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# Methylglyoxal May Affect Hydrogen Peroxide Accumulation in Manuka Honey Through the Inhibition of Glucose Oxidase

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ABSTRACT Although hydrogen peroxide ($H_2O_2$) is one of the major antibacterial factors in most honeys, it does not accumulate in medical-grade manuka honey. The goal of this study was to investigate the effect of artificially added methylglyoxal (MGO) on $H_2O_2$ accumulation in natural non-manuka honeys. $H_2O_2$ concentrations in the honey solutions were determined using a fluorimetric assay. Two, the most potent $H_2O_2$ producers honeydew honeys were mixed with MGO at final concentrations of 250, 500, and 1000 mg/kg, and incubated for 4 days at 37°C. Subsequently, $H_2O_2$ concentrations were determined in 50% (wt/vol) MGO supplemented honey solutions. *In vitro* crosslinking of the enzyme glucose oxidase (GOX) after incubation with MGO was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Tested honeys at a concentration of 50% (wt/vol) accumulated up to $495.8 \pm 9.1$ $\mu$M $H_2O_2$ in 24 h. The most potent producers were the two honeydew honeys, whose 50% solutions accumulated $306.9 \pm 6.8$ and $495.8 \pm 9.1$ $\mu$M $H_2O_2$, respectively. Levels of $H_2O_2$ increased significantly over time in both honey solutions. Contrary to this, the MGO-treated honeys generated significantly lower amounts of $H_2O_2$ ($P < .001$), and this reduction was dose dependent. In addition, MGO-treated GOX formed high molecular weight adducts with increasing time of incubation accompanied by loss of its enzymatic activity. High levels of MGO in manuka honey, by modifying the enzyme GOX, might be responsible for suppressing $H_2O_2$ generation. These data highlight the detrimental effect of MGO on significant proteinaceous components of manuka honey.

**KEY WORDS:** • *honey* • *hydrogen peroxide* • *methylglyoxal*

Currently, honey is considered to be a therapeutic agent, and its successful application in the treatment of chronic wounds and burns has promoted further research into its antibacterial properties. The antibacterial activity of honey has been extensively studied and several honey antibacterial compounds, including hydrogen peroxide ($H_2O_2$), methylglyoxal (MGO), and bee defensin 1, have been identified.[1–3] $H_2O_2$ is one of the major antibacterial components,[4,5] and is produced by glucose oxidase (GOX)-mediated conversion of glucose under aerobic conditions in diluted honey. $H_2O_2$ accumulation is highest in the range of 30–50% honey.[6] Bee-derived GOX is found as a regular but quantitatively variable component of honeys,[7] and consequently, $H_2O_2$ levels may also differ from honey to honey.[8]

Interestingly, some honeys, such as manuka honey derived from the nectar of New Zealand manuka trees (*Leptospermum scoparium*) do not accumulate detectable levels of $H_2O_2$ upon dilution.[9] It has been documented that the pronounced antibacterial activity (also referred to as ''non-peroxide'' activity) of manuka honey directly originates from MGO.[2] The reason why $H_2O_2$ is not generated in these honeys has never been elucidated biochemically.

Several factors affecting $H_2O_2$ accumulation in honey have been identified to date: (1) inactivation of the enzyme GOX by exposure to heat, (2) degradation of $H_2O_2$ by a catalase enzyme of pollen origin, or (3) low levels of GOX in the honey. Nevertheless, honeys with non-peroxide antimicrobial activity are more closely associated with floral sources, being generally derived from *Leptospermum* species. Therefore, other factor(s) in manuka honey may need to be considered responsible for the absence of $H_2O_2$ accumulation.

Based on current knowledge about the destructive effects of MGO on some proteins in manuka honey,[10] we hypothesize that the inability to produce high levels of $H_2O_2$ in manuka honey could be associated with high reactivity of MGO with the GOX enzyme. Accordingly, we investigated the effect of artificially added MGO on $H_2O_2$ accumulation in natural non-manuka honeys, which are capable of generating high levels of $H_2O_2$. Furthermore, we examined the effect of MGO on *in vitro* crosslinking of GOX and its biological activity.

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Table 1. Hydrogen Peroxide Concentrations in Different Honey Solutions After 24-h Incubation at 37°C

| *Honey sample* | *Principal botanical origin* | *Sample age (year)* | *Geographic origin* | *Hydrogen peroxide concentration (μM)* |
|---|---|---|---|---|
| Honeydew 1 | *Abies alba* Mill | 2 | Bardejov (Slovakia) | 495.8 ± 9.1 |
| Honeydew 2 | *A. alba* Mill | 2 | Banska Stiavnica (Slovakia) | 306.9 ± 6.8 |
| Acacia | *Robinia pseudoacacia* | 1 | Šahy (Slovakia) | 36.3 ± 0.7 |
| Manuka | *Leptospermum* spp. | 3 | New Zealand | 78.9 ± 1.9 |
| Artificial | N/A | 1 | N/A | 16.1 ± 1.2 |

N/A, not applicable.

Honey samples ($n = 3$) were obtained from several regions in Slovakia (Table 1). Commercially available manuka honey with unique manuka factor 15 (UMF 15+), containing around 250 mg/kg MGO, imported from New Zealand, was purchased from Nature's Nectar. Artificial honey was prepared as described elsewhere.[11]

The honeys with the highest levels of $H_2O_2$ were selected for incubation with MGO (Sigma-Aldrich). Two undiluted honeydew honeys were mixed with MGO at final concentrations of 250, 500, and 1000 mg/kg, and incubated for 4 days at 37°C. Subsequently, 50% (w/v) solutions were prepared from MGO-supplemented honeys, and incubated at 37°C for periods of 2, 5, and 24 h.

Each honey sample (0.5 g) was dissolved with distilled water to a final volume of 1 mL until completely fluid. The 50% (w/v) liquid solutions obtained were filtered through a 0.22-$\mu$m PES filter (Millipore) and incubated at 37°C for 24 h. $H_2O_2$ concentrations in the honey solutions after each indicated time of incubation were determined using a $H_2O_2$/peroxidase fluorimetric kit (Cell Biolabs, Inc.) according to the manufacturer's instructions. The fluorescence of the formed product, resorufin, was measured at an excitation wavelength of 530 nm using a 590 nm emission line with a Synergy HT (BioTek Instruments) microplate reader. Each honey sample and standard was tested in triplicate. The results are presented as mean ± SD. The data were statistically analyzed using an unpaired Student's *t*-test; *P*-values lower than .05 were considered significant.

GOX type II from *Aspergillus niger* was purchased from Sigma-Aldrich. In case of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analyses, *in vitro* crosslinking of GOX enzyme was performed at 37°C with increasing time; 2, 24, and 48 h. GOX was dissolved in ultrapure water to a final concentration of 1 mg/mL and was incubated with or without MGO at concentrations corresponding to those found in manuka honey (5, 10, and 15 mM). After treatment, the mixture aliquots (15 $\mu$L) were fractionated on 8% SDS-PAGE gels using a Mini-Protean II electrophoresis cell (Bio-Rad). The proteins were stained with SERVA Blue G (Serva) for 60 min and then destained overnight with tap water.

For determination of enzymatic activity, GOX was dissolved in phosphate-buffered saline (PBS) (pH 7.2) to a final concentration of 0.1 mg/mL, and supplemented with 1, 2.5, and 5 mM MGO. Samples were incubated at 37°C for 7 days. To exclude the possibility of MGO interference with fluorimetric assay, samples containing same amounts of MGO, but lacking GOX, were prepared and incubated under given conditions.

Activity of GOX was examined upon $H_2O_2$ development. The reactions were performed according to Graf and Peniston[12] with several modifications. Briefly, 29.85 and

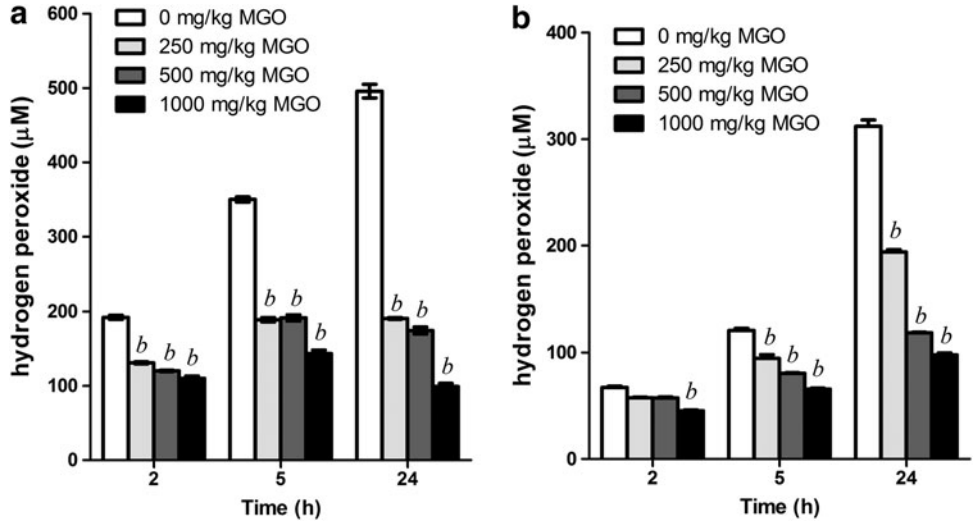


**FIG. 1.** Hydrogen peroxide ($H_2O_2$) concentrations in honeydew honey solutions (50% w/v) treated with methylglyoxal (MGO) at different concentrations after 24 h of incubation at 37°C. **(a)** Honeydew honey (Bardejov); **(b)** honeydew honey (Banska Stiavnica). $^bP < .001$ versus control (0 mg/kg MGO).

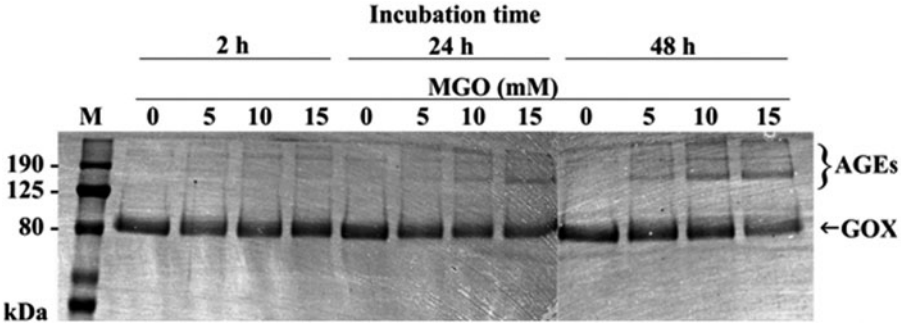


**FIG. 2.** *In vitro* crosslinking of glucose oxidase (GOX) by MGO. GOX at concentration of 1 mg/mL was incubated with or without MGO at concentrations of 5, 10, and 15 mM for an indicated time period. After treatment, the mixture aliquots (15 $\mu$L) were fractioned on 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels. The gels were stained with Serva Blue G. High molecular weight GOX adducts (AGEs) were detected after 48 h of incubation with MGO. M, molecular weight marker; AGEs, advanced glycation end products.

59.7 $\mu$g of glucose was mixed with 1.88 $\mu$g of GOX in 1 mL containing 1 × PBS (pH 7.2) and incubated for 90 min at 37°C. The level of $H_2O_2$ was subsequently evaluated using the $H_2O_2$/peroxidase fluorimetric kit as described above except that samples and standards were tested in duplicate. In case of controls containing no GOX, the analyzed volume corresponded to the samples with 0.1 mg/mL of enzyme.

In this work, we present data showing that artificially added MGO affected $H_2O_2$ generation in natural honeys with high levels of $H_2O_2$. We documented that observed inhibition of $H_2O_2$ production is due to structural changes of MGO-treated GOX.

We used four natural honeys for monitoring of $H_2O_2$ generation after dilution. Tested honeys at a concentration of 50% (w/v) accumulated up to 495.8 ± 9.1 $\mu$M $H_2O_2$ in 24 h. The most potent producers were the two honeydew honeys, whose 50% solutions accumulated 306.9 ± 6.8 and 495.8 ± 9.1 $\mu$M $H_2O_2$, respectively. Significantly lower levels of $H_2O_2$ were found in manuka and acacia honey (Table 1). In our very recent study, we documented that high concentrations of MGO present in manuka honeys modify defensin 1, an antibacterial bee-derived peptide and also the dominant protein in honey—MRJP1.[10] Due to these MGO-induced structural modifications, defensin 1 lost its antibacterial activity. To evaluate whether MGO has a detrimental effect on the function of GOX, we incubated honeydew honeys with MGO at different concentrations commonly found in manuka honeys. As seen in Figure 1, levels of $H_2O_2$ increased significantly over time in both honey solutions. Contrary to this, the MGO-treated honeys generated significantly lower amounts of $H_2O_2$, and this reduction was dose dependent. Increasing concentrations of MGO in artificial honey did not affect the $H_2O_2$ assay (data not shown). After 24 h of incubation, the untreated honeydew honeys contained three- and fivefold higher concentrations of $H_2O_2$ than honeys treated with 1000 mg/kg MGO.

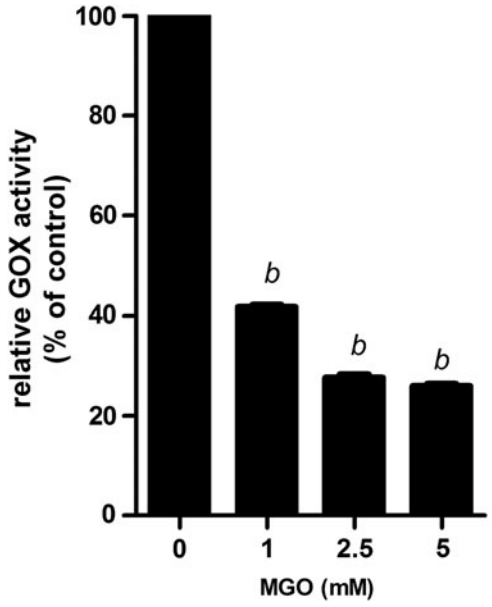


**FIG. 3.** Effect of MGO on enzymatic activity of GOX. GOX (0.1 mg/mL) was incubated with 1, 2.5, and 5 mM MGO at 37°C for 7 days. Activity of GOX was examined upon $H_2O_2$ development using the $H_2O_2$/peroxidase fluorimetric assay. Results were expressed as the percentage of relative GOX activity. $^bP < .001$ versus control (0 mM MGO).

To elucidate observed reduction of $H_2O_2$ levels in MGO-treated honeys, we incubated enzyme GOX with different concentrations of MGO for increasing amounts of time. We found that GOX not exposed to MGO migrated as a single band (~80 kDa), while MGO-treated GOX after 48 h formed high molecular weight adducts—advanced glycation end products (AGEs) (Fig. 2). Similarly, GOX exposed to the sugars (glucose, fructose, and ribose) exhibited a clear alteration in electrophoretic behavior and formed products that failed to enter the gel.[13] In addition to MGO-induced structural changes of GOX, we determined the catalytic activity of GOX after incubation with/without MGO, which was examined by $H_2O_2$ development. A 58% and 70% reduction of enzyme activity was observed after incubation of GOX with 1 and 5 mM MGO, respectively (Fig. 3).

It has recently been found that MGO at concentration of 10 mM caused significant inhibition of antioxidant enzymes possible due to modification of protein molecules.[14] On the other hand, incubation of human catalase with 10 mM MGO did not affect the enzyme activity.

Catalase is a variable natural component of honey.[15] The enzyme that originates in the pollen, has a destroying effect on $H_2O_2$. Therefore, catalase may also take part in the elimination of $H_2O_2$ in manuka honey, but the potential

negative effect of MGO on catalase in manuka honey remains to be investigated.

In conclusion, our data, along with those from our previous study, suggest that MGO induces modification of significant proteinaceous components of honey, including those contributing to its antibacterial activity.

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## AUTHOR DISCLOSURE STATEMENT

The authors declare that there are no conflicts of interest.

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