

THE THIOBARBITURIC ACID TEST OF 4, 5-UNSATURATED DIGALACTURONIC ACID*

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Waravdekar and Saslaw (1957) reported a sensitive color test for deoxyribose. In this test, malondialdehyde produced from 2-deoxyribose by periodate oxidation is coupled with thiobarbituric acid to give a color with an absorption maximum at 532m μ . Weissbach and Hurwitz (1959) found that the thiobarbituric acid test provides an excellent method also for the determination of 2-keto-3-deoxyaldonic acid. In this case the product of periodate oxidation is formyl pyruvic acid and the color has an absorption maximum at 545-550m μ rather than 532m μ . They considered that no other compounds, except 2-keto-3-deoxy sugar acid, react in the thiobarbituric acid to form the same color. However, 4, 5-unsaturated oligogalacturonides, which are formed from pectic substances by heating the neutral solution or by the action of the lyases specific for pectic substances (pectin *trans*-eliminase, Albersheim et al. 1960; pectate *trans*-eliminase, Hasegawa and Nagel 1962), and 4, 5-unsaturated dimannuronic acid (T sujino 1963) also form a color having an absorption maximum at 545-556m μ in the thiobarbituric acid test. According to Albersheim et al (1960), 4, 5-unsaturated oligogalacturonides give the same test even when the procedure of periodate oxidation is omitted. In the present study it has been found that, although the color produced in the thiobarbituric acid test of 4, 5-unsaturated digalacturonic acid (Okamoto et al. 1964) is far less than that formed in the case of 2, 5-diketo-3-deoxygluconic acid, it may be increased by raising the temperature of periodate oxidation. Some other factors influencing the thiobarbituric acid test of 4, 5-unsaturated digalacturonic acid have also been studied. The results obtained are described here.

EXPERIMENTAL AND RESULTS

Conditions for Periodate Oxidation of 4, 5-Unsaturated Digalacturonic Acid

Temperature. Crystalline calcium salt of 4, 5-unsaturated digalacturonic acid was prepared as described in the preceding paper (Okamoto and Ozawa 1958) and used as a 0.5mM aqueous solution. Portions of this solution (0.4 ml) were pipetted into glass tubes and mixed with 0.025N-NaIO₄ in 0.125N-H₂SO₄ (0.5 ml). The mixtures were incubated at varying temperatures (0, 30, 55, 80 and 100°C). After various periods of time, 1 ml of 2% sodium arsenite in 0.5N-HCl and 4 ml of

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0.3% thiobarbituric acid were successively added at an interval of two minutes. The mixture was heated in a boiling water bath for 10 minutes and then cooled in a bath of cold water. The optical densities of the colored solutions were measured at $548m\mu$ on a Beckman DU spectrophotometer with cells of 1 cm. The results obtained are shown in Fig. 1.

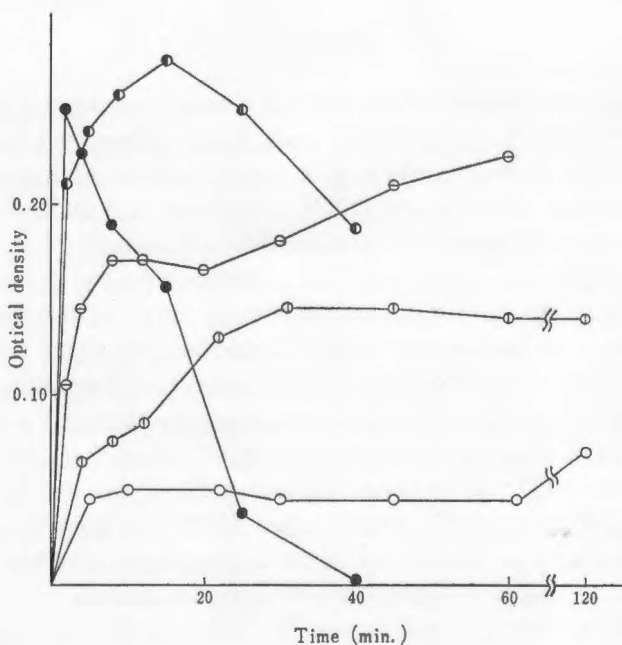


Fig. 1. Effect of temperature of periodate oxidation on the color production in the thiobarbituric acid test of 4,5-unsaturated digalacturonic acid. ○, 0°C; ◐, 30°C; ◑, 55°C; ◒, 80°C; ●, 100°C.

It can be seen from Fig. 1 that the amount of color increased as the temperature of periodate oxidation was raised. Maximum color production was observed at 80°C for 15 minutes. At 80 and 100°C, the color-forming substance appears to be rapidly destroyed.

Other factors. Concentration of H_2SO_4 .—A portion of the above 4,5-unsaturated digalacturonic acid solution (0.4 ml) was mixed with 0.025N- $NaIO_4$ in 0.125, 0.25 or 0.50N- H_2SO_4 (0.5 ml) and heated at 80°C for 15 minutes. The subsequent procedures were followed as above. The concentration of H_2SO_4 was found having no effect on the color production in the range 0.125 to 0.50N.

Concentration of $NaIO_4$.—The thiobarbituric acid tests of 4,5-unsaturated digalacturonic acid were carried out as above with the exception that 0.025, 0.0375 and 0.050N- $NaIO_4$ in 0.125N- H_2SO_4 were used. Difference due to concentration of $NaIO_4$ was not observed.

Furthermore, ammonium oxalate (0.2%), brom phenol blue of Clark and Lubs (E_{310} , 0.070), 0.05M-borax—0.1N-HCl (pH 8.0) and phosphate buffer (0.1M,

pH 7.0) added in place of 4, 5-unsaturated digalacturonic acid were found not to produce any color showing absorption at $548\text{m}\mu$ (the conditions for periodate oxidation; 80°C , 15 minutes, 0.025N-NaIO_4 in $0.125\text{N-H}_2\text{SO}_4$). The colored solution obtained from 4, 5-unsaturated digalacturonic acid as above was stable for at least 4 hours at 30°C .

Thiobarbituric Acid Test of the Degraded Products of Pectic Substances

Acid soluble pectic acid and oligogalacturonide were obtained from "citrus pectin", which was purchased from Nippon Kako Co. Ltd, by hydrolysis with hydrochloric acid. Galacturonic acid and digalacturonic acid were removed from

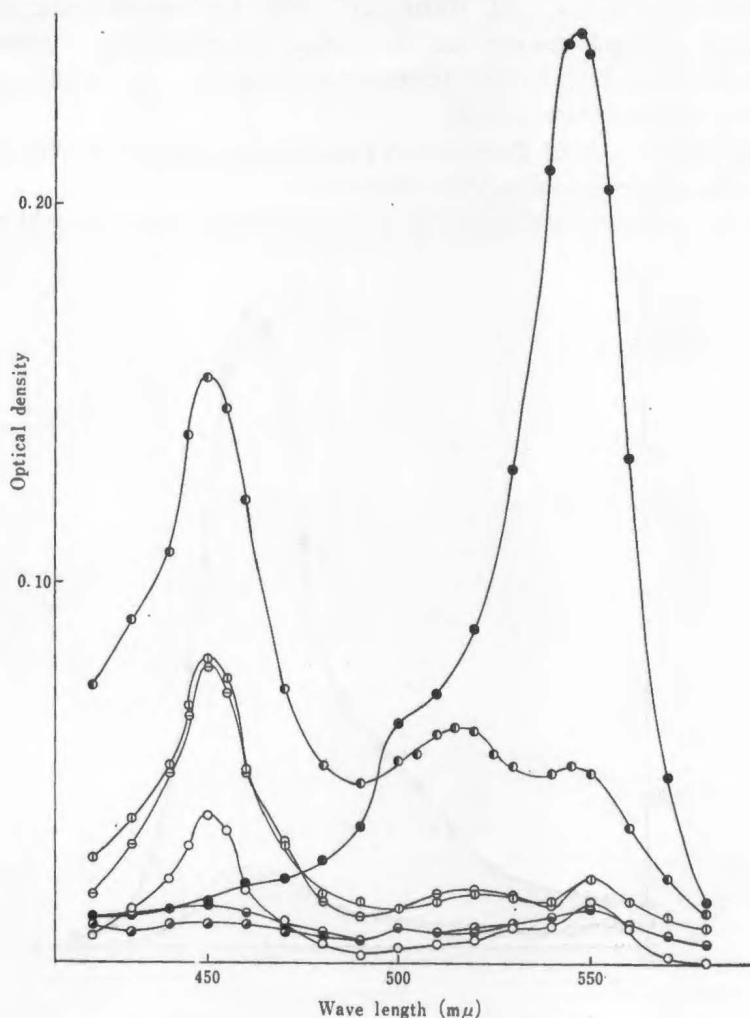


Fig. 2. Absorption spectra of the colors produced from the degraded products of pectic substances in thiobarbituric acid test. ○, citrus pectic acid; ⊖, acid soluble pectic acid; ⊕, oligogalacturonide; ⊙, trigalacturonic acid; ⊖, digalacturonic acid; ⊙, galacturonic acid; ●, 4, 5-unsaturated digalacturonic acid.

the latter by paper chromatography. Citrus pectic acid, acid soluble pectic acid, oligogalacturonide, trigalacturonic acid, digalacturonic acid or galacturonic acid (Ozawa 1952, 1955) was dissolved in dilute sodium hydroxide solution and used as a 0.1% solution, pH 7.0. The thiobarbituric acid tests were made on 0.4 ml of these solutions and 0.5 mM calcium 4, 5-unsaturated digalacturonate. After the addition of 0.5 ml of 0.025 N-NaIO₄ in 0.125 N-H₂SO₄, these were oxidized at 80°C for 15 minutes. The subsequent procedures were followed as described in the preceding section. Absorption spectra of the colored solutions are shown in Fig. 2.

Every solution has a peak at 550 m μ . Especially trigalacturonic acid gives considerably strong absorption, though the peak is not so intense as that of 4, 5-unsaturated digalacturonic acid. Citrus pectic acid, acid soluble pectic acid, oligogalacturonide or trigalacturonic acid has another absorption peak at 450 m μ . This absorption band could not be used for the determination of these substances because their optical densities were variable.

Thiobarbituric Acid Test of 4, 5-Unsaturated Digalacturonic Acid with the Periodate Oxidation Step Omitted

The thiobarbituric acid test of 4, 5-unsaturated digalacturonic acid was made

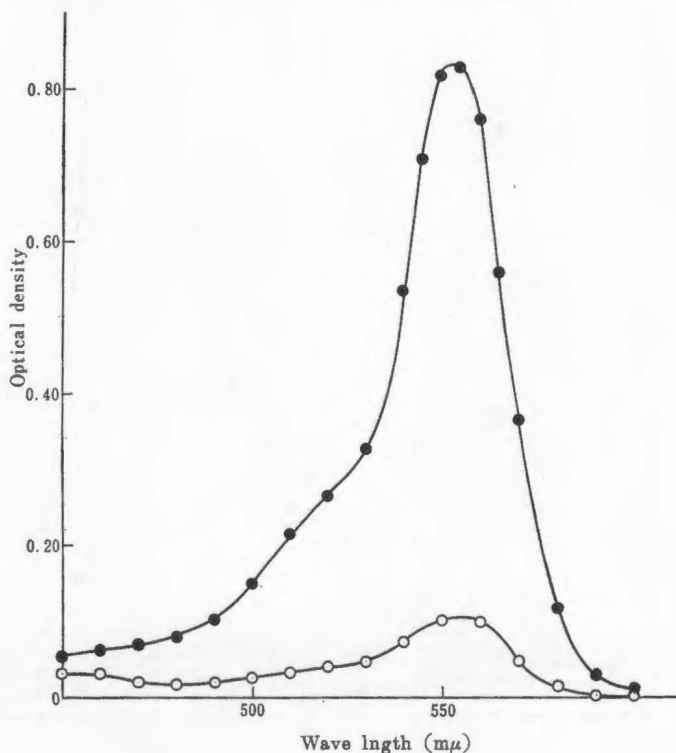


Fig. 3. Absorption spectra of the color produced from 4, 5-unsaturated digalacturonic acid in the thiobarbituric acid tests with and without periodate oxidation step. ○, test with periodate oxidation step omitted, optical density found $\times 1,088$; ●, test involving periodate oxidation step.

with the periodate oxidation step omitted according to the method of Albersheim et al. (1960). Calcium 4, 5-unsaturated digalacturonate (1 mM, 0.2 ml), phosphate buffer (pH 7.0, 0.2M, 0.2 ml), HCl (0.5M, 2 ml) and thiobarbituric acid (0.01M, 4 ml) were successively pipetted into a glass tube and heated in a boiling water bath for 30 minutes. After cooling the spectrum was measured and compared with that obtained by the above-mentioned method, in which 4, 5-unsaturated digalacturonic acid (0.5 mM, 0.4 ml) was previously oxidized at 80°C for 15 minutes with NaIO_4 (0.025N in 0.125N- H_2SO_4 , 0.5 ml).

As shown in Fig. 3, an absorption peak at 550 $m\mu$ was also obtained by the method with periodate oxidation step omitted. But its optical density was only one eighth that obtained by the ordinary method. As 4,5-unsaturated digalacturonic acid gives the same peak absorption as 2-keto-3-deoxy-aldonic acid, its product of periodate oxidation would be formyl pyruvic acid.

The thiobarbituric acid tests were made on 0.4 ml of 5-formyl-2-furancarboxylic acid (2 mM) and 2-furancarboxylic acid (2 mM) respectively, both of which were produced from 4, 5-unsaturated digalacturonic acid by acid hydrolysis (Okamoto and Ozawa 1965). Both compounds gave no thiobarbituric acid test, whether they were previously oxidized with periodate or not. A mixture of 5-formyl-2-furancarboxylic acid (1 mM), 2-furancarboxylic acid (1 mM) and formic acid (0.5 mM) gave also a negative thiobarbituric acid test.

SUMMARY

1) The amount of color produced in the thiobarbituric acid test of 4, 5-unsaturated digalacturonic acid increased as the temperature of periodate oxidation was raised from 0 to 80°C. Maximum color production was at 80°C for 15 minutes.

2) Even when the procedure of periodate oxidation was omitted, 4, 5-unsaturated digalacturonic acid gave positive thiobarbituric acid test, forming the color with an absorption peak at 550 $m\mu$. But the optical density was only one eighth that obtained by the ordinary method.

3) It was found that 2-furancarboxylic acid and 5-formyl-2-furancarboxylic acid prepared from 4, 5-unsaturated digalacturonic acid by acid hydrolysis gave negative thiobarbituric acid test.

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