Lebensm.-Wiss. u.-Technol., 30, 748–753 (1997)

Nature and Origin of the Antibacterial Substances in Honey

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(Received August 21, 1996; accepted March 10, 1997)

The nonperoxide antibacterial activity of honey and honey fractions was tested with Staphylococcus aureus *and* Micrococcus luteus *bacterial species. Antibacterial activity correlated significantly with the honey acidity but did not correlate with honey pH. There were small differences between the antibacterial activities of different honey types: rhododendron, eucalyptus and orange honeys had a relatively low activity, whereas dandelion, honeydew and rape honeys had a relatively higher activity. These results suggest that a part of the antibacterial activity might be of plant origin. However, the antibacterial activity of sugar-adulterated honeys was the same as that of control honeydew honeys produced in the same apiary suggesting that the major part of the antibacterial activity of honeydew honey is of bee origin.*
Ten different honeys were fractionated into four fractions using column chromatography or vacuum distillation: acidic; basic; nonvolatile, nonpolar; and volatile. The antibacterial activity of the different fractions tested was: acids > bases = nonpolar, nonvolatiles > volatiles. This order was the same using either Staph. aureus *or* Micrococcus luteus *as test strains.*
An exception was manuka honey from New Zealand where almost the entire activity was found in the acidic fraction.

Keywords: honey; antibacterial substance; fractionation; nonperoxide activity

Introduction

The antibacterial action of honey was reported for the first time in 1982 (1). Different aspects of the antibacterial properties of honey have recently been extensively reviewed (2). There are two sorts of antibacterial agents, or so called 'inhibines'. One of them is heat- and light-sensitive and has its origin in the H_2O_2 produced by honey glucose oxidase (3–5). Some workers believe that hydrogen peroxide is the main antibacterial agent in honey (3, 6, 7). However, other authors find that the nonperoxide activity is more important (8–13). The argument for the latter is that in ripe honey glucose oxidase is inactive and honey contains only a small amount of peroxide, not sufficient to inhibit bacterial growth. However, when eaten or diluted, peroxide can be produced causing an antibacterial action. The nonperoxide antibacterial activity is insensitive to heat and light (8, 9, 13) and remains intact after storage of honey for long periods (8, 10). The main honey substances are sugars, which exert an antibacterial action by their osmotic effect (2). However, antimicrobial tests used in different studies are usually carried out at concentrations where the sugars are not osmotically active. It has been claimed that honey contains lysozyme, a well known antibacterial agent (11). However, in another study no lysozyme activity was found (8). The antibacterial flavonoid pinocembrin is present in honey but its concentration and contribution to the nonperoxide antibacterial activity of honey is small (14). In New Zealand honeys, mainly manuka and viper's bugloss honey, several aromatic acids with antibacterial activity have been isolated (2, 15). Another investigation claimed that low honey pH, in addition to the high honey osmomolarity, was responsible for the antibacterial activity (16). Some workers have isolated volatile substances with antibacterial activity (17, 18), but their quantitative contribution to the antibacterial action of honey was not examined. Other workers found nonperoxide activity of honey to be extractable by organic solvents but were not able to identify the chemical nature of the substances (12, 19, 20). A major part of the antibacterial activity has been postulated to be of bee origin (10).
However, in two unifloral New Zealand honeys the main antibacterial substances were shown to have a flower origin (2, 15). Determination of the antibacterial activity of honey can be quantitative and can be used as an additional quality criterion for honey (21). Thus, the chemical identity, quantitative contribution, and origin of the different honey antimicrobial substances remain to a great extent unknown.
The purpose of the present study was to clarify these problems by fractionating the honey into the major antibacterial substances and using an antibacterial test reflecting only the nonperoxide antimicrobial activity (8). *Staphylococcus aureus* and *Micrococcus luteus* were utilized in a quantitative turbidometric assay as test

0023-6438/97/070748 + 06 $25.00/0/fs970259

strains because they are known to be sensitive to honey antibacterial substances and are widely used for testing antibacterial action.

Materials and Methods

Samples

Honeys analysed in this study were either market samples of foreign origin or Swiss samples of known origin.

Sugar-adulterated honey

In two apiaries, each with 10 and 12 bee hives, two colonies per apiary were fed with 500 g/L saccharose solution during the honeydew flow in summer. During sugar feeding the bees take the sacharose, invert it into fructose and glucose, enrich it with their own secretion and store it in the supers as a 'sugar-adultered honey'. The adulterated and control honeys were harvested separately.

Routine analysis methods

Honey moisture content (measured with an Abbé refractometer, Zeiss, Germany), pH, acidity, and invertase and diastase (Phadebas method) activities were all determined with standard methods of the Swiss Food Manual (22). Free acidity is expressed in meq/kg, invertase in Hadorn units (invertase number) and diastase in Schade units (diastase number). H_2O_2 production was determined as described by Bodganov (8).

Turbidity test

The following liquid medium was used for the turbidity test: 10 g/L pepton (Merck Art. Nr. 10 7213), 10 g/L Lab-Lemco (Oxoid Art. Nr. L29) and 1 g/L glucose. Test strains were two catalase-negative strains, *Staphylococcus aureus* 6538P and *Micrococcus luteus* ATCC 4698 (old name for *Sarcina lutea*). Suspensions with 0.2 absorption units at 520 nm were used for inoculation of bacteria growth tests. A spectronic 20 spectrometer, capable of measuring 20 mL test tubes directly at 520 nm, was used for measuring the turbidity of the bacterial suspensions. Twenty millilitre sterile test tubes containing honey solutions were incubated in a thermostatable shaking incubator at 37 °C. The 'honey-sugar' standard was a solution of 40 g fructose, 35 g glucose, 7 g maltose, 0.2 g KCl and 17.8 g H_2O per 100 g. Growth medium of honey (sample) (20 g/100 mL) and 'honey-sugar' (control) were mixed with 10 mL liquid growth medium (each sample in duplicate) and the absorbance read at 520 nm (E_1). The incubation medium was buffered for all honeys to pH values lying in the range between 6.5 and 7.0, as in a preliminary test optimal bacterial growth was found for the whole range between pH 5 and 7. One drop of bacteria suspension was added and mixed using a Vortex. The tubes were incubated in a shaking water-bath at a constant shake speed for maximal bacterial growth for 12 h (*Staphylococcus*) or 36 h (for *Lutea*). Turbidity was then read at 520 nm (E_2) and $\Delta E = E_2 - E_1$ calculated. Honey fractions were tested against both strains, while in the other experiments only tests with *Staph. aureus* were conducted. Addition of 10,000 units catalase (Sigma, Art. Nr. C 9447) to destroy all possible peroxide present in the assays with both bacteria (8) had no effect on bacterial growth. Thus only nonperoxide antibacterial activity is measured under these conditions (8). It was shown earlier that inhibition of bacterial growth by the 'honey-sugar' standard is about 10% compared to a control without sugar (8). The control inhibition values before and after passage through the different fractionation steps (see below) were the same, so that the above control incubation was used both for whole honey tests and for the honey tests after fractionation.

Honey fractionation

The columns used for honey fractionation were: (i) C-18 1000 mg SPE (Solid Phase Extraction, Baker Art. Nr. 7020-07) disposable columns; (ii) 2 cm^3/column 50 mesh (Dowex 50 W × 8) strong acidic citation exchanger; (iii) 2 cm^3/column 50 mesh (Dowex 1 × 8) strong basic anion exchanger.

The SPE columns were mounted on Baker-10 SPE extraction manifold with vacuum. Biorad polypropylene disposable columns Nr.731-1550 were used for the ion exchange fractionation, without the use of vacuum.

Honey water solutions (50 g/100 mL) were used for all fractionation steps. The initial pH of each honey solution was measured. Antimicrobial tests were carried out with the initial honey solutions and with the honey solutions after each fractionation step. Loss of antibacterial activity in the honey after a fractionation step was assumed to be due to the removal of antibacterial substances by this step. Before performing the antibacterial test, the honey concentration of the solution after each fractionation step was adjusted to 20 g/100 mL and the pH of the solution was set at the pH of the honey solution before fractionation. Honey solution depleted of all the fractions by subsequent fractionation steps on the same honey had only about 10% of the original antibacterial activity. This means that our fractionation procedure removed almost the entire antibacterial activity. A scheme of the fractionation procedure is shown in **Fig 1**.

Removal of volatile substances. Honey solution (50 g/100 mL) was heated at 60 °C in a Rotavapor under vacuum (15 mg Hg) for 2 h in order to remove all volatile substances. Vacuum steam distillation has been used for isolating honey volatiles and testing their antibacterial effect (17, 18). It leads to a complete dehydration of honey and to a removal of all volatile compounds were capable of producing hydrogen bonds with water. A preliminary experiment was performed

with our vacuum distillation with one blossom and one honeydew honey and more than 150 different volatile compounds were detected in the distillate. Controls for fractionation of the volatile compounds were also done with all honeys: honeys were heated for 2 h without vacuum and no effect on the antibacterial activity was seen.

Removal of nonpolar nonvolatile substances. Baker C-18 columns were activated with one volume of ethanol, followed by one volume of water. The honey solution was then passed through under constant vacuum.

Removal of bases. The cation exchange column was converted into the H-form by passing 2 mL of 2 mol/L HCl. It was then washed with water until the eluate was neutral, and the honey solution, where bases are in a cation form, was then passed through.

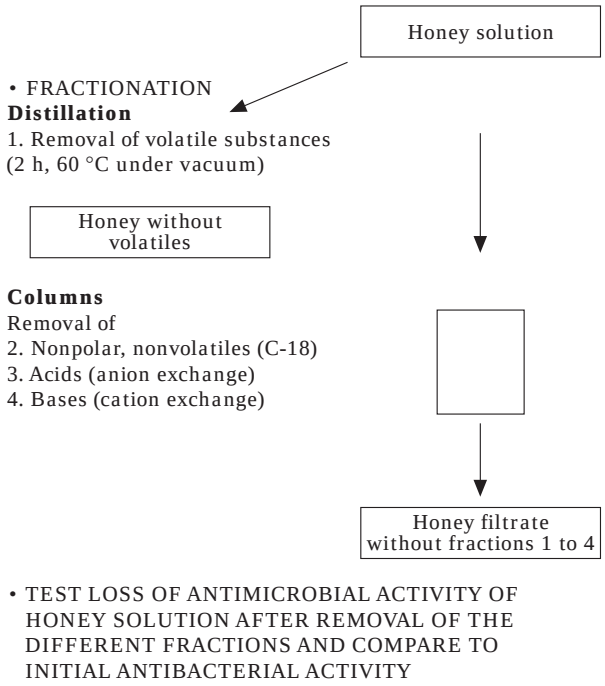

Fig. 1 Scheme of the fractionation and testing of different antimicrobial fractions

Removal of acids. The anion exchange column was converted into the OH-form by passing through 2 mL 2 mol/L NaOH. It was then washed with water until the eluate was neutral. The honey solution was then set at pH 11, and the solution, where acids are in their dissociated anion form, passed through the column. Controls for fractionation of acids were done with all honeys: a shift of the initial honey pH to pH 11 had no effect on the antibacterial activity, as a back titration to the original pH resulted in a honey solution having its initial antibacterial activity.

Expression of antimicrobial activity

Whole honey. Results were calculated by the turbidities of the incubation mixtures at the end of the bacterial test. They were expressed in % inhibition compared to the absorbance of the control (control = 0% inhibition).

Honey fractions. Increased bacterial growth after removal of a certain honey fraction (see above) was attributed to the removal of antibacterial substances by this fractionation step, e.g. a honey having an initial bacterial inhibition of 90% has a 80 % inhibition value after removal of the volatile fraction. This means, that the fractionation inhibition value I_f due to volatile fraction is 90 – 80 = 10%. The relative inhibition $I_{f\text{-}rel}$ attributed to each fraction is calculated as:

$$I_{f\text{-}rel} = I_f \cdot 100/I_t \qquad \text{Eqn [1]}$$

where I_t is the sum of the different I_f values.

Results and Discussion

Correlation between acidity, pH and antibacterial activity

Table 1 summarizes the results of the different unifloral and polyfloral honeys for the following parameters: pH, free and total acidity, and inhibition of growth of *Staph. aureus*. The linear correlation analysis between pH, and free and total acidity on the one hand and bacterial inhibition on the other yielded the following results

Table 1 pH, acidity and inhibition of growth of *Staph. aureus* in different honeys

		pH		Free acid		Total acid		% Inhibition	
Honey	*n*	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x
Acacia	7	3.9	0.3	1.14	0.33	1.97	0.30	57	31
Blossom	30	4.1	0.5	1.44	0.61	2.27	0.92	56	22
Chestnut	7	5.4	0.6	0.58	0.30	1.01	0.43	56	26
Dandelion	2	4.4	0.1	0.65	0.08	0.89	0.11	66	5
Eucalyptus	4	4.4	0.5	1.10	0.44	1.78	0.54	40	8
Lavender	5	3.4	0.2	2.18	0.13	3.80	0.72	64	9
Orange	3	3.8	0.1	0.99	0.27	1.71	0.40	47	8
Rape	7	3.9	0.1	0.93	0.37	2.01	0.92	74	18
Rhododendron	3	3.7	0.1	0.86	0.24	1.51	0.48	37	8
Sunflower	4	3.7	0.1	1.49	0.18	2.51	0.38	58	26
Honeydew	10	4.4	0.3	2.24	0.71	2.96	1.09	67	19

Mean values ($\bar{x}$) and standard deviation (s_x) for *n* samples of unifloral honey.

(n = 81 cases), summarized in **Table 2**: bacterial inhibition correlates significantly with free and total acidity, but not with honey pH. This is in accordance with other results of this paper that the main part of the nonperoxide activity is found in the acid fraction (see below). As the acids are of bee origin (23), these results can be interpreted that a part of the antibacterial activity has a bee origin.

The low honey pH, besides the osmotic effect of the sugars, was postulated to be the main antibacterial factor of honey (16). However, there are quite a few honeys (honeydew, chestnut) having pH values of 5 and more which also inhibit bacterial growth. In our test the pH was varied from 5 to 7 and optimal bacterial growth found at all conditions (see **Methods**). It can thus be concluded that although honey acids exert the main antibacterial action, honey pH could additionally act as an antibacterial factor.

Table 2 Correlation between antibacterial activity, pH and acidity

Parameter	pH *vs.* inhibition	Free acidity *vs.* inhibition	Total acidity *vs.* inhibition
r	0.06	0.35	0.31
P	0.58	0.001	0.005

r = coefficient of correlation; *P* = probability. Parameters were calculated for *n* = 82 honeys of different origin (see **Table 1**).

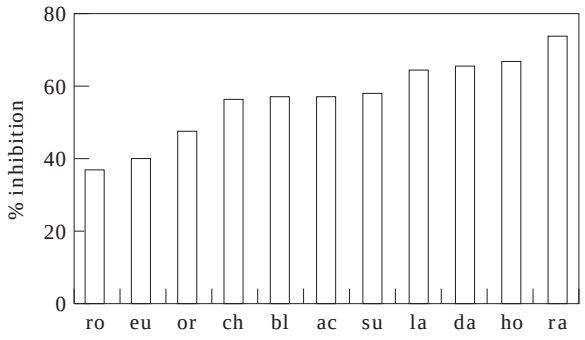

Fig. 2 Nonperoxide activity of different honeys against *Staph. aureus*. ro = rhododendron; eu = eucalyptus; or = orange; ch = chestnut; bl = mixed blossom; ac = acacia; su = sunflower; la = lavender; da = dandelion; ho = honeydew; ra = rape

Antibacterial activity of honeys of different origin

If the antibacterial substances in honey originate from plants, differences in the inhibitory capacity of different unifloral honeys should be expected. The bacterial inhibition of nine unifloral and two mixed (different blossom and honeydew origins) honeys are shown in **Fig. 2**, using the average values in **Table 1**. There were slight differences between the different honeys: rhododendron and eucalyptus honeys had the lowest activity, while honeydew and rape honeys had the highest activity.

However, there is a considerable variation within each honey type (see standard deviations in **Table 1**), therefore between-honey differences were not statistically significant. Differences in antibacterial activity of unifloral honeys have been reported (2). However, a great variation in the activities of unifloral honeys was found. Also, in the reported studies it is often not clear which part of the antibacterial activity is being measured.

Antibacterial activity of sugar-adulterated honeys

If the antibacterial activity originates from the bee, then one would expect that sugar-adulterated honey would have the same antibacterial activity as genuine honey produced under the same conditions. In **Table 3** the quality criteria of two genuine honeydew honeys are compared with those of two sugar-fed honeys, produced at the same time in the same apiary. In the sugar-adulterated honeys the adulteration indicators prolin and ash were about one-third of the values of the control honeydew honeys, which means that the honeys contained a major portion of the sugar fed to the bees. The nonperoxide antibacterial activity, but also the peroxide accumulation capacity, in both adulterated honeys was about the same as that of the control honeys. Thus it is evident that the greater part of both types of antibacterial activity of honeydew honeys is of bee origin. These results corroborate the conclusions of another study in our laboratory where a highly significant correlation between the diastase and the invertase activity, both originating from the bees, and bacterial inhibition was found (21).

Sugar feeding experiments of this type during the flow of different unifloral honey sources is necessary in order to quantify the relative amount of bee- and plant-derived antimicrobial activity.

Table 3 Antibacterial activity in honeys produced under sugar feeding

Honey	% Inhibition *Staph. aureus*	H_2O_2 (µg/g/h)	Prolin (mg/kg)	Ash (g/kg)
Honeydew 1	91	56	1670	4.5
Honeydew 1 + sugar feeding	81	56	760	1.2
Honeydew 2	95	77	1200	4.2
Honeydew 2 + sugar feeding	96	49	480	1.5
Mean honeydew[a]	93	66	1430	4.3
Mean honeydew + sugar-feeding	91	52	620	1.3

[a] Mean value of honeydew honeys 1 and 2.

Table 4 Relative distribution of antibacterial activity in different honey fractions

	% Antibacterial activity in different fractions[a]							
	acidic		basic		nonpolar		volatile	
Honey	*St.*	*Mic.*	*St.*	*Mic.*	*St.*	*Mic.*	*St.*	*Mic.*
Manuka N.Z.	100	75	0	10	0	5	0	10
Sunflower It	58	46	13	15	16	25	13	15
Rape CH	25	40	7	33	63	22	5	5
Lavender Fr	25	27	34	30	23	29	18	14
Mountain CH	24	25	60	25	8	25	8	24
Blossom S. America	62	73	13	20	9	7	16	0
Honeydew CH	45	46	26	15	26	15	2	24
Honeydew CH	32	31	37	31	19	31	12	6
Honeydew CH	43	26	22	26	19	26	15	23
Honeydew Europe	43	32	25	31	26	37	6	0
Average	46	42	24	24	21	22	10	12
Standard deviation	23	18	17	8	17	10	6	9
Minimum	24	25	0	10	0	5	0	0
Maximum	100	75	60	33	63	37	18	24

[a] Values of individual honeys.
St = Staphylococcus aureus; *Mic = Micrococcus luteus.*

Relative distribution of antimicrobial activity among different honey fractions

We fractionated 10 different honeys into four basic substance groups: volatile; nonvolatile and nonpolar; acidic; and basic substances (see **Fig. 1**). The relative inhibition of each honey fraction was tested against *Staph. aureus* and *Micrococcus luteus*. The results are summarized in **Table 4**. The acidic fraction had the greatest inhibitory activity, while the volatiles were the weakest bacterial inhibitors. The relative distribution of the antibacterial activity in the different fractions was about the same when both bacterial species were tested. On average, the following relative distribution of antibacterial activity was observed: 44% acids, 24% bases, 21% nonpolar, nonvolatile, and 11% volatiles.

When differences between the distribution of activity among the different groups were tested by a *t*-test, only the difference between the volatile activity on the one hand and the acidic ($P = 0.000$) and the basic fraction activity ($P = 0.05$) on the other proved to be significantly different. This is due to the variation of distribution among the fractions of the different honey types. In manuka honey 90% of the activity was found in the acidic fraction; in the rape honey the major part of the activity was in the nonpolar fraction; and in one Swiss blossom honey the basic fraction had the highest activity.

Conclusions

The nonperoxide antibacterial activity in honey was found to correlate significantly with the acid content of honey, but not with its pH.

There are differences in the activity of different unifloral honeys: rhododendron and eucalyptus honeys had the lowest activity, while honeydew and rape honeys had the highest activity. However due to the considerable variation of the antibacterial activity within honey types the differences were not statistically significant.

From experiments with sugar-adulterated honey it can be concluded that the antibacterial activity of honeydew honeys was of bee origin.

By fractionation into different substance classes the following relative distribution of nonperoxide antibacterial activity was found: acids > bases = nonpolar, nonvolatiles > volatiles. This order was the same using *Staph. aureus* and *Micrococcus luteus* as test strains.

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