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ORIGINAL PAPER

Honeybee glucose oxidase—its expression in honeybee workers and comparative analyses of its content and H_2O_2-mediated antibacterial activity in natural honeys

Marcela Bucekova · Ivana Valachova · Lenka Kohutova · Emanuel Prochazka · Jaroslav Klaudiny · Juraj Majtan

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Abstract Antibacterial properties of honey largely depend on the accumulation of hydrogen peroxide (H_2O_2), which is generated by glucose oxidase (GOX)-mediated conversion of glucose in diluted honey. However, honeys exhibit considerable variation in their antibacterial activity. Therefore, the aim of the study was to identify the mechanism behind the variation in this activity and in the H_2O_2 content in honeys associated with the role of GOX in this process. Immunoblots and in situ hybridization analyses demonstrated that *gox* is solely expressed in the hypopharyngeal glands of worker bees performing various tasks and not in other glands or tissues. Real-time PCR with reference genes selected for worker heads shows that the *gox* expression progressively increases with ageing of the youngest bees and nurses and reached the highest values in processor bees. Immunoblot analysis of honey samples revealed that GOX is a regular honey component but its content significantly varied among honeys. Neither botanical source nor geographical origin of honeys affected the level of GOX suggesting that some other factors such as honeybee nutrition and/or genetic/epigenetic factors may take part in the observed variation. A strong correlation was found between the content of GOX and the level of generated H_2O_2 in honeys except honeydew honeys. Total antibacterial activity of most honey samples against *Pseudomonas aeruginosa* isolate significantly correlated with the H_2O_2 content. These results demonstrate that the level of GOX can significantly affect the total antibacterial activity of honey. They also support an idea that breeding of novel honeybee lines expressing higher amounts of GOX could help to increase the antibacterial efficacy of the hypopharyngeal gland secretion that could have positive influence on a resistance of colonies against bacterial pathogens.

Keywords Glucose oxidase · Honey · Antibacterial activity · Hydrogen peroxide · Gene expression · *Apis mellifera*

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M. Bucekova
Faculty of Natural Sciences, Comenius University, Mlynska Dolina, 842 15 Bratislava, Slovakia

M. Bucekova · I. Valachova · E. Prochazka · J. Majtan
Institute of Zoology, Slovak Academy of Sciences, Dubravska cesta 9, 845 06 Bratislava, Slovakia

L. Kohutova · J. Klaudiny
Institute of Chemistry, Slovak Academy of Sciences, Dubravska cesta 9, 845 38 Bratislava, Slovakia

J. Majtan (✉)
Department of Microbiology, Faculty of Medicine, Slovak Medical University, Limbova 13, 833 03 Bratislava, Slovakia
e-mail: juraj.majtan@savba.sk

Introduction

The vast majority of the population of a honeybee hive consists of workers that perform most tasks in the colony. A particular task is usually performed by workers of a certain age: cell cleaning and capping by the youngest bees, nursing (brood and queen care/feeding) by 4–16-day-old bees, food handling and comb building by middle-aged bees and ventilating, guarding and foraging by older bees between 15 and 30 days (Winston 1987). One of the important tasks is foraging of nectar from flowering plants by forager bees and its transfer to nestmates at the colony. The nectar can be directly used for feeding purposes or processed by regurgitation and passing among processor bees (to mix it with secreted enzymes) until it is placed in comb cells. Here, after final evaporation of water, it is processed into honey that represents a food storage possessing besides nutritional also antibacterial properties (De Grandi-Hoffman and Hagler 2000; Seeley 1992).

The antibacterial activity of honey has been extensively studied, and several honey antibacterial compounds have been identified. Hydrogen peroxide (H_2O_2) is one of the major antibacterial components and is produced by glucose oxidase (GOX)-mediated conversion of glucose to gluconic acid under aerobic conditions in diluted honey (White et al. 1963). Although H_2O_2 at concentrations found in honey does not kill bacteria, it is able to interact with bacterial cell proliferative signals, and thus, it affects bacterial growth even at high dilutions of the honey (Brudzynski 2006). Moreover, it has recently been demonstrated that honey H_2O_2 exerts DNA-degrading activities to bacterial cells (Brudzynski et al. 2011).

GOX (FAD oxidoreductase, EC 1.1.3.4) is an 85-kDa enzyme that is expressed in hypopharyngeal glands of the processor and forager bee and secreted into the nectar during the preparation of honey. It was not detected in hypopharyngeal glands of the nurse bee (Ohashi et al. 1999). On the contrary, recent studies (Li et al. 2008; Santos et al. 2005) showed that GOX is also expressed in the hypopharyngeal glands of the nurse and winter worker bees. Thus, GOX is secreted into the nectar and obviously also larval food where it acts as one means of sterilizing the food. This way, GOX may provide immunological protection at the colony level and prevent some larval diseases (Yang and Cox-Foster 2005). The level of GOX in honey could be one of the factors participating in prevention of bacterial and fungal infections in colonies as well as a mean/marker in selection of honeys with high antibacterial activities.

The levels of H_2O_2 in honey may differ from honey to honey without relation to its botanical and geographical origin (Brudzynski et al. 2011). The following factors have been proposed to affect the total concentration of H_2O_2 in honey: (i) inactivation of GOX due to exposure to excess heat or light (White et al. 1963), (ii) chemical scavenging of H_2O_2 (White et al. 1963), (iii) destruction of H_2O_2 by catalase occurring in nectar and pollen (Brudzynski 2006), (iv) modification of GOX by methylglyoxal (Majtan et al. 2014) and (v) autooxidation of polyphenols/flavonoids (Brudzynski 2006). Another plausible explanation for the variation in H_2O_2 content in honey could be differences either in levels of bee-derived GOX or GOX enzymatic activity that could be significantly affected by compounds present in honey or honeybee protein diets (Alaux et al. 2010).

The aims of this study were to (i) evaluate simultaneously the content of bee GOX and H_2O_2 in honey samples of different botanical and geographical origins; (ii) localize the *gox* expression in honeybee workers performing different tasks: cleaner, nurse, processor and forager bee; (iii) examine *gox* expression in workers during their behavioural development; and (iv) characterize the antibacterial activity of honey samples against Gram-negative bacteria (*Pseudomonas aeruginosa*).

Materials and methods

Honeybee samples

Apis mellifera carnica workers of particular ages (2–30 days) for qPCR were obtained from two naturally mated queen colonies in the apiary of the Institute of Apiculture in Liptovský Hrádok, Slovakia, in July of 2012. Experimental bees were 11–19 h old when were colour-marked on their thorax to denote their age.

For in situ hybridization, *A. mellifera carnica* cleaner, nurse, processor and forager bees were obtained from naturally mated queen colonies in the apiary of Mr. Dedinsky (Bratislava, Slovakia) between June and September of 2013.

Honey samples

Honey samples (n=20) were received from beekeepers throughout several regions of Slovakia between May and August of 2013 (Table 1). Honey samples were stored in glass or plastic containers at room temperature in the dark. Identification of the floral source of the honey was performed by the beekeepers based on the availability of flora for nectar foraging, location of the apiary and organoleptic characteristics of the honey.

Microorganisms

The antibacterial activity of honey samples was assessed against isolate of *P. aeruginosa*. Bacterial clinical isolate from non-healing wound was collected from the Department of Clinical Microbiology of the Hospital Čadca (Čadca, Slovakia) and transported to the Department of Medical Microbiology, Slovak Medical University (Bratislava, Slovakia).

The reason to use Gram-negative bacteria for testing antibacterial activity of honeys is based on fact that defensin-1, a bee-derived antibacterial peptide in honey, is effective solely against Gram-positive bacteria. Thus, we can evaluate the antibacterial activity of generated H_2O_2 in honey samples without contribution of defensin-1.

Ultrafiltration of honey samples

Each honey sample (2.5 g) was dissolved with deionized water to a final volume of 5 ml until completely fluid. The liquid solution obtained was filtered through a 0.22-μm PES filter (Millipore, MA, USA) and then concentrated by centrifugation at 5,000g at room temperature in a Vivaspin 6 concentrator tube (Sartorius, Germany), with an exclusion limit of 10 kDa, to a final volume of 1 ml. The Vivaspin retentate was subsequently stored at −20 °C.

Table 1 Honey samples analyzed in the study

Honey sample	Floral source	Geographic origin in Slovakia	Time of harvesting
1	*Robinia pseudoacacia*	Veľký Krtíš	June
2	*Robinia pseudoacacia*	Lamač	June
3	*Robinia pseudoacacia*	Lamač	June
4	*Robinia pseudoacacia*	Borský Mikuláš	June
5	*Robinia pseudoacacia*	Borský Mikuláš	June
6	*Robinia pseudoacacia*	Borský Mikuláš	June
7	*Tilia platyphyllos*	Jarovce	June
8	*Tilia platyphyllos*	Slovenská Lupča	June
9	*Castanea sativa*	Lamač	June
10	*Helianthus annuus*	Jarovce	June
11	*Brassica napus*	Jarovce	June
12	*Abies alba* (honeydew)	Slovenská Lupča	August
13	*Abies alba* (honeydew)	Čergov	August
14	Mixed flora	Čergov	July
15	Mixed flora	Podpolanie	May
16	Mixed flora	Vysoká nad Uhom	June
17	Mixed flora	Vysoká nad Uhom	July
18	Mixed flora	Vysoká nad Uhom	August
19	Mixed flora	Inovec	May
20	Mixed flora	Inovec	August

Anti-honeybee GOX polyclonal antibody

An affinity-purified polyclonal anti-honeybee GOX antibody was purchased from GenCust Europe (Dudelnag, Luxembourg). Two New Zealand rabbits immunized with a synthetic peptide corresponding to the C terminus of bee GOX (CDEFVEDSDDYWN) provided antibodies that were then affinity-purified using this peptide.

Detection of GOX in honeys and honeybee gland extracts by immunoblotting

The retentates of the honey samples from ultrafiltration and gland extracts of unspecified hive bees (5 glands/200 μl of PBS) were electrophoresed on SDS-PAGE gels using a Mini-Protean II electrophoresis cell (Bio-Rad, CA, USA). The proteins were transferred onto a 0.22-μm nitrocellulose Advantec® membrane (Sigma-Aldrich, UK) in 48 mM Tris, 39 mM glycin and 20 % methanol using the semi-dry blotting procedure. The membrane was blocked for 1 h in a TBST buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 0.05 % Tween 20) containing 5 % non-fat dried milk and then incubated overnight with a rabbit polyclonal antibody against honeybee GOX diluted 1:2,000 in TBST-blocking buffer. After washing with TBST, the membrane was incubated for 2 h in blocking buffer containing goat anti-rabbit HRP-linked antibodies (Promega, WI, USA) diluted 1:2,500. Immunoreactive bands were detected in solution containing dissolved SigmaFast 3,3-diaminobenzidine tablets (Sigma-Aldrich, UK). In the case of honey samples, the signal intensity of GOX bands was quantified by densitometry (Quantity One, Bio-Rad, USA) and expressed as the relative volume intensity (intensity*mm^2).

Determination of antibacterial activity

The antibacterial efficacy of various honey samples analyzed was evaluated by the minimum inhibitory concentration (MIC) assay. Briefly, one bacterial colony was suspended in PBS buffer, pH 7.2, and the turbidity of the suspension was adjusted to 10^8 colony forming units (CFU)/ml and diluted with Mueller-Hinton broth (MHB) medium to a final concentration of 10^6 CFU/ml. Ten-microlitre aliquots of suspension were inoculated into each well of sterile 96-well polystyrene plates (Sarstedt, Germany). The final volume in each well was 100 μl, consisting of 90 μl of sterile medium or diluted honey and 10 μl of bacterial suspension. After 18 h of incubation at 37 °C, bacterial growth inhibition was determined by monitoring the optical density at 490 nm. The MIC was defined as the lowest concentration of honey inhibiting bacterial growth. All tests were performed in triplicate and were repeated three times.

A serial twofold dilutions of each honey were prepared from 40 to 50 % (*w*/*v*) honey solution, resulting in a final concentrations of 50, 40, 25, 20, 12.5, 10, 6.25, 5, 3.12, 2.5 and 1.25 %.

Determination of H_2O_2 concentration in honey

The maximum levels of accumulated H_2O_2 that occurred in honey solutions were found in solutions diluted to concentrations between 30 and 50 % (Bang et al. 2003). Therefore, the levels of H_2O_2 were determined in 40 % honey solutions. Each honey sample (0.8 g) was dissolved with 1.2 g of distilled water until completely fluid. The 40 % (*w*/*w*) liquid solutions obtained were incubated at 37 °C for 4 h. Hydrogen peroxide concentrations in the honey solutions after each indicated time of incubation were determined using a hydrogen peroxide/peroxidase fluorimetric kit (Cell Biolabs Inc, CA, USA) 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 microplate reader (BioTek Instruments, VT, USA). Each honey sample and standard was tested in duplicate.

In order to prove that GOX, a heat-labile enzyme, is responsible for H_2O_2 generation, 40 % honey solutions were heat-treated at 95 °C for 5 min and incubated at 37 °C for 4 h. Hydrogen peroxide concentrations in heat-treated honey samples were determined as described above. Changes in H_2O_2 concentrations in untreated and heat-treated honey samples were expressed as a percentage in reduction of H_2O_2 levels.

Comparison of gox transcript levels in heads of workers by qPCR

RNA isolation and cDNA synthesis

Total RNA and complementary DNA (cDNA) from pooled head samples were prepared as previously described (Kohutova et al. 2013). Briefly, each sample was prepared from the heads of four workers of a particular age. RNA was isolated by guanidine thiocyanate-acid phenol extraction followed by spin column purification. Three sets of samples from bees in the age range of 2–30 days were prepared from a colony. First-strand cDNA was synthesized from 1.6 μg of total RNA in a 20-μl reaction using oligo $(dT)_{18}$ primer.

Reference gene selection

Reference genes for studying expression in heads of workers performing various tasks were selected from among five candidate reference genes (Table 2) we have recently found to be stably expressed in the heads of nurses (Kohutova et al. 2013). The most suitable reference genes were selected on the basis of their expression stabilities evaluated in pooled head samples of bees aged 2–30 days (for particular ages, see Fig. 3) in two colonies. This was accomplished employing the programs geNorm^{PLUS} (part of qbase^{PLUS} software, version 2.5.1) (Hellemans et al. 2007; Vandesompele et al. 2002) and NormFinder, version 0.953 (Andersen et al. 2004). Input data used for these programs were generated previously from qPCR expression analyses of the genes (Kohutova et al. 2013). The data used for geNorm^{PLUS} were raw Cq values of genes, and the data used for NormFinder were relative quantities of genes, calculated from Cq values by the ΔCq method.

GeNorm^{PLUS} evaluated the expression stability of all tested genes as high (criterion *M* value ≤0.5) and recommended usage of two reference targets (ranks 1 and 2) for each colony. NormFinder ranked the genes equally or similarly as geNorm^{PLUS} in the colonies A and B, respectively, and determined the most suitable combinations of two reference genes to be *cyclophilin-gapdh* in the colony A and *cyclophilin-psa1* in the colony B (Table 2). As a normalization factor suitable for both colonies, the geometric mean of three genes with the best overall ranking (considering both colonies)—*cyclophilin*, *psa1* and *gapdh*—was selected.

Table 2 Expression stability of candidate reference genes in heads of workers aged 2–30 days

Gene	Colony A				Colony B			
	geNorm^{PLUS}		NormFinder		geNorm^{PLUS}		NormFinder	
	Stability Value M	Rank	Stability value	Rank	Stability Value M	Rank	Stability value	Rank
cyclophilin	**0.246**	**1**	**0.112**	**1**	0.218	3	**0.102**	**1**
psa1	**0.268**	**2**	0.129	2	**0.202**	**1**	**0.131**	**3**
tctp1	0.283	3	0.140	3	0.282	4	0.208	4
gapdh	0.286	4	**0.151**	**4**	**0.206**	**2**	0.123	2
actin	0.291	5	0.175	5	0.405	5	0.328	5

psa1 proteasome subunit alpha type-1, *tctp1* translationally controlled tumor protein 1, *gapdh* glyceraldehyde-3-phosphate dehydrogenase, *actin* actin-related protein 1

Bold letters mark the most suitable reference genes recommended by geNorm^{PLUS} and NormFinder programs

qPCR and data analysis

The qPCR amplification strategy and amplification conditions were the same as described by Kohutova et al. (2013). Briefly, each sample was analyzed in duplicate in a reaction of 15 μl containing 5 μl of diluted cDNA (1:50 in water) and 0.4 μM primers (Table 3). The PCR reactions were performed with Hot FirePol EvaGreen qPCR mix plus (no ROX) (Solis BioDyne, Estonia) on a CFX96 thermocycler (Bio-Rad, USA) using the following conditions: 95 °C for 15 min and 40 cycles at 95 °C for 15 s, 58 °C for 20 s, and 69 °C for 20 s. The specificity of amplification was monitored by melting curve analysis, electrophoretic determination of amplicon sizes and sequencing of the PCR products. No-reverse transcriptase and no-template control reactions showed none or not significant contaminations. The Biorad CFX Manager 3.0 software was employed for the determination of Cq values and the estimation of relative normalized transcriptional levels of *gox* gene in samples of all sets of a colony related to the lowest value.

In situ hybridization

Primers for the synthesis of a digoxigenin (Dig)-labelled probe were designed based on the honeybee *gox* cDNA sequence as follows: sense primer, 5′-TACCCGACCTTCAA CCAGAC-3′; antisense primer, 5′-ATTCTCCCCAATCCTC CTTG-3′. The 706-bp amplicon was re-amplified using the PCR Dig Probe synthesis kit (Roche Applied Science, Germany) to synthesize the Dig-labelled antisense DNA probe. The Dig-labelled probe was purified using a PCR Purification kit (Promega, WI, USA) and stored at −20 °C. The hypopharyngeal glands, salivary glands and mandibular glands, fat bodies and guts were dissected from honeybees performing a particular task, fixed in 4 % paraformaldehyde (*w*/*v*) at 4 °C overnight and subjected to the whole-mount in situ hybridization procedure as described elsewhere (Kim et al. 2006).

Statistical analysis

One-way ANOVA was used for evaluation of data from qPCR analysis. The Pearson correlation test was used for correlation analysis between antibacterial activity/relative content of GOX and H_2O_2 production in honeys. The data are expressed as mean values with the standard error of the mean (SEM). Data with P values smaller than 0.05 were considered as statistically significant. All statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

Results

Localization of *gox* expression in workers performing distinct tasks

Extracts of mandibular, hypopharyngeal, head salivary and thorax salivary glands were separated on SDS-PAGE, blotted onto nitrocellulose and immunostained with anti-honeybee GOX antibody. A specific immunoreactive band with a size of around 85 kDa was detected only in extracts of hypopharyngeal glands suggesting that the antibody has high specificity, and there was no cross-reaction with other proteins in the extracts (Fig. 1).

Using the specific cDNA probe, we detected strong expression of *gox* mRNA in the hypopharyngeal glands of all selected bees (Fig. 2). The expression level in hypopharyngeal glands remained strong throughout the behavioural development of the worker bees from cleaners to foragers. No expression of *gox* was detected in mandibular, head salivary and thorax salivary glands of the worker bees. Similarly, no *gox*

Table 3 Genes and primers used for qPCR

Gene	Accession number	Primer sequence (5′→3′)	Amplicon size (bp)	E (%)[a]	R^2[b]
gox	NM_001011574.1	GAGGGCGGAAAATCATCAGACC AGGATTACCCGAGATCACCTGC	151	94.0	1
cyclophilin	XM_393381.4	GTATTCTTTCCATGGCTAATGCTG GCCTCCAATTTTCTAACAACATCC	145	90.8	1
gapdh	XM_393605.4	TTGTTGACTTAACAGTTAGACTTGG TTTAGCATCAAAAATACTGGCATGG	170	98.0	1
psa1	XM_625029.3	ATGATGATCTATTACCTGTGAGTCG GTGGTCCTTGATCATCATATCCAG	132	100.3	1

gox glucose oxidase, *gapdh* glyceraldehyde-3-phosphate dehydrogenase, *psa1* proteasome subunit alpha type-1

[a] qPCR reaction efficiency calculated by the standard curve method

[b] Coefficient of determination for the standard curve

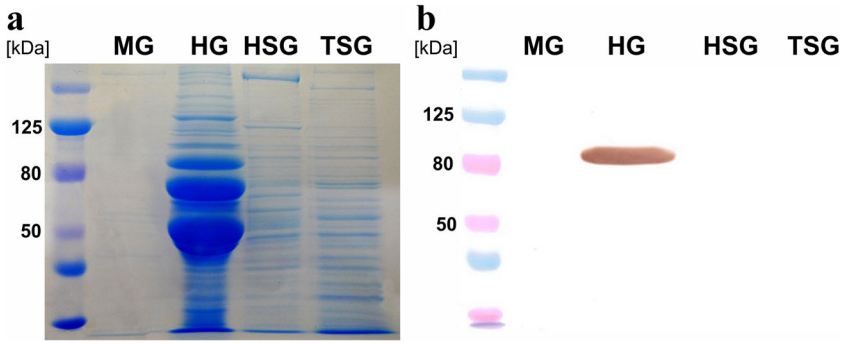

Fig. 1 Immunospecificity of an anti-GOX polyclonal antibody evaluated by immunoblot assays. Gland extracts of unspecified hive bees (5 glands/200 μl of PBS) were electrophoresed on a 12 % SDS-PAGE gel **a** stained with Coomassie Blue R or **b** immunoblotted with a purified rabbit polyclonal antibody against honeybee GOX. *MG* mandibular glands, *HG* hypopharyngeal glands, *HSG* head salivary glands, *TSG* thorax salivary glands

expression was detected in the fat body and gut (data not shown).

Gox expression in heads of workers of various ages

Transcript levels of *gox* using *cyclophilin*, *psa1* and *gapdh* as reference genes were compared in three sets of pooled head samples from bees of different ages in two colonies (A and B). In our previous work, we found that these colonies differed in age-related division of labour (Kohutova et al. 2013). The comparisons of transcription levels of *gox* in the heads of workers of colonies A and B normalized to the geometric means of the reference genes are shown in Fig. 3.

In both colonies, *gox* expression showed similar patterns in relation to the assumed division of labour: The expression was progressively increasing with ageing of the youngest bees including cleaners and nurses and reached the highest levels in nectar processors/foragers bee (normalized transcription levels 3.6–4.5 times higher than those in 2-day-old bees). Between the examined colonies, partial differences can be observed in *gox* expression levels among workers assumed to deal with nectar processing and foraging.

Comparison of GOX content and H_2O_2 production in honey samples

The content of GOX in the honey samples was examined in their ultrafiltration retentates in which only substances with molecular weights (MWs) of more than 10 kDa including GOX should be present. Bands of GOX with sizes of around 85 kDa were detected on the immunoblots using an anti-honeybee GOX antibody for all honey samples (data not

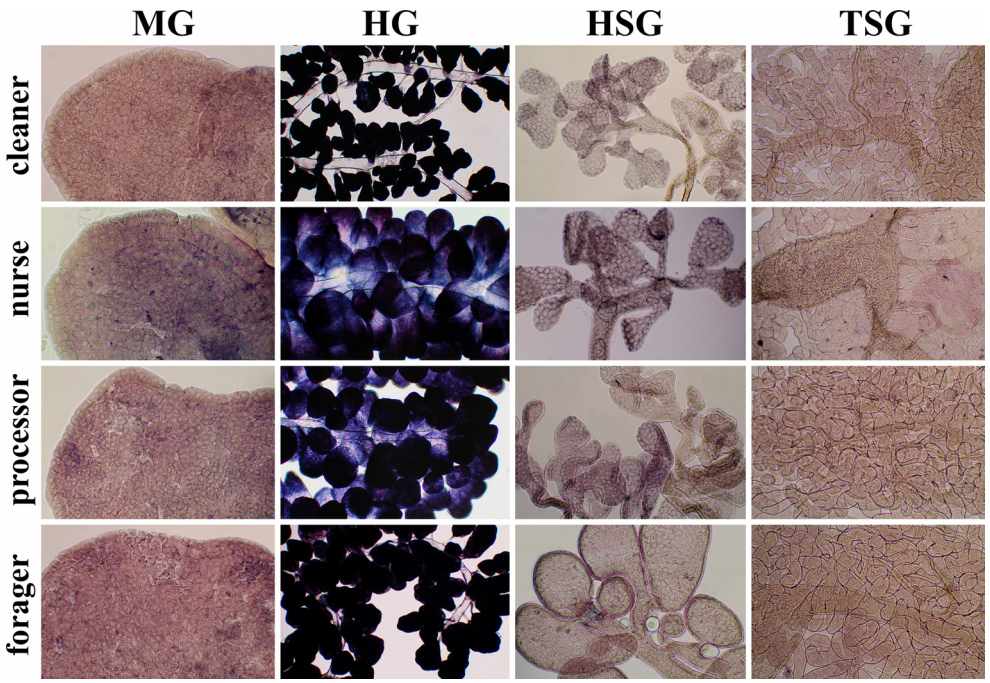

Fig. 2 Localization of *gox* expression in different glands of workers specialized in different tasks performed within a colony at different ages. The expression was localized in glands by in situ hybridization through a microscope with × 100 magnification. Workers performing the following tasks were used in the study: cleaner, nurse, processor and forager. *MG* mandibular glands, *HG* hypopharyngeal glands, *HSG* head salivary glands, *TSG* thorax salivary glands. Note that nurse and processor bees contain larger acines in the HG as compared to cleaner and forager bees

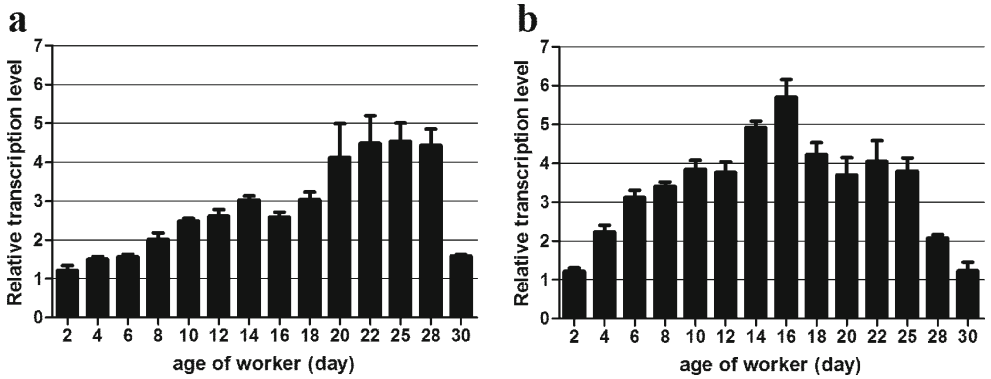

Fig. 3 Relative transcription levels of *gox* in pooled head samples of workers of various ages from colony A (**a**) and colony B (**b**). Three reference genes (*cyclophilin*, *psa1* and *gapdh*) were used for normalization. Data are expressed as mean values±standard error of the mean (SEM). qPCR analyses were performed on three independent experiments, with each qPCR performed in duplicate

shown). The retentates of the 20 honey samples analyzed, with various botanical and geographical origins, showed differences in their GOX content (Fig. 4a). Differences in the GOX content were observed among the honey samples of the different and also the same botanical origin. They were also observed among honey samples of different geographical origin and harvesting time. These observations indicate that the amount of GOX in honey depends on the GOX production capacity of honeybees in colonies.

The intensity of GOX bands and the level of generated H_2O_2 showed a significant correlation (n=20, r=0.745, P=0.0002) almost in all honey samples (Fig. 4c). Two samples (No. 12 and 13) of honeydew honeys contained a relatively low level of GOX but were able to produce a high level of

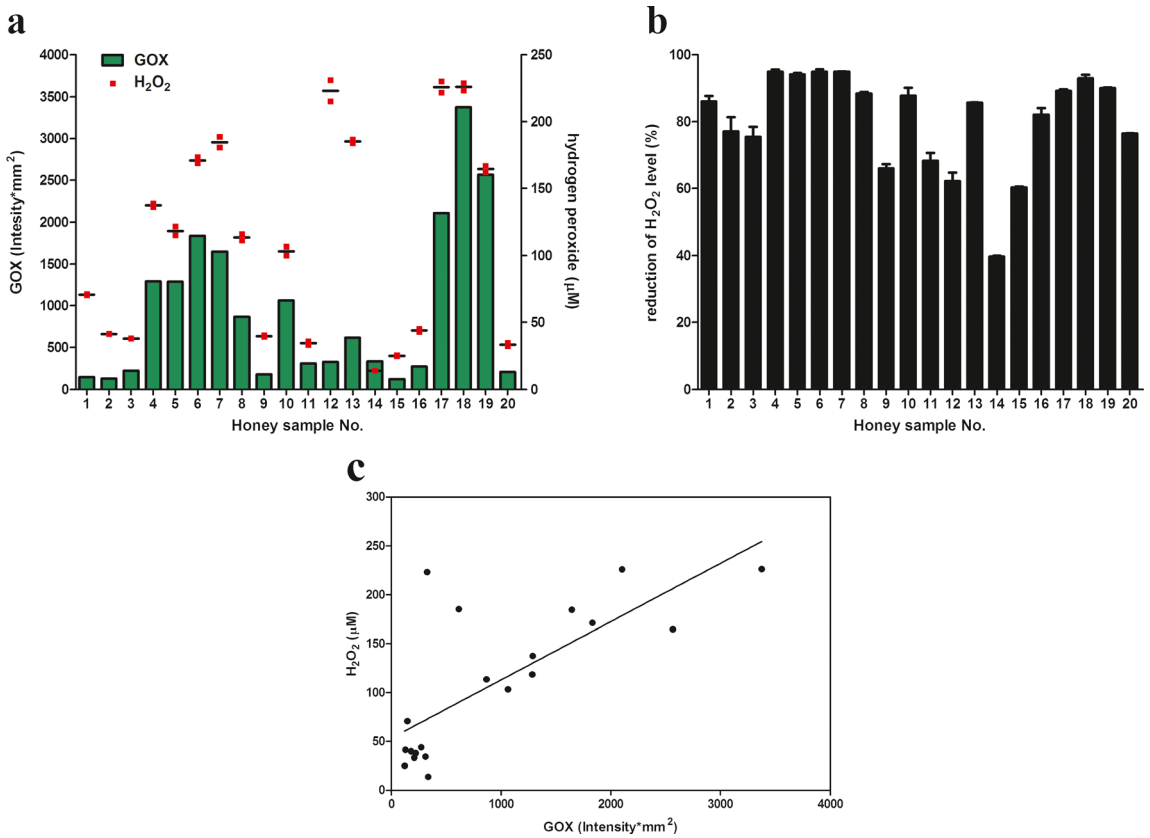

Fig. 4 Content of GOX and H_2O_2 production in the honey samples. GOX levels in equal volumes of honey retentates (15 μl) were determined by immunobloting using a polyclonal antibody against GOX. **a** The signal intensity of immunoreactive bands was quantified by densitometry and expressed as a relative volume intensity (intensity*mm^2). Accumulation of H_2O_2 was determined in 40 % (*w*/*w*) honey solutions after a 4-h incubation at 37 °C using a hydrogen peroxide/peroxidase fluorimetric kit. Data are expressed as mean values±SEMs. **b** Reduction of H_2O_2 generation in heat-treated 40 % honey samples. **c** Relationship between H_2O_2 and GOX content in natural honeys expressed as a relative volume intensity (intensity*mm^2). A Pearson correlation test was used for correlation analysis

H_2O_2 (~200 μM) (Fig. 4a). Levels of H_2O_2 in heat-treated honey samples were significantly reduced in all honeys (Fig. 4b) suggesting that GOX is a principal factor responsible for H_2O_2 generation.

Antibacterial activity of honey samples

The MIC values of 20 natural honeys against the tested bacterial strain are shown in Fig. 5a. Honey samples were differentially effective against a Gram-negative *P. aeruginosa* bacterial isolate. The MICs of honeys for *P. aeruginosa* ranged from 5 to 40 %. Honey samples Nos. 17, 18 and 19 with an unspecified mixed floral origin possessed the strongest antibacterial activity against tested bacteria.

The results of the Pearson *r* correlation test comparing the level of accumulated H_2O_2 and antibacterial activity of each honey sample revealed that there was a significant concentration-dependent correlation between the production of H_2O_2 and antibacterial activity against *P. aeruginosa* (n=20, r=−0.673, P=0.0012) (Fig. 5b).

Discussion

In this work, we demonstrated that *gox* is constitutively and solely expressed in the hypopharyngeal glands of workers performing various tasks. The least expression is in cleaners, and the highest and high amounts occurred in processors and foragers, respectively, which both secrete GOX into the nectar during its processing into honey. Thus, our results indicate that GOX is secreted by nurses into larval jelly and by processors and foragers into honey. Further, we present data confirming that GOX is a regular component of natural honeys and show that the amount of GOX and its enzymatic activity determined as the amount of generated H_2O_2 in diluted honeys varied significantly in the different honeys examined. Our results correspond with the results of others who demonstrated the presence of GOX in the hypopharyngeal glands of foragers (Ohashi et al. 1999), nurses and winter worker bees (Li et al. 2008; Santos et al. 2005) and expression of *gox* in the heads of nurses and foragers collecting pollen (Liu et al. 2011).

Analyses of honey samples revealed that the amount of GOX varied significantly from honey to honey and that this variation seems to have a multifactorial character. The content of GOX varied between honeys of different floral sources but also within the same floral source from different geographical origins. Interestingly, the greatest amount of GOX was found in honeys with an unspecified mixed botanical source. One of the possible explanations is that the content of GOX in honey was affected by pollen nutrition in the honeybee colony. It has been shown that polyfloral pollen diets enhanced some immune functions compared with monofloral diets, in particular GOX activity in honeybee heads, suggesting that diversity in floral resources can have a direct effect on the expression of immune and antibacterial factors including GOX (Alaux et al. 2010). On the other hand, the variation in GOX content may be associated with genetic diversity of honeybees (various genotypes). Our previous studies documented that the levels of bee antimicrobial peptide defensin-1 vary in larval jelly and honey samples, and the obtained results indicated that the variations are determined by genetic factors (Klaudiny et al. 2012; Majtan et al. 2012). Variations in the amount of major royal jelly protein 1 in natural honeys have also been described (Bilikova and Simuth 2010). It is likely that the levels of more bee-derived proteins/peptides in honey, including those contributing to honey antibacterial activity (e.g. GOX), could be influenced by honeybee genetic/epigenetic factors.

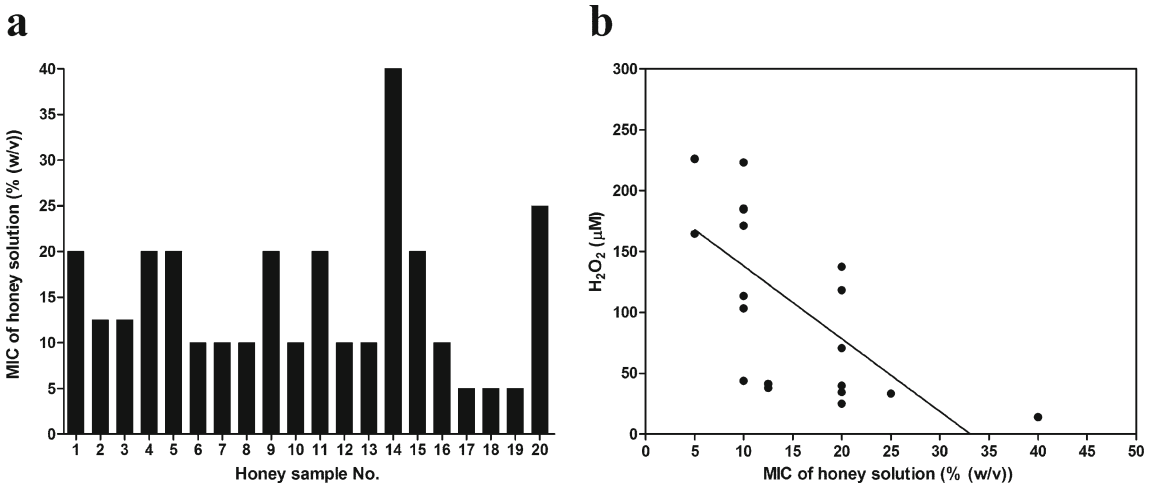

Fig. 5 Antibacterial activity of honeys (n=20) against *Pseudomonas aeruginosa* isolate. **a** The activity was determined by a minimum inhibitory concentration (MIC) assay. The MIC was defined as the lowest concentration of honey solution in percent inhibiting bacterial growth. **b** Relationship between H_2O_2 content and antibacterial activity of natural honeys against *P. aeruginosa*. A Pearson correlation test was used for correlation analysis

Another factor affecting the level of H_2O_2 might be catalase, an enzyme which efficiently hydrolyzes H_2O_2 to oxygen and water. Although the total concentration of catalase depends on the amount of pollen grains in honey (Brudzynski et al. 2011), no data are available regarding the exact concentration of catalase in honey. In addition, it has been shown that the amount of catalase necessary to destroy the antibacterial activity of honey was found to be unexpectedly high (White et al. 1963). In present study, we showed a significant strong correlation between the relative amount of GOX and generated H_2O_2. Therefore, we assumed that the natural content of catalase in honey did not significantly interfere with the presentation of hydrogen peroxide in honeys. According to our findings, H_2O_2 accumulated in every honey upon dilution, which correlates with the constitutive presence of GOX in honeys. However, not all diluted honeys (e.g. manuka honey) are able to form H_2O_2 (Kwakman et al. 2011). The reason manuka honey, a pronounced medical-grade honey, does accumulate only negligible amounts of H_2O_2 has been revealed just recently (Majtan et al. 2014). We documented that methylglyoxal, a major antibacterial factor of manuka honey, induces a modification of significant proteinaceous components (e.g. GOX and defensin-1) of honey resulting in a loss of their biological activities (Majtan et al. 2014; Majtan et al. 2012).

Until now, a very few studies have attempted to determine the concentration of H_2O_2 in different honeys and to evaluate its effect on the overall antibacterial activity of honeys (Brudzynski 2006; Chen et al. 2012). Brudzinsky and coworkers (2006) tested 42 Canadian natural honeys and found that all honeys exhibited antibacterial activity, with the higher efficacy against Gram-negative *Escherichia coli* than Gram-positive *Bacillus subtilis*, and that these antibacterial activities were correlated with H_2O_2 production in the honeys. Similarly, other study by Chen et al. (2012) showed a greater correlation between antibacterial activity against *Staphylococcus aureus* and amount of H_2O_2 in Australian honey samples. These findings are in agreement with our results where a significant correlation between antibacterial activity and H_2O_2 production was observed. However, Slovak natural honeys were more effective against Gram-positive bacteria than Gram-negative *P. aeruginosa* (data not shown). In our previous work (Majtan et al. 2012), antibacterial bee-derived peptide defensin-1 was shown to be a regular component of each honey, and it significantly contributes to the overall antibacterial activity against Gram-positive bacteria, but it is ineffective against Gram-negative bacteria.

Two samples of honeydew honey tested in this study showed a high capacity of H_2O_2 production (over 180 μM) even though the amount of GOX in these samples was low. Honeydew honey is known for the high content of phenolic acids and flavonoids possessing antioxidant and pro-oxidant properties (Alvarez-Suarez et al. 2013). In the presence of transitional metal, polyphenols have been shown to be involved in the generation of substantial amounts of H_2O_2 (Akagawa et al. 2003; Long et al. 2010). Therefore, specific plant-derived polyphenols in honey could be an additional source of H_2O_2 as documented for our two honey samples. Thus, honeydew honey might be an ideal candidate for clinical applications (Cernak et al. 2012; Vlcekova et al. 2012).

In conclusion, our data constitute the first direct evidence that *gox* is constitutively expressed in hypopharyngeal glands of workers performing various tasks, and GOX is a regular but quantitatively variable component of natural honeys. Expression of GOX seems to progressively increase in the hypopharyngeal glands with the age of worker bees, reaching the highest levels in processor bees that are responsible for the processing of nectar into honey. We showed that the level of GOX in honeys positively correlated with H_2O_2 accumulation and, thus, that GOX affects the total antibacterial activity of honey. The assumed dependency of GOX levels in honeys, and also larval jellies from bee genetic/epigenetic factors, needs to be investigated and confirmed. Breeding of novel honeybee lines expressing higher amounts of GOX could be employed in such research. Such lines should contain workers producing secretions of hypopharyngeal glands with increased antibacterial efficacy that could have a positive effect on a resistance of colonies against bacterial pathogens.

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