butanol	100	−0.005
1-butanol	1000	−0.010
2-propanol	100	−0.005
dimethyl sulfoxide	100	−0.005
galacturonic acid	100	0.000
Na_2EDTA	100	−0.008
glucose	100	−0.001

make it unlikely that interferences will be encountered in most types of samples. Any interference resulting from the dione reaction could be corrected for by using a blank without enzyme.

Comparison with Other Methodology. Our procedure offers a rapid, sensitive, and selective method for the determination of methanol. The average relative standard deviation among replicate samples (the 21 groups in Table I) is 1.15%. Table III indicates a negligible if not nonexistent interference due to other simple primary alcohols and some other substances of which one might be suspicious.

Methanol is routinely determined by methods such as high-performance liquid chromatography (HPLC) (Gandelman and Birks, 1982), gas chromatography (GC) (Knuth and Hoglund, 1984), and spectrophotometry (Vijayalakshmi et al., 1976; Dion and Miron, 1982; Chernaya and Kleshchevnikova, 1983). Another method utilizing alcohol oxidase, immobilized onto an oxygen electrode (Guibault et al., 1983), is also available.

The HPLC procedure requires a postcolumn reactor that must either be constructed or purchased. The GC procedure has a very low sensitivity for methanol and is better suited for higher alcohols. HPLC and GC methods each require specific columns and detectors. The spectrophotometric methods each require oxidation with permanganate, a step for which we present a simpler alternative. The immobilized alcohol oxidase method is not specific for methanol.

ACKNOWLEDGMENT

This work was supported in part by the Citrus Products Technical Committee, Los Angeles, CA.

Registry No. MeOH, 67-56-1; alcohol oxidase, 9073-63-6; pectin, 9000-69-5; low-methoxyl pectin, 9049-34-7.

LITERATURE CITED

Belcher, R.; Nutten, A. G. *Quantitive Inorganic Analysis*, 2nd ed.; Butterworths: London, 1960; pp 221, 258.

Chernaya, A. V.; Kleshchevnikova, V. N. *Zavod Lab.* **1983**, *49*, 25–27.

Dion, R.; Miron, M. *Ann. Biochim. Clin. Que.* **1982**, *21*, 8–10.

Fishman, M. L.; Pfeffer, P. E.; Barford, R. A.; Doner, L. W. *J. Agric. Food. Chem.* **1984**, *32*, 372–378.

Gandelman, M. S.; Birks, J. W. *J. Chromatogr.* **1982**, *242*, 21–31.

Guilbault, G. G.; Danielsson, B.; Mandenius, C. F.; Mosbach K. *Anal. Chem.* **1983**, *55*, 1582–1585.
Knuth, M. L.; Hoglund, M. D. *J. Chromatogr.* **1984**, *285*, 153–160.
McComb, E. A.; McCready, R. M. *Anal. Chem.* **1952**, *24*, 1630–1632.
Vijayalakshmi, M. A.; Sarris, J.; Varoquaux, P. *Lebensm.-Wiss. Technol.* **1976**, *9*, 21–23.
Wood, P. J.; Siddiqui, I. R. *Anal. Biochem.* **1971**, *39*, 418–428.

Received for review December 2, 1985. Accepted March 7, 1986. Throughout this paper reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

Purine Degradative Pathway of the Yam and Sweet Potato

Godson O. Osuji[1] and Robert L. Ory*

The degradation of purines by nitrogen-deficient plants was investigated in the carbohydrate staples yam tubers (*Dioscorea spp.*) and sweet potatoes (*Ipomoea batatas*). The activities of adenosine deaminase, xanthine oxidase, uricase, allantoinase, allantoicase, and allantoate amidohydrolase were detected in extracts of the tubers, showing that purine degradation operates in these nitrogen-deficient plants as in the case of nitrogen-rich plants. The presence of allantoate amidohydrolase in the tubers suggested that much ammonia was lost from the pathway. Stored tubers also had increased activities of some enzymes of the pathway and experienced high losses of nitrogen. These results indicate that the nitrogen deficiency of root tubers was in part attributed to the ammonia lost via the purine degradative pathway. The implications of these reactions are discussed as they affect storage of the tubers for food uses.

INTRODUCTION

The yam (*Dioscorea spp.*) and sweet potato (*Ipomoea spp.*) are staple food crops for millions of people (Coursey, 1967; Walter et al., 1984), especially in West Africa. They are a major source of carbohydrate and a minor source of nitrogen nutrition. Recent studies to understand the causes for the low nitrogen contents of yams showed that the proteins of the tubers were degraded to low molecular weight proteins during tuber storage (Osuji, 1981; Osuji and Umezurike, 1983) and the tuber accumulates large quantities of ureide nitrogen as allantoin (Ueda and Sasaki, 1956). The deposition of ureides in root tubers (known to be N deficient) is unusual because thus far the ureides are known to be important intermediates in the metabolism of nitrogen-rich plant species (Fosse, 1926; Mothes and Engelbrecht, 1952, 1954; Krupk and Towers, 1959; Reinbothe and Mothes, 1960). Thus, the deposition of ureides in root tubers could be a clue for a general understanding of the causes of low nitrogen contents of tubers.

It was considered necessary, therefore, to understand the purine degradative pathway in root tubers and to determine whether the nitrogen deficiency and allantoin accumulation are attributable to activities of the enzymes of the pathway, especially in stored yam tubers. Yam tubers are traditionally stored for several months to be used as food in many parts of the tropics. Further support for such a study of purine degradation in root tubers was based upon the fact that ureides are known to be metabolized mainly by plant roots (Brunel and Capelle, 1947; Kushizaki et al., 1964; Ishizuka et al., 1970) and nitrogen fixation and ureide synthesis are coupled via purine degradation (Fujihara and Yamaguchi, 1978a, 1978b, 1980).

The purpose of this investigation was to understand the purine degradative pathway of N-deficient plants. The purine pathway intermediates contain 50% N by weight, and their degradation would be critically important in nitrogen economy of the plant. The results of such a study should indicate the mechanism for regulation of the pathway and whether nitrogen deficiency in root tubers is attributed to activities of enzymes of the pathway, especially in stored yam tubers.

MATERIALS AND METHODS

Sweet potato [*Ipomoea batatas* (L.) Lam.] (Jewel cv.) was purchased from a local grocery. Yam tubers (*Dioscorea spp.*) were provided by the National Root Crops Research Institute, Umudike, Nigeria; some were purchased from open markets in Benin City, Nigeria. The tubers were used about 1 month after harvest. For the effects of tuber storage on purine degradation, healthy yam tubers were placed in paper bags and stored in cupboards at room temperature. Tubers that sprouted during storage were not used for the enzyme assays.

Homogenization of Tubers. The cork layer of each tuber was carefully removed by scraping to minimize loss of the outer tissues since this layer contains much of the tuber protein (Walter et al., 1984; Eka, 1985). The tuber was cut into small pieces (approximately 1 in.^{3}) and frozen in 50-g portions with solid CO_2. The frozen pieces were transferred to a Waring blender containing 100 mL of ice-cold 0.1 M K_2HPO_4 and 0.1 mL of β-mercaptoethanol. Tuber pieces were homogenized at No. 1 speed for 3 min; the homogenate was squeezed through two layers of cheesecloth, and the filtrate was centrifuged at 20000*g* for 10 min. The pellet was discarded, and the supernatant liquid was dialyzed against three changes of deionized water for 24 h to remove low molecular weight sugars that

U.S. Department of Agriculture, Agricultural Research Service, Southern Regional Research Center, New Orleans, Louisianna 70179.
[1]Visiting Fulbright Research Professor. Present address: Department of Biochemistry, Anambra State University of Technology, Enugu, Nigeria.

0021-8561/86/1434-0599$01.50/0