COPPER-DEPENDENT HYDROXYL RADICAL DAMAGE TO ASCORBIC ACID

Formation of a thiobarbituric acid-reactive product

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1. Introduction

Reactivity with 2-thiobarbituric acid (TBA) has been widely used as a test to detect oxidative deterioration in lipid products. Solutions of amino acids, carbohydrates and DNA develop TBA-reactivity after iron-dependent free radical damage [1,2]. In these test systems it appears that a TBA adduct is formed with malondialdehyde (MDA) which is released from precursor molecules during the acid heating stage of the test [2,3]. Several other aldehydes as well as the bile pigments give similar chromogenic adducts with TBA [4,5]. However, these can be distinguished from the MDA-TBA adduct following chromatographic separation or characterisation of their fluorescent spectra [6].

Considerable evidence from independent studies has shown that ascorbic acid can act both as an antioxidant and as a pro-oxidant. Its antioxidant properties are often seen at high concentrations when it is acting as a radical scavenger. Pro-oxidative properties are usually associated with its metal-ion reducing capabilities. Both iron and copper salts are reduced by ascorbate in a reaction involving a single electron transfer. These reduced metal ions can then autoxidise in solution with the formation of oxygen radicals.

The interaction of copper (II) salts and ascorbic acid to produce reactive radicals has been widely investigated in lipid, DNA, virus and whole cell systems [7–10]. However, there has not been a study to see what happens to the ascorbate in such systems. In the reaction described here, copper salts reduced by ascorbate autoxidise with the ultimate formation of hydroxyl radicals which damage the ascorbate to produce a TBA-reactive product.

2. Materials and methods

2.1. *Reagents*

Superoxide dismutase bovine erythrocyte (spec. act. 2900 units/mg protein), catalase bovine liver thymol-free, albumin human fatty acid-free, 1,10-phenanthroline were obtained from Sigma. All other chemicals were of AnalaR grade where available and obtained from BDH Ltd.

2.2. *Methods*

Freshly prepared 10 mM ascorbic acid (0.2 ml) was added to 1.6 ml chelex-treated distilled water, followed by the addition of 0.2 ml cupric chloride. The resulting pH of the reaction mixture was 3.5. After incubation at 37°C for 15 min, TBA-reactivity was developed. Studies with inhibitors were performed by adding 0.2 ml inhibitor to 1.4 ml water before addition of 0.2 ml 1.0 mM copper salt and 0.2 ml 4 mM ascorbic acid.

2.3. *Development of thiobarbituric acid reactivity*

Concentrated glacial acetic acid (1.0 ml) was added to each tube, followed by 1.0 ml 1% TBA in 0.05 M NaOH. The tubes were heated for 15 min at 100°C, cooled and the resulting TBA adduct measured at 550 nm.

2.4. *Thin-layer chromatography*

Aqueous samples of the TBA adducts were directly applied to Merck concentration plates (silica gel 60) and separated in the solvent system chloroform: methanol:acetic acid, 60:20:10 (by vol.).

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2.5. *Spectrofluorimetry*

The relative fluorescence intensity of each TBA adduct was measured against a standard 3×10^{-6} M rhodamine B set to 100 units at excitation 480 nm, emission 580 nm, slit/width 10 nm and sensitivity × 1.

3. Results

3.1. *TBA-reactivity of ascorbate–copper (II)*

Ascorbate in the presence of copper (II) ions reacts to form a TBA-reactive adduct with an absorbance maximum at 550 nm (fig.1). At a final reaction mixture concentration of cupric ions 0.1 mM, greatest TBA-reactivity was observed with 1.0 mM ascorbate.

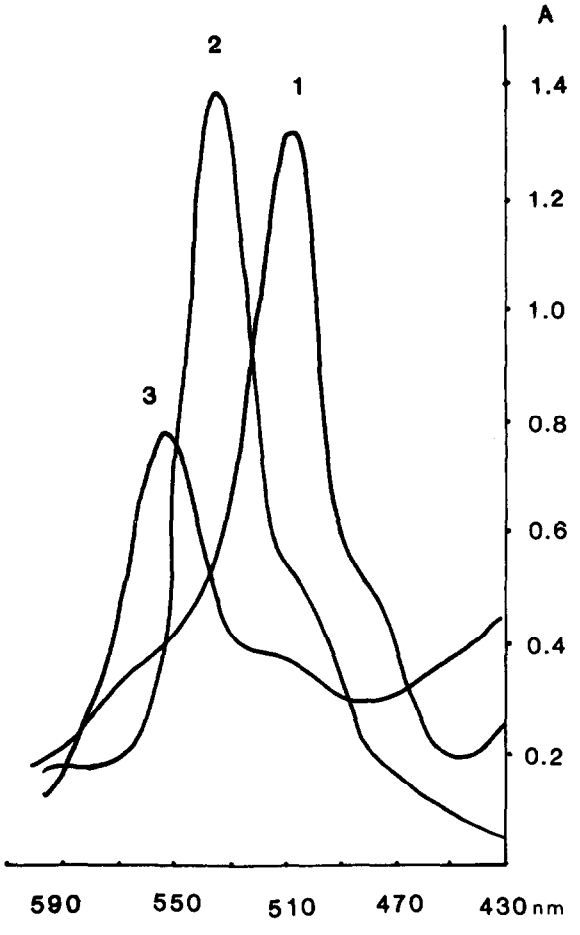

Fig.1. Scan difference spectra of: (1) furfuraldehyde 1 mM; (2) malondialdehyde 40 μM; (3) copper (II) 0.1 mM plus ascorbate 1.0 mM, following TBA colour development as in section 2.3.

Table 1
Thin-layer chromatographic separation of TBA adducts

TBA adducts formed from the following reactants:	R_F
Furfuraldehyde	0.74
Malondialdehyde	0.71
Cupric ions–Ascorbate	0.21

Aqueous solutions of the TBA adducts were directly separated on silica gel plates under the conditions in section 2.4. The concentration of furfuraldehyde was 1.0 mM, malondialdehyde (prepared by the hydrolysis of 1,1,3,3-tetramethoxypropane) 40 μM and the ascorbate derived product from 0.1 mM Cu(II) and 1.0 mM ascorbate. The R_F-values are a mean of 3 separate experiments. The TBA-test was performed on each sample as in section 2.3

This gave an absorbance at 550 nm of 0.74. As the concentration of ascorbate was increased above this, TBA-reactivity began to fall. TBA-reactivity was enhanced at acid pH-values but greatly reduced at neutral and alkaline pH. Substitution of iron (III) salts for the copper (II) salts over the same concentration range and under the same reaction conditions did not yield a TBA-reactive product.

The ascorbate–copper (II) derived TBA-reactive product gave a single discrete zone when separated chromatographically on silica gel (table 1). This was clearly distinguishable from MDA and furfuraldehyde prepared TBA adducts. Adjustment of the TBA adducts from MDA, furfuraldehyde and the copper–ascorbate reaction to an absorbance value of 0.10 at 532, 510 and 550 nm, respectively, gave relative fluorescence intensity units of 130, 6.5 and 9.5.

3.2. *Effect of free radical scavengers on the copper-dependent damage to ascorbate*

The hydroxyl radical scavengers formate, mannitol and thiourea inhibited copper-dependent damage to ascorbate (table 2).

Similarly, catalase was also inhibitory, implicating hydrogen peroxide as a key intermediate in the reaction. Superoxide dismutase, however, had little effect. This latter effect, like that of albumin and boiled catalase, can probably be ascribed to non-specific protein binding of copper [11]. The copper chelator, 1,10-phenanthroline and the non-specific metal chelator EDTA, both inhibited formation of the TBA-reactive product. The specific iron (III) chelator desferrioxamine slightly inhibited the reaction.

Table 2

Inhibitor added	Amount of TBA-reactive material formed A_{550}	% Inhibition of ascorbic degradation
Control (no inhibitor)	0.30	–
Mannitol, 10 mM	0.0	100
Formate, 10 mM	0.0	100
Thiourea, 0.5 mM	0.0	100
Urea, 0.5 mM	0.28	7
1,10-phenanthroline, 0.2 mM	0.01	97
EDTA, 0.2 mM	0.0	100
Desferrioxamine, 0.2 mM	0.13	57
Superoxide dismutase, 0.05 mg/ml	0.23	23
Catalase, 0.05 mg/ml	0.02	93
Catalase, 0.05 mg/ml (boiled)	0.19	37
Albumin, 0.1 mg/ml	0.21	30

Effect of inhibitors on damage to ascorbate induced by copper (II) salts. The concentrations stated above were the final concentrations of each reagent in the reaction mixture. The results are a mean of 3 separate experiments which were reproducible. The superoxide dismutase contained 2900 units/mg protein (units defined as in [13]) and the catalase 25 000 units/mg protein (units defined as in [14]). Inactivation of catalase was made by heating at 1000°C for 5 min and cooling before use

4. Discussion

Free radical damage to polyunsaturated lipids, DNA, certain amino acids and carbohydrates can bring about the formation of TBA-reactive intermediates. These intermediates, when heated under optimal conditions of acidity, break down to liberate malondialdehyde, which in the presence of TBA forms a characteristic adduct showing absorbance at 532 nm and fluorescence at 553 nm.

Under the conditions described here ascorbic acid (AH_2) serves as both a cupric ion reductant as well as a substrate for the resulting free radical damage.

$$AH_2 + Cu(II) \rightarrow AH^{\cdot} + Cu(I) + H^{+} \quad (1)$$

$$AH^{\cdot} + Cu(II) \rightarrow A + Cu(I) + H^{+} \quad (2)$$

Studies with hydroxyl radical scavengers implicate the $OH^{\cdot}$ radical as the species most likely to be responsible for this damage (table 2). The $OH^{\cdot}$ radical is probably formed by the reaction sequence shown in eq. (3–5). Autoxidation of Cu(I) will lead to the formation of superoxide radicals which rapidly dismutate to form hydrogen peroxide. This is then decomposed in a Fenton-type reaction by cuprous ions to give the hydroxyl radical ($OH^{\cdot}$).

$$Cu(I) + O_2 \rightleftharpoons Cu(I) - O_2 \rightarrow Cu(II) + O_2^{\cdot -} \quad (3)$$

$$2\,O_2^{\cdot -} + 2\,H^{+} \rightarrow H_2O_2 + O_2 \quad (4)$$

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + OH^{\cdot} + OH^{-} \quad (5)$$

Catalase inhibits hydroxyl radical formation by removal of hydrogen peroxide in reaction (5) but superoxide dismutase is without effect since it serves only to enhance the rate of reaction (4).

Although iron(III) salts are readily reduced by ascorbate and the resulting ferrous ions autoxidise with the formation of hydroxyl radicals [12], their substitution for copper in this system did not result in the formation of TBA-reactive material.

The TBA-reactive product formed following copper damage to ascorbate is not due to the liberation of malondialdehyde since it shows different chromatographic, spectrophotometric and spectrofluorimetric properties. The reaction between cupric ions and ascorbate provides a simple system for generating hydroxyl radicals and detecting their damage to a biological molecule.

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