Modified Thiobarbituric Acid Assay for Measuring Lipid Oxidation in Sugar-Rich Plant Tissue Extracts

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Use of the thiobarbituric acid (TBA) assay for thiobarbituric acid-reactive substances (TBARS) of lipid oxidation in extracts of plant materials was examined. Sucrose, fructose, glucose, lactose, citrus pectin, and bovine serum albumin (BSA) interfered with the TBA assay reaction. To correct for the interference caused by sugars (the major interference), a modified procedure was developed using standard curves for both malondialdehyde (MDA) and sucrose. Molar absorbance (MA) of adducts from TBA–MDA and TBA–sucrose reactions increased when the acidity in the reaction mixture increased and the time of heating and the temperature of heating for the reaction increased. Visible absorbance spectra showed that the adducts from the TBA–sucrose reaction were a mixture of at least two components. Comparisons between the basic and modified procedures indicated that the modified procedure was a more accurate measurement of TBARS of lipid oxidation in apple peel, tomato and pepper fruits, and tomato leaves.

INTRODUCTION

The thiobarbituric acid (TBA) assay is widely used to measure thiobarbituric acid-reactive substances (TBARS) of lipid oxidation (Buege and Aust, 1978; Gray, 1978) due to its simplicity, although high-performance liquid chromatography methods have been developed (Lee and Csallany, 1987; Tatum et al., 1990). TBA reacts with malondialdehyde (MDA), a product of lipid oxidation, to give a red, fluorescent 1:2 MDA/TBA adduct with maximum absorbance at 532 nm (Janero, 1990; Sinnhuber et al., 1958). In plant tissues, lipid oxidation in membrane systems takes place during senescence (Dhindsa et al., 1981). Since this oxidation appears to be an integral part of this process (Leshem et al., 1986), TBA assays of MDA or TBARS are being used increasingly in plant senescence research (Heath and Packer, 1968; Upadhyaya et al., 1989; Upadhyaya et al., 1985). MDA has been reported to inactivate ribonuclease and other enzymes in plant tissues (Chio and Tappel, 1969) and DNA in other systems (Nair et al., 1986).

In the TBA assay, absorbance at 532 nm is taken as a measure of MDA or lipid oxidation (Askawa and Matsushita, 1979; Bernheim et al., 1947; Frenkel and Neff, 1983). However, TBA also reacts in a similar way with other products of lipid oxidation such as alkanals, alkenals, and dienals (Knight et al., 1988), and some researchers recently reported that MDA may not be the major TBA-reactive component of lipid breakdown (Ichinose et al., 1989; Kosugi et al., 1989). In addition, many substances other than products of lipid oxidation have been reported to interfere with the TBA assay. These substances include monosaccharides and disaccharides, especially sucrose (Baumgartner et al., 1975; Albro and Corbett, 1986), and analogues of MDA such as acetaldehyde (Wills, 1964; Knight et al., 1988). Sucrose reacts with TBA to produce a chromogen with a maximum absorbance at 453 nm when incubated at a temperature of 37 °C (Wilbur et al., 1949; Baumgartner et al., 1975).

The TBA assay was developed for use in animal system research, and little consideration has been given to the different conditions that may exist in plant systems. In particular, little knowledge exists about potential interferences that may exist when the assay is used for plant extracts. This study was conducted to examine the use of the TBA assay for plant extracts, especially in sugar-rich plant extracts, and to determine modifications that may be needed to improve its specificity and accuracy.

MATERIALS AND METHODS

Chemicals. Bovine serum albumin (BSA), glutamic acid, glycine, fructose, lactose, and thiobarbituric acid (crystal) were from Sigma. Sucrose, glucose, mannitol, and glycerin were from Fisher. Malonaldehyde bis(dimethyl acetal), 98%, and trichloroacetic acid (TCA), 98% were from Aldrich. Starch (soluble) was from Mallinckrodt. Pectin (citrus) was from Matheson Coleman & Bell.

Plant Material. Three plant species were used. Cortland apples (*Malus domestica* Bork.) were grown at the University of Massachusetts Horticultural Research Center and harvested on different dates. Apples were peeled, and the peel was stored at −25 °C for three months. Frozen peels were used for measurements. Heinz 1350 tomatoes (*Lycopersicon esculentum*) and Large Bell green peppers (*Capsicum frutescens*) were grown in the greenhouse under standard conditions, and leaves and fruit were sampled for measurements on fresh tissue.

Ten grams of plant tissue were homogenized with 50 mL of cold acetone, and filtered through Whatman no. 4 paper. The filtrate was brought to 75 mL with water and kept at 4 °C until used for measuring TBARS and total soluble sugars.

Basic Procedure. The basic procedure of TBA assay was that of Heath and Packer (1968). Two milliliters of 20% TCA aqueous solution and 1 mL of 0.67% TBA aqueous solution were added to 2 mL of the extract from plant tissues. The mixture then was heated in a boiling water bath for 15 min, cooled quickly with running tap water, and centrifuged at 15 000 rpm for 15 min. The clear supernatant was brought to 10 mL with distilled water. The absorbance at 532 nm was recorded, and the absorbance for the nonspecific turbidity at 600 nm was subtracted. Two milliliters of distilled water instead of the extract, treated with the same procedure, was used as a blank.

A linear standard curve of MDA was prepared by the following procedure. Malonaldehyde bis(dimethyl acetal) [24.6 mg] was dissolved in 100 mL of distilled, deionized water to give a stock solution. Working standards were made by diluting the stock solution 1:75, 1:100, 1:150, 1:200, and 1:300 with 0.01 N HCl. The working solutions were prepared fresh daily. The molar absorbance (MA, 1.61×10^5) was close to the reported molar extinction coefficient of 1.57×10^5 (Albro et al., 1986) at 532 nm. The

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Table I. Effects of Selected Compounds on the TBA Assay[a]

compound	concn range	absorbance max, nm	MA 440 nm	MA 532 nm
sucrose	1–10 mM	440	147	8.4
fructose	1–10 mM	440	141	8.1
lactose	10–40 mM	370	11.4	
		440	1.8	0.14
glucose	10–50 mM	440	1.2	0.09
pectin	0.1–1%	440	0.081–0.73[b]	0.006–0.062[b]
BSA	0.1–0.4%	370	0.094–0.35[b]	
		530	0.005–0.014[b]	0.005–0.015[b]
MDA	0.75–5.0 μM	532	4991	161 000

[a] Data are the means of four replications. [b] Presented as optical density of the given concentration ranges at the wavelengths.

concentration of TBARS in terms of MDA equivalent was calculated as follows:

$$\text{MDA equivalent (nmol/mL)} = [(A_{532} - A_{600})/157\,000]10^6$$

Modified Procedure. The modified procedure was the same as the basic procedure except that the absorbance was measured at three instead of two wavelengths, i.e., at 532, 600, and 440 nm, and that a standard curve of sucrose was used to rectify the interference of soluble sugars in samples. By using a series of concentrations of sucrose from 2.5 to 10 μmol/mL, the standard curve of sucrose was prepared by following the basic procedure except that the absorbance was read at 532 and 440 nm. The absorbance of sucrose at 440 nm was proportional to the concentration of sucrose being measured and could be used for crude measurement of total soluble sugars in samples. The concentration of TBARS expressed in MDA equivalent (nmol/mL) was calculated as follows:

$$[[(A_{532} - A_{600}) - [(A_{440} - A_{600})(\text{MA of sucrose at 532 nm/MA of sucrose at 440 nm})]]/157\,000]10^6$$

However, A_{600} is very small, and often can be omitted from the above formula.

RESULTS AND DISCUSSION

Some soluble sugars, amino acids, polyhydroxy compounds—pectin, starch, and protein—were chosen for the test because these compounds are common components in plant tissues or, due to their structure, are possible interfering substances in the TBA assay. Among the 11 compounds tested under the conditions described above, five gave no detectable chromophore: 0.05–1% starch, 10–40 mM glycerin, 10–40 mM mannitol, 10–40 mM glycine, and 10–40 mM glutamate. Two (sucrose and fructose) significantly interfered with the TBA–MDA reaction, and the other four (lactose, glucose, pectin, and BSA) interfered only slightly (Table I). Reaction products of all of the tested sugars except lactose had high maximum absorbance at about 440 nm, which is different from the previously reported maximum absorbance at 453 nm (Wilbur et al., 1949; Baumgartner et al., 1975). Lactose had a maximum absorbance at 370 nm, but had a secondary absorbance peak at 440 nm. Sucrose and fructose both had a high MA of about 1.5×10^2, which was about 100 times higher than the MA of lactose or glucose in this experiment. Although the MA for sucrose is about 10^3 times (≈147/161 000, Table I) lower than that of MDA, the concentration of sugars may be about 10^3–10^4 times (40–400 mM/5–70 μM, Table V) higher than those of TBARS in plant tissues. Thus, the interference of sugars cannot be ignored. The MA for sugars in this experiment was much higher than reported previously (Albro et al., 1986; Wilbur et al., 1949; Baumgartner et al., 1975). One reason for such differences in maximum absorbance and MA could be the difference of the reaction temperature.

Table II. Influences of Temperature and Acidity on the TBA–Sucrose Reaction[a]

reaction conditions	MA at 532 nm	rel value	MA at 440 nm	rel value
68 °C; TCA, 0.488 M[b]	0	0	20 ± 2.1	14
88 °C; TCA, 0.488 M	3.2 ± 0.3	39	103 ± 5.3	71
98 °C; TCA, 0.488 M	8.1 ± 0.5	100	145 ± 4.8	100
98 °C; TCA, 0.122 M	0	0	25 ± 2.0	17
98 °C; TCA, 2.745 M	13.4 ± 0.8	165	228 ± 5.1	157
98 °C; HCl, 0.488 M	11.4 ± 0.6	140	161 ± 5.2	111

[a] Sucrose was present at 7.5 μmol/mL, and solutions were heated for 15 min in a water bath. Data were presented as means ± SE of three replications. [b] 0.488 M TCA is the normal concentration used for the assay.

Most previous experiments were conducted at 37 °C, but in this experiment the temperature was 95–100 °C except where noted. Other data in this experiment (Table II) showed that reaction temperature influenced not only the absolute value of MA, but also the relative value of MA at different wavelengths. As temperature increased, the absorbance at 532 nm increased more rapidly than that at 440 nm. This suggested that at high temperature, sucrose interfered with TBA assay more significantly than at low temperature.

Pectin had a maximum absorbance at 440 nm, and it produced the chromophore at room temperature by reaction with TBA. BSA gave a high absorbance peak at 370 nm and a low absorbance peak at 530 nm. Prior to this study, protein had not been reported to interfere in TBA assay. Although most fresh plant tissues do not contain large amounts of protein (Leveille et al., 1983; Souci et al., 1986), some, e.g., seeds of legume, may contain considerable amounts [10–25%] (Leveille et al., 1983). In extracts with high soluble pectin or protein concentrations, corrections in addition to that described here may be necessary in a TBA assay. However, since sucrose gave very high interference and it is in quite high concentrations [5–15%] (Souci et al., 1986) in many plant tissues, a series of tests using sucrose as the interfering compound examined factors affecting TBA assays of plant extracts.

Several factors were found to be important for TBA assay. The MA of both 532 and 440 nm increased as time of heating increased. However, after 15 min these increases had leveled off. The MA was also influenced by temperature of heating and acidity of reaction mixture. The higher the temperature or the acidity, the higher the value of MA (Table II). Replacement of TCA by an equal concentration of HCl gave an increased MA for the TBA–sucrose reaction (Table II), but equal replacement did not significantly change the MA of TBA–MDA reaction (TCA, 100 ± 3.6; HCl, 99 ± 3.3). These results are consistent with the possibility that the pyrolysis products of sucrose react with TBA to produce a red chromogen (Baumgartner et al., 1975), possibly a MDA-like product (Matsuo et al., 1987), and suggest that acidic conditions may influence the pyrolysis of sucrose.

A precipitate of the chromogen from the TBA–sucrose reaction can form (Wilbur et al., 1949). Under the conditions of this experiment, a precipitate was often observed when the final concentration of sucrose was higher than 7.5 μmol/mL. After this precipitate was redissolved in distilled water, the absorbance curve in the visible spectrum was different from that of its supernatant and that of the uncentrifuged chromogen solution, producing a higher absorbance at wavelengths longer than 460 nm (Figure 1). Thus, the precipitate interfered more at 532 nm than did the supernatant chromogen. These results indicated that the chromogen of the TBA–sucrose

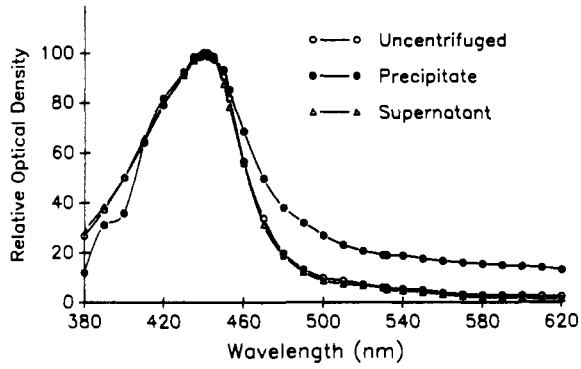

Figure 1. Relative optical densities of the uncentrifuged sucrose-reaction mixture, sucrose-reaction precipitate, and sucrose-reaction supernatant.

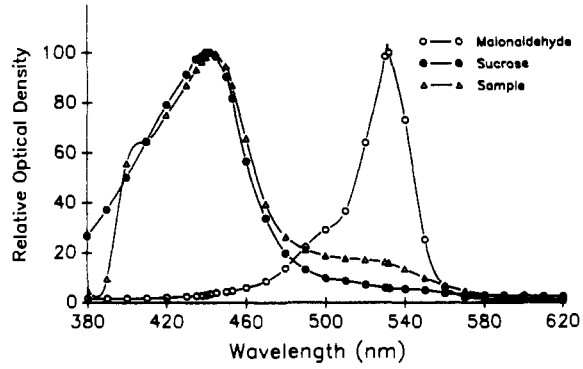

Figure 2. Relative optical densities of MDA and of the TBA reaction product of an apple peel extract and uncentrifuged reaction products of sucrose.

Table III. Effects of Additions to an Apple Peel Extract, of MDA and Sucrose, Alone or in Combination, on the Measurements of TBARS and Sucrose Concentrations Using the Modified TBA Procedure

additions to the apple extract	measured increase in concn	
	TBARS, nmol/mL	sucrose, μmol/mL
0.5 nmol/mL MDA	0.54 ± 0.03	
1.5 nmol/mL MDA	1.34 ± 0.06	
2.5 μmol/mL sucrose		2.5 ± 0.11
5.0 μmol/mL sucrose		4.4 ± 0.26
0.5 nmol/mL MDA + 2.5 μmol/mL sucrose	0.53 ± 0.03	2.6 ± 0.13
0.5 nmol/mL MDA + 5.0 μmol/mL sucrose	0.56 ± 0.04	5.1 ± 0.17
1.5 nmol/mL MDA + 2.5 μmol/mL sucrose	1.46 ± 0.05	2.7 ± 0.14
1.5 nmol/mL MDA + 5.0 μmol/mL sucrose	1.45 ± 0.05	5.1 ± 0.19

[a] Data are presented as means ± SE of three replications.

reaction was a mixture of at least two components. One component had a relatively low solubility in water and precipitated when its concentration was high, i.e., when the concentration of sucrose was higher than 7.5 μmol/mL, while the other had relatively high solubility in water and existed only in the supernatant. This interpretation was supported by the data in Table II: absorbance at different wavelengths increased at different rates when temperature of heating or acidity of the reaction mixture increased. The absorbance of a sample of apple peel extract was different from that of the pure TBA–sucrose reaction (Figure 2). The small increase at about 530 nm of the absorbance curve of the apple sample apparently was due to TBARS in the sample.

Because six compounds, especially sucrose and fructose, had significant absorbance at 532 nm (as a tail [about 5%] of the absorbance at 440 nm, Table I) and they are abundant in plant tissues, they may interfere with measurement of TBARS in plant tissue extracts. This raises the problem of how to correct for these interferences in a practical assay. Since it is very difficult to exclude all of these interfering compounds from plant tissue extracts, a modified procedure was developed, as described in Materials and Methods.

Table IV. Comparisons of Various Solvents Used To Extract Apple Peel Tissue for the TBA Assay[a]

solvent	% recovery	
	MDA	sucrose
ethanol (100%)	94 ± 6	95 ± 5
ethanol/water (3/1 v/v)	71 ± 5	97 ± 5
acetone (100%)	45 ± 5	73 ± 5
acetone/water (3/1 v/v)	46 ± 3	96 ± 4
water (100%)	20 ± 3	72 ± 6
1% TCA in water	19 ± 3	68 ± 4
propyl alcohol (100%)	10 ± 4	88 ± 7
chloroform (100%)	2 ± 1	1 ± 1
hexane (100%)	0	0

[a] Sucrose (3 mL, 500 mM) and MDA (0.5 mL, 1.5 μM) were added to 7.5 g of apple peel before extraction. Recovery is calculated as percent of the added MDA or sucrose that was measured by the modified TBA procedure at 532 nm (MDA) or 440 nm (sucrose). Data are presented as means ± SE of three replications.

Since compounds other than those tested in this experiment may interfere with the TBA assay of plant extracts, internal standards of sucrose and MDA were added to apple extract samples. The systems of sample plus standard solution of MDA or sucrose or both behaved similarly to the pure system of standard solution of MDA or sucrose (Table III). This suggests that there was no significant interaction between MDA and sucrose, or between the components of a sample and exogenous MDA and sucrose.

TBARS can be extracted by different solvents (Wilbur et al., 1949; Ichinose et al., 1989; Heath and Packer, 1968). However, the effectiveness of different solvents for the extraction of TBARS and interfering substances in plant tissues has not been reported before. We found considerable differences among solvents and solvent combinations in extraction recovery of TBARS and sucrose from apple peels (Table IV). Ethanol (100%) gave the highest recovery for both TBARS and sucrose, while hexane extracted no detectable TBARS or sucrose from apple peels. A relatively polar solvent may be needed for this method. Acetone, routinely used as the solvent for TBA assay, recovered only 45% and 73%, respectively, of TBARS and sucrose. However, acetone gave the best recovery (96–100%) for peroxides in extraction from plant tissues (data not shown). Whether the low recovery of MDA in many solvents is due to the instability of MDA in the presence of some strong oxidants that exist in plant tissues, e.g., H_2O_2 (Kostka and Kwan, 1989), or due to the solvent itself is not known. It appears that the solvent of choice depends on what substances you wish to extract and possibly, the tissue you are extracting.

Table V compares results of basic and modified procedures for samples from an experiment using Cortland apples. In this case, the sample tissue contained 10–13% soluble sugars, which accounted for about one-third of the total absorbance at 532 nm, since the measured concentration of TBARS by the modified procedure was about 35% less than that measured by the basic procedure.

The modified procedure also was used to measure extracts from tomato and green pepper plants (Table V). The amount of total soluble sugars was calculated from the absorbance at 440 nm, which could be slightly higher

Table V. Comparisons of the Basic and Modified TBA Assays for Measuring TBARS Concentrations in Peel from Cortland Apples Harvested on Different Dates, in Tomato Leaves and Fruit, and in Green Pepper Fruit[a]

plant tissue	TBARS, nmol/g fw: basic assay (A)	TBARS, nmol/g fw: modified assay (B)	B/A, %
Cortland apple (9/6/89)	83.4 ± 3.3	52.1 ± 4.7	63
Cortland apple (9/13/89)	67.1 ± 8.7	39.1 ± 5.0	58
Cortland apple (9/18/89)	86.8 ± 2.6	54.7 ± 2.0	63
Cortland apple (10/2/89)	101.0 ± 5.0	66.7 ● 6.7	66
tomato leaves	7.9 ± 0.9	4.6 ± 0.8	58
tomato fruit (green)	7.8 ± 0.8	1.7 ± 0.3	22
tomato fruit (red)	16.5 ● 1.4	5.1 ± 0.6	31
pepper fruit (red)	22.6 ± 1.7	4.9 ± 0.6	22

[a] Soluble sugar concentrations were 10–13% fw in apple peel, 1.3% in tomato leaves, 2.8% and 4.6% in green and red tomato fruit, respectively, and 7.1% in pepper fruit, as measured by the TBA reaction. Data are presented as means ± SE of four replications.

than the actual amount (Leveille et al., 1983; Souci et al., 1986) in plant tissues due to interference at 440 nm by pectin or other substances. The concentrations of TBARS and soluble sugars in these plant tissues were much lower than those in apple peels, and the concentrations of sugars also were much more variable (Table V). In these samples, the sugars accounted for up to 78% of the "TBARS" calculated from the basic procedure, a difference that could lead to misinterpretations of the results. Thus, use of the basic procedure for measuring extracts from sugar-rich plant tissues may greatly overestimate the TBARS concentrations and use of the modified procedure could overcome this problem.

Soluble sugars could be the primary interference in TBA assay of many plant tissues. In most vegetable and fruit crops, soluble sugar concentrations change greatly during maturation, ripening, and senescence (Rhodes, 1980). A simple method to correct for the interference by such varying compounds could be important.

Although the specificity to MDA of the TBA assay has been questioned, Pompella et al. recently (1987) reported that TBA assay has comparable reliability with other methods in measuring lipid oxidation both in vivo and in vitro. Use of the modified procedure described here could improve its accuracy when applied to plant tissues. Plaisance (1917) reported that TBA could be a qualitative reagent for ketohexose. We do not suggest use of the modified procedure as a quantitative method for measuring total soluble sugars in plant tissues. However, it could be used for a simple semiquantitative measurement of total soluble sugars in plant tissues.

ABBREVIATIONS USED

A, absorbance; BSA, bovine serum albumin; MA, molar absorbance; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive substances; TBA, thiobarbituric acid; TCA, trichloroacetic acid.

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Registry No. TBA, 504-17-6; MDA, 542-78-9; sucrose, 57-50-1; fructose, 57-48-7; glucose, 50-99-7; lactose, 63-42-3; pectin, 9000-69-5.