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The Role of Simple Carbohydrates in the Suppression of Hydroxyl Free Radicals in γ-Irradiated Papaya Juice

James Gray & Howard F. Mower*

Department of Biochemistry and Biophysics, John A. Burns School of Medicine, University of Hawaii at Manoa, Honolulu, Hawaii 96822, USA

and

Cancer Research Center, University of Hawaii, 1236 Lauhala Street, Honolulu, Hawaii 96813, USA

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ABSTRACT

Hydroxyl free radical (HO˙), reactions with a series of trapping agents, such as 2-deoxyribose, 2-hydroxybenzoic acid or dimethylpyrroline N-*oxide, are effectively blocked when dilute solutions of papaya juice are added to irradiation mixtures prior to exposure to* ^{60}Co *γ-rays.*

Our studies to determine the agents present in papaya juice responsible for the HO˙ quenching activity suggest that glucose, fructose and sucrose, present in papaya juice in total concentrations of ~0·5–0·7 molar, block HO˙ reactions and produce malonaldehyde and other sugar derivatives when the juice is exposed to γ-rays.

Papaya contains a sequestered invertase which is released when the fruit is crushed during juice extraction. Heat treatment of the fruit before juice preparation inactivates this enzyme, and the juice of this fruit contains lower total amounts of the simple sugars and is a proportionally poorer quenching agent than the unheated juice.

Synthetic mixtures of the simple sugars at concentrations equal to those in papaya juice mimic the inhibition of HO˙ reactions and the production of malonaldehyde by papaya juice.

* To whom correspondence should be addressed.

INTRODUCTION

The infestation of some fruit, for example, papaya, by insect eggs and larva restricts their importation into areas that are free of the contaminating insects. Low dose γ-irradiation of fruit is an effective means of controlling many forms of insect contamination, while not affecting the quality of the fruit (Moy & Nagai, 1985). A detailed irradiation chemistry of papaya is not at present known, so that complete assurance of the harmless long term ingestion of the irradiated fruit cannot be made at this time. Since papaya juice is 85–90% water, the primary irradiation reaction in this fruit is probably the cleavage of water into hydrogen atoms and hydroxyl free radicals (Czapski, 1971; Schwarz, 1981). The latter are highly reactive radicals which cause chemical reaction with virtually every collision with most carbon–hydrogen bonds. $HO^{\cdot}$ reacts in this manner with the simple carbohydrates $[10^{10} > k_2\,(HO^{\cdot}) > 10^9]$ (Bucknall *et al.*, 1978). The ensuing carbon free radical on the sugar molecule undergoes a variety of reactions depending on the nature of the sugar, pH, etc. Malonaldehyde is a common product formed, among others, when many of the simple sugars are γ-irradiated. In this paper we present evidence that the quenching of hydroxyl free radicals by papaya juice, as described in a previous publication (Webman *et al.*, 1989), is primarily a consequence of their high simple sugar content.

METHODS

Irradiation of papaya juice and carbohydrate solutions was done using the ^{60}Co source of the Hawaii Research Irradiator. The irradiator is currently operating at 1·7 Krad min^{-1}. Samples were irradiated for variable times from 5 to 30 min.

Papaya juice was obtained from fresh ripe fruit through a small incision in the skin of the fruit. By the application of gentle pressure, ~0·5–1·0 ml of juice was collected and clarified by centrifugation at room temperature, in a desk-top clinical centrifuge at ~3/4 max speed for 5 min. Heat inactivation of invertase in some papaya juice preparations was done by heating the fruit prior to juice extraction for 2 min in a microwave oven as described by Chan and Kwock (1975).

Carbohydrate analysis of papaya juice was done using a Dionex Pulsed Amperometric Detector (PAD), model UEM-1 (Hardy *et al.*, 1988). The chromatography was done using isocratic conditions with a mobile phase of 300 mM NaOH. The flow rate was 1·0 ml/min using a Dionex PAC HPIC AS6 ion exchange column with a HPIC AG6 guard column. The PAD

detector was set at: E 1 at 0·1 V, E 2 at 0·6 V, E 3 at −0·8 V, t1 at 300 ms., t2 at 120 ms, t3 at 300 ms. The output range was 300 nA, and the zero was set after sample injection by the 'auto-off' function of the detector. The output of the detector was monitored by a Hewlett Packard 3390A recording integrator which was set at the following settings: Zero, 10, Attenuation, 8, Peak Width, 0·03, Threshold, 11, Area Reject, 9000. The juice sample was diluted 10^4 times with water, and 10 μl of this solution was injected on the column.

HO˙ formation was measured by reaction of these radicals with salicylic acid (Halliwell & Gutteridge, 1984). Irradiation mixtures routinely contained 0·5 ml 10 mM salicylic acid, water and variable amounts of centrifuged undiluted papaya juice or equivalent solutions of glucose and fructose to give a total volume of 1·0 ml. Following irradiation, 0·125 ml 10% trichloroacetic acid, 0·25 ml. 10% sodium tungstate and 0·25 ml freshly prepared 5% sodium nitrite were added. The mixture was vortexed and allowed to stand at room temperature for 20 min. One millilitre of 1·0M KOH was then added and, following mixing, the optical density determined at 510 nm. Identical but unirradiated solutions were used as 'blanks' in each spectroscopic measurement. A standard curve was constructed using 2,3-dihydroxybenzoic acid [Aldrich Chemical Co. No. 12620–9] without irradiation.

Malonaldehyde formation was measured by reaction with 2-thiobarbituric acid to form a pink-colored condensation product with an absorption maximum at 532 nm. (Gutteridge, 1981). One millilitre mixtures containing 0·2 ml, 0·2M phosphate buffer at various pH values, 0·1 ml papaya juice or an equivalent amount of glucose and fructose were irradiated as described above. One millilitre of 2-thiobarbituric acid, [Sigma Chemical Co T-5500] 1% (w/v) in 0·05M NaOH, was then added followed by 1 ml glacial acetic acid. The solutions were heated at 100°C for 25 min., cooled and the optical density determined at 532 nm. For each irradiated solution, an unirradiated but otherwise identical mixture was used as a 'blank' in each optical density measurement. Malonaldehyde bis(dimethyl acetal) [Aldrich Chemical Co. No. 10838–3] was used to construct a standard curve. The molar extinction coefficient at λ_{max}, 532 nm, was $1{\cdot}62 \times 10^5$. A similar value has been reported previously. (Aust, 1985).

RESULTS

When aqueous solutions of salicylic acid were exposed to ^{60}Co γ-rays, 2,3-dihydroxybenzoic acid was formed in amounts proportional to the radiation dose (Fig. 1). Five millimolar solutions of salicylic acid formed

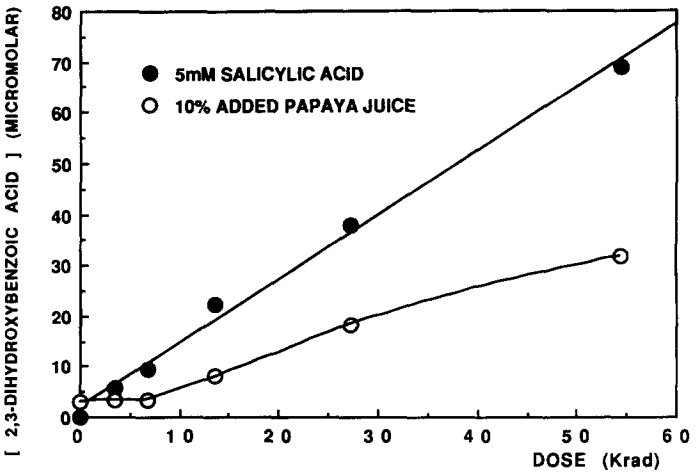

Fig. 1. Formation of 2,3-dihydroxybenzoic acid from salicylic acid by ^{60}Co γ-ray irradiation in the presence and absence of 10% papaya juice.

routinely $\sim 70\,\mu M$ 2,3-dihydroxybenzoic acid after exposure to 51 Krad γ-rays. Addition of papaya juice to the irradiation mixtures decreased the amount of 2,3-dihydroxybenzoic acid formed (Figs. 1, 3, 4). The optical absorption curves of salicylic acid solutions after irradiation and color development were identical to those obtained using unirradiated solutions of authentic 2,3-dihydroxybenzoic acid. Other isomers of dihydroxybenzoic acid gave different optical absorption curves, suggesting that the 2,3-dihydroxy isomer is the principal product formed by $HO^{\cdot}$ reaction with salicylic acid under the conditions of this experiment. The molar concentration of 2,3-dihydroxybenzoic acid was related to optical density at 510 nm, after color development, by the following expression; $(O.D._{510}) = 8{\cdot}5 \times 10^{-3} + 3{\cdot}2 \times 10^{3}$ [2,3-dihydroxybenzoic acid]. A similar result has been reported previously (Halliwell & Gutteridge, 1984).

The PAD electrochemical detector response was linear for glucose, fructose and sucrose concentrations up to a detector response of $\sim 4 \times 10^{7}$ area units. The molar response differed somewhat for each sugar as shown by the following; [Glc] = 0·05 (Area units $\times 10^{-7}$), [Fru] = 0·06 (Area units $\times 10^{-7}$), [Suc] = 0·08 (Area units $\times 10^{-7}$). The retention times for these sugars are, Glc, 2·85 min, Fru, 3·36 min, Suc, 4·55 min (all $\pm$0·05 min). A typical chromatogram of a papaya juice obtained from fruit with and without thermal inactivation of invertase prior to juice extraction, is shown in Fig. 2. Glucose, fructose and sucrose accounted for all the major chromatographic peaks observed in the papaya juice analyzed in this study. Table 1 summarizes the simple sugar content of this juice.

The quenching of $HO^{\cdot}$ by increments of unheated papaya juice or by

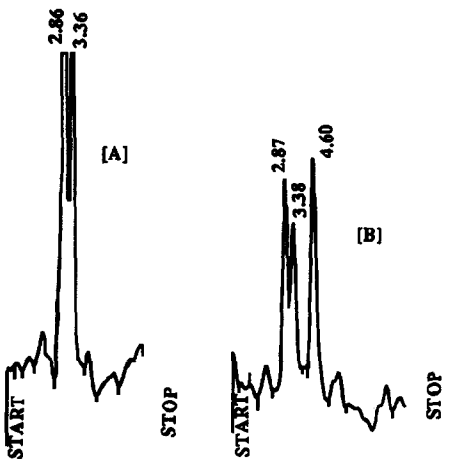

Fig. 2. HPLC trace of carbohydrates in papaya juice from heated and unheated fruit.

TABLE 1
Carbohydrate Analysis of Papaya Juice by Anion Exchange Chromatography and Pulsed Amperometric Detection

Juice preparation	*Glucose*[a]	*Fructose*[a]	*Sucrose*[a]	*Total*[a]
Without prior heat treatment (+ invertase)	0·325	0·424	0·0	0·749
With prior heat treatment (− invertase)	0·226	0·058	0·234	0·517

[a] Molar concentration.

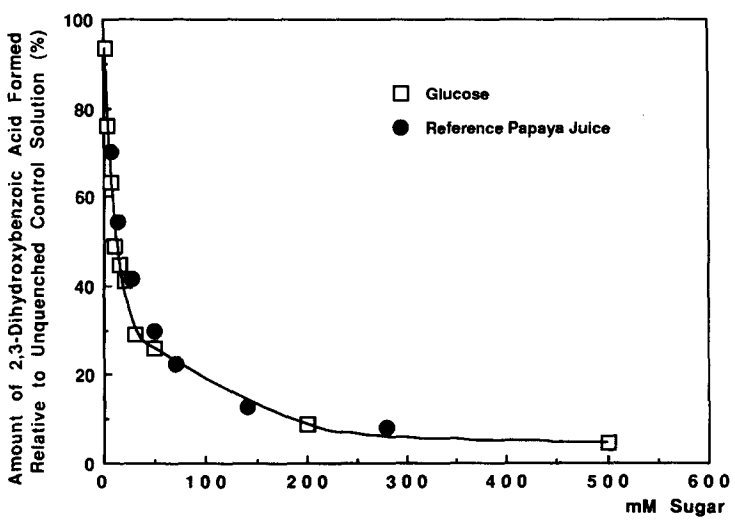

Fig. 3. Inhibition of 2,3-dihydroxybenzoic acid formation by graded amounts of glucose or papaya juice.

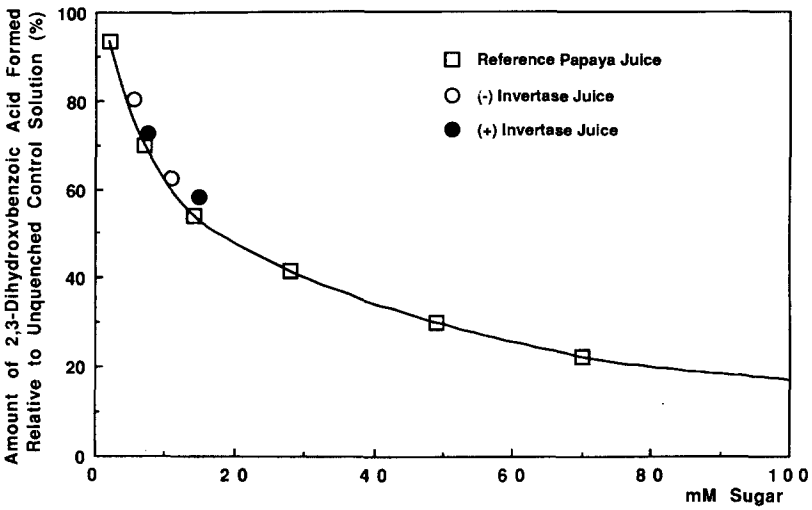

Fig. 4. Effect of thermal destruction of invertase on the quenching of $HO^{\cdot}$ by papaya juice.

glucose solutions is shown in Fig. 3. Papaya juice obtained from fruit which has been preheated to inactivate the enzyme invertase contains less sugar and has proportionally lower quenching activity than the unheated juice as shown in Fig. 4.

Malonaldehyde formation in γ irradiated solutions at different pH values of papaya juice and in solutions of glucose/fructose mixtures is shown in Fig. 5. Little malonaldehyde is formed below pH 9·5, and both mixtures

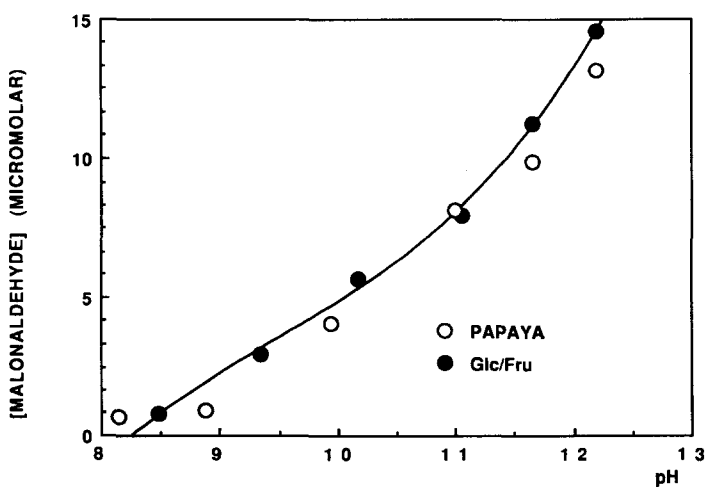

Fig. 5. Malonaldehyde formation at different pH values in irradiated solutions of papaya juice and in equimolar mixtures of glucose and fructose of equivalent sugar concentration.

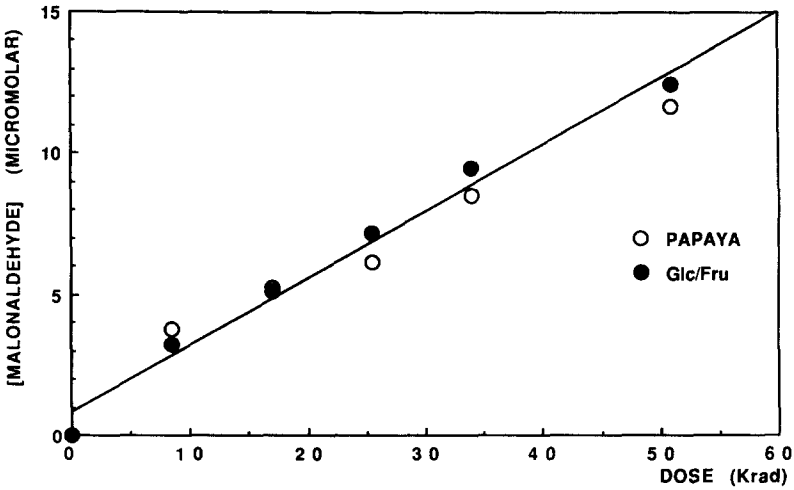

Fig. 6. Malonaldehyde formation (pH 12·2) at different γ-ray doses in irradiated solutions of papaya juice and in equimolar mixtures of glucose and fructose of equivalent sugar concentration.

show a linear increase with pH above this value. The irradiation dose response of malonaldehyde formation in these solutions at pH 12·2 is shown in Fig. 6. Both solutions show a linear increase with irradiation time (γ-ray dose) at this pH and the same malonaldehyde production at each dose.

DISCUSSION

Hydroxyl free radicals are highly reactive radicals, postulated to be one of the most important agents of irradiation induced cellular damage and mediators of oxygen toxicity in living systems. These radicals react 'on first contact' with most C—H bonds to abstract and combine with H$^{\cdot}$ to form water. The carbon atom is converted in this process to C$^{\cdot}$ which usually is a more stable, slower reacting radical. We believe that in papaya juice most of these intermediate carbon free radicals are glucose, fructose or sucrose free radicals. These sugar radicals may undergo several possible reactions to form stable non-radical compounds. They may: (1) Rearrange with the unpaired electron appearing at a different carbon than the one at which it was first formed. This process usually results in the formation of a carbonyl at one of the sugar carbons. (2) React with H$^{\cdot}$, present in the reaction mixture as a result of the primary radiolytic cleavage of water, changing C$^{\cdot}$ to C—H. (3) React by transfer of one unpaired electron from one C$^{\cdot}$ to the other C$^{\cdot}$ and form, by this disproportion reaction, C:$^{-}$ and C^{+}. The former reacts with

H^+ to form C—H, while the latter reacts with loss of H^+ to form C=C in the sugar molecule. (4) Undergo C—C bond cleavage, at high pH, promoted by ionization of the sugar OH groups adjacent to C$^\cdot$. This reaction results in malonaldehyde formation as well as the production of dihydroxyacetone, and possibly 2-keto glyceraldehyde. At pH values above ~9 this becomes an important reaction of the intermediate C$^\cdot$ formed by the HO$^\cdot$/sugar reaction. Since glucose/fructose mixtures of the same concentration as in the papaya juice form nearly identical amounts of malonaldehyde as does the juice with the same pH dependency (Figs 5 and 6), it seems likely that these sugars are the exclusive agents that react with HO$^\cdot$ in this juice.

We have also examined other fruit juices for their ability to quench HO$^\cdot$ and correlated this activity with their sugar content. No case has been found in which a juice had quenching activity greater than a glucose/fructose/sucrose solution whose concentrations were the same for these sugars as measured in the juice. In some juices, however, the quenching activity was less than of the corresponding sugar solution. The cause of this discrepancy is not known, but it is possible that these juices contain substances such as metal chelates that increase HO$^\cdot$ formation by their reaction with superoxide, the solvated electron, the hydrogen atom and hydrogen peroxide.

Sugar free radicals undergoing reactions 1 to 4 described above can form di and tri carbonyl derivatives, which together with malonaldehyde, glyoxal, formaldehyde and hydrogen peroxide are postulated to be responsible for the toxicity of irradiated sugar solutions. (Molin & Ehrehberg, 1964; Berry *et al.*, 1965; Schubert & Saunders, 1971). While solutions of these products inhibit the growth of mammalian cells in culture, they have also been reported to have anticancer, antiviral and antibacterial properties. (references in Schubert & Saunders 1971).

There is much interest in the development of post-irradiation detection markers which could be used as a measure of the past radiation exposure of a food. (Stevenson & Crone, 1990). The development of such a substance for fruit will probably be based on the identification of those products derived from HO$^\cdot$ reaction with the sugars.

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