ELSEVIER

Food Chemistry 84 (2004) 145–147

Food Chemistry

www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

On the nature of non-peroxide antibacterial activity in New Zealand manuka honey

M.J. Snow, M. Manley-Harris*

Chemistry Department, University of Waikato, Private Bag 3105, Hamilton, New Zealand

Received 5 December 2002; received in revised form 1 May 2003; accepted 1 May 2003

Abstract

Some conclusions, which exist in the literature about the nature of non-peroxide antibacterial activity in manuka honey, have been revisited. The stability of non-peroxide antibacterial activity in manuka honey at basic pH was investigated. At pH 11 antibacterial activity was immediately and irreversibly destroyed. This indicates that it is not possible to carry out chromatography of honey solutions at elevated pH with the intent to isolate the active fraction. The effect of 10-fold excess of catalase upon the antibacterial assay was examined. No statistical difference in the outcome was observed between the normal amount of catalase and the 10-fold excess. This indicates that non-peroxide antibacterial activity in manuka honey is not likely to be due to residual hydrogen peroxide.

Keywords: Manuka; Honey; Antibacterial activity; Non-peroxide

1. Introduction

Manuka honey is derived from the manuka tree (*Leptospermum scoparium*) a native of New Zealand. All honeys contain peroxide, which imbues them with antibacterial properties but certain manuka honeys exhibit antibacterial activity, which cannot be attributed to the peroxide present (Allen, Molan, & Reid, 1991; Molan & Russell, 1988) and which may constitute the major part of the total antibacterial activity. This non-peroxide activity is an extremely marketable property of manuka honey and samples, which have been certified by a microbial assay, command a premium price. Several studies have attempted to ascertain the nature of the component or components that give rise to non-peroxide antibacterial activity in manuka honey, none have been successful thus far. During the course of the aforementioned studies some conclusions have been drawn, which have been revisited as part of our own investigation into the nature of this activity.

Bogdanov (1997) attempted to determine the fraction of the honey that contained the non-peroxide activity. This was done by a process of elimination, in which fractions were removed and the activity of the residue tested. Extraction of acids at pH 11 on an anion exchange column resulted in loss of activity, even though pH was readjusted after chromatography, and Bogdanov therefore concluded that the activity resided in the acidic fraction of the honey.

Weston (2000) stated that hydrogen peroxide is the only antibacterial substance of any consequence in honey and that other substances such as propolis-derived phenolics are insignificant in comparison to hydrogen peroxide. He also stated that the level of hydrogen peroxide is essentially determined by the amount of plant derived catalase in a honey, and that, based on the findings of White, Subers, and Schepartz (1963) and Dustmann (1971), hydrogen peroxide is generated by glucose oxidase in samples of honey or fractions thereof as they are diluted and prepared for antibacterial assays and thus that the amount of catalase added to these samples in present methods (Allen, Molan, & Reid, 1991; Molan & Russell, 1988) is insufficient to destroy all of the hydrogen peroxide produced in this way.

This paper reports the outcome of an investigation of the stability of non-peroxide activity in manuka honey at alkaline pH and an investigation of the affect of varying the amount of catalase used in the antibacterial assay.

* Corresponding author. Fax: +64-7-838-4219.
E-mail address: manleyha@waikato.ac.nz (M. Manley-Harris).

0308-8146/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0308-8146(03)00258-9

2. Experimental

2.1. Materials

The following materials were obtained from the companies indicated: Nutrient Agar (Becton Dickinson), Trypticase soy broth (Difco), Catalase (Sigma lot 128H7252, 1850 units/mg, 1 unit decomposes 1 mmol H2 O2 /min, pH 7, 25 °C), *Staphylococcus aureus* culture (Environmental Science & Research Ltd., Porirua, N.Z.), phenol (BDH). Active Manuka honey #1 was a standard sample donated by the Waikato Honey Research unit, Active Manuka Honeys #s 2–4 were kindly donated by Comvita Ltd, N.Z. from their UMF10 product line.

2.2. Assay of antibacterial activity

Staphylococcus aureus was cultured by placing a bead culture in trypticase soy (Ts) broth and incubating overnight at 37 °C. Agar was prepared, by dissolving nutrient agar powder (23 g) in distilled water (1 l), and divided into 250 ml conical flasks each containing 150 ml, which were sterilised by autoclaving at 121 °C for 20 min. Cultured *Staphylococcus aureus* (100 μl)(OD540 0.5) was added to the warm nutrient agar, which was poured into plates that were allowed to set in the refrigerator for at least 6 h. Wells were punched in the set agar with an agar punch in a regular grid pattern.

Honey samples were tested at a concentration of 25% for antibacterial activity. Catalase solution was made by dissolving catalase (0.02 g) in distilled water (10 ml). The honey (2.00 g) was dissolved in distilled water (2.00 ml) and divided into two new vials containing 1.00 ml of sample each. Then either 1.00 ml of distilled water (giving total activity) or catalase solution (giving non-peroxide activity) was added to the two sample vials.

Three replicates of phenol standards ranging from 2 to 6% and three to five replicates of the samples being tested were introduced into recorded random wells in the agar plate. The plate was incubated at 37 °C over night allowing the bacteria to grow where possible. After incubation digital calipers were used to measure the diameter of the area of inhibition around the wells. A standard curve was produced by plotting the square of the diameter against the concentration of the phenol standards. The relative activities of the honey samples were obtained as ''phenol equivalents'' by taking the square of the diameter of the area of inhibition and comparing it to the phenol standard curve.

2.3. Stability to alkaline conditions

Honey solutions were made alkaline by addition of NaOH solution (0.1 M) and after being held at the requisite pH were titrated back to pH 7 using HCl (0.1 M) before assay.

3. Results and discussion

To ascertain the precision of the antibacterial assay a standard sample was tested in all 32 wells of each of five different plates. The results are shown in Fig. 1. There is a very narrow 95% confidence interval and the results for all five plates overlap within the 95% confidence interval, which indicates that there was little variation within and between plates. When the data for all the plates was combined, the total and non-peroxide activity were indistinguishable at the 99.9% confidence level, Fig. 2, which shows that for this particular honey sample all activity can be attributed to non-peroxide activity.

Exposure to alkaline pH had a very marked affect upon both non-peroxide and total activity, Fig. 3. Honey solutions in deionized water are intrinsically

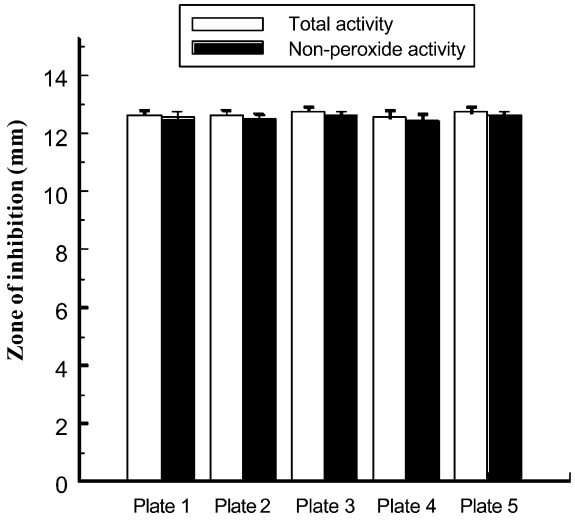

Fig. 1. Average zone of inhibition of antibacterial assay with error bars indicating the 95% confidence limit.

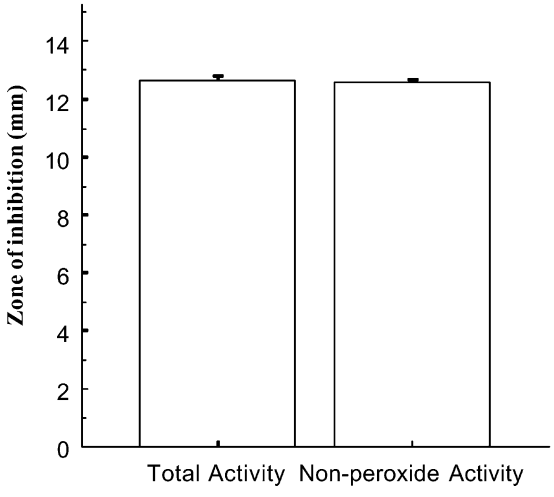

Fig. 2. Pooled results for five plates in the antibacterial assay with error bars indicating the 99.9% confidence limit.

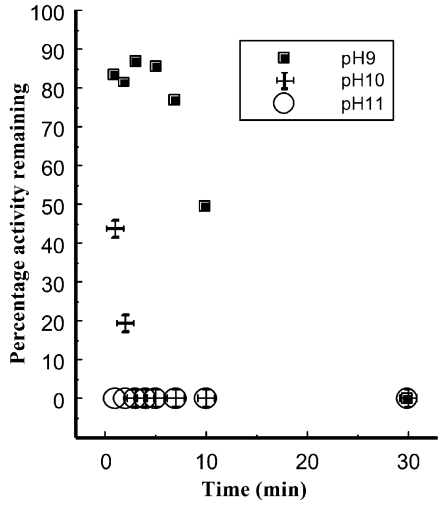

Fig. 3. Effect of pH upon non-peroxide activity. For clarity the results for only one honey are shown.

Table 1
Square of zone of inhibition (mm^2) obtained with normal and 10-fold excess of catalase used in antibacterial assay of non-peroxide activity

Replicate	Normal assay	Tenfold excess of catalase	
		(1)	(2)
1	254.40	219.04	245.55
2	227.71	234.40	259.53
3	228.92	228.01	227.71
Mean	237.01	227.15	244.26
Standard deviation	15.08	7.71	15.95

acidic and have a pH reading of 3–4. These solutions retained their activity (100%) over a period of 30 min. Similarly solutions, in which the pH was adjusted to 7, retained activity. At pH 9 the activity remained relatively stable over 5 min then gradually declined until 30 min. At pH 10 the decline was more rapid and all activity was gone at 3 min.

At pH 11 immediate (within 1 min) loss of all activity occurred. It should be noted that the loss of activity is irreversible since the solutions were titrated back to neutral pH before assay. These results call into question the possibility of liquid chromatography at high pH. If all or even a large proportion of the activity is lost by simply holding the solution at high pH, it would seem inappropriate to conclude that the disappearence of activity was due to its failure to elute from the column (Bogdanov, 1997).

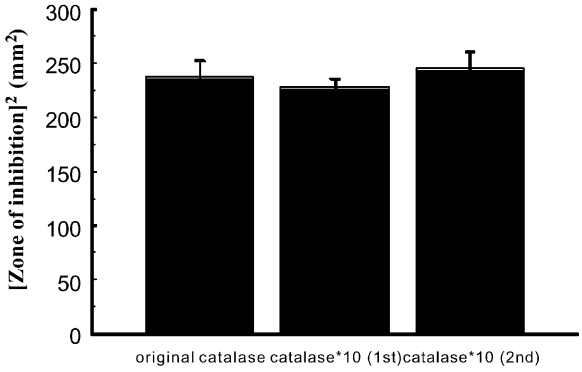

Fig. 4. Effect upon the antibacterial assay of changing the catalase concentration with error bars indicating the standard deviation.

To test the hypothesis that non-peroxide activity is due to excess peroxide that has not been destroyed by the catalase, antibacterial assays were performed using a 10-fold excess of catalase. The results are shown in Table 1 and illustrated in Fig. 4.

Analysis of the data in Table 1 shows that there is no significant difference at the 95% confidence level (when comparing the difference between the means and the pooled random error) between the assay carried out using the normal amount of catalase and the assay carried out using a 10-fold excess of catalase. If the outcome of the antibacterial assay for non-peroxide activity were due to the unreacted peroxide (Weston, 2000), it seems unlikely that addition of a large excess of catalase would not affect the result. We therefore conclude that the usual amount of catalase is sufficient for the assay and that the activity, which is manifested after the addition of catalase, is genuinely non-peroxide activity.

References

Allen, K. L., Molan, P. C., & Reid, G. M. (1991). A survey of the antibacterial activity of some New Zealand honeys. *Journal of Pharmacy and Pharmacology*, *43*, 817–822.

Bogdanov, S. (1997). Nature and origin of the antibacterial substances in honey. *Lebensm-Wiss. U.-Technology*, *30*(1), 748–753.

Dustmann, J. H. (1971). Catalase activity in honey of heather plant (Ericaceae) flow. *Z. Lebensm.-Unters.-Forsch*, *145*(5), 294–295.

Molan, P. C., & Russell, K. M. (1988). Non-peroxide antibacterial activity in some New Zealand honeys. *J. Apic. Res.*, *27*(1), 62–67.

Weston, R. J. (2000). The contribution of catalase and other natural products to the antibacterial activity of honey: a review. *Food Chemistry*, *71*, 235–239.

White, J. W. Jr., Subers, M. H., & Schepartz, A. I. (1963). The identification of inhibine, the antibacterial factor in honey, as hydrogen peroxide and its origin in a honey glucose–oxidase system. *Biochem. Biophys. Acta*, *73*, 57–70.