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Characterisation of Antibacterial Substances in Honey

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Two types of antimicrobial agents (inhibines) were identified by microbiological and chemical methods. The one is the well known H_2O_2 honey system, the other consists of heat-resistant substances. In contrast to the H_2O_2 production capacity of honey, these inhibines are relatively insensitive to heat and light and also did not change their activity after storage for 6 months at room temperature. The anticmicrobially active flavonone pinocembrine was present in 11 out of 12 honey samples of different origin. No lysozyme activity could be detected under our experimental conditions.

Introduction

The existence of the antibacterial properties of honey is well documented. Two sorts of antibacterial agents (inhibines) are thought to coexist. The inhibines, which are heat- and light sensitive (1–6), have their origin in the H_2O_2 system of honey (3). The capacity of honey to produce H_2O_2 is determined by H_2O_2 synthesis through glucose oxidase (3) and H_2O_2 breakdown through catalase (7). Nowadays it is generally accepted that the H_2O_2 system of honey plays a major role in the antibacterial action of honey. On the other hand other workers (8, 9) have postulated the existence of volatile, heat-stable substances of unknown origin. In one work (10) lysozyme, which is also known to posess antibacterial properties, was found in honey. Honey has antimicrobial activity towards a number of gram-positive and -negative bacteria (5, 6, 11). In our work we used as test strain *Staphylococcus aureus*, because it has been used mostly in honey inhibine studies as it is particularly sensitive to honey antimicrobial action.

The purpose of this work was to clarify the role of the different honey inhibines, especially of the heat-stable ones, and to elucidate their chemical structure.

Materials and Methods

Materials

Agar medium Nr. 1 (pH 7.0) was from Oxoid. For the turbidity tests the following liquid medium (pH 7.0) was used: 1% pepton, 1% Lab-Lemco, (both Oxoid) and 0.1% glucose. Glucose oxidase (125,000 U/gm) and catalase (400,000 U/gm) were from Sigma. The lysozyme test was from DIFCO. A "honey-sugar" standard was a water solution, containing 40% fructose, 35% glucose and 7% maltose. Thin layer chromatography (tlc) was done on silica gel and polyamid 11 plates (Merck). The flavonoid standards were commercial preparations. Pinocembrine was a kindly gift of Prof. E. Wollenweber (Darmstadt). The tlc spraying reagents were: 2% $ZrOCl_2$ and 1% flavon-reagent (Fluka) in methanol. We examined fresh honeys of known swiss origin, as well as commercial samples. The dark honeys were of honey dew – and sweet chestnut flower origin, the light honeys were of different floral origin.

Methods

For the agar diffusion method 50% honey solutions in 40 mM Sodium-Phosphate buffer (pH 7.0) were poured in the agar wells and after an incubation for 24 hours at 37°C the inhibitory zones were measured. Standards, containing 0.1–2.5 µg glucose oxidase/ml 20% "honey-sugar" standard in the above buffer were run with each incubation. The inhibine values (0–50) are relative concentration units, calculated from a standard curve (glucose oxidase concentration versus diameter of inhibitory zone). Under these conditions a 50% "honey sugar" standard did not inhibit bacterial growth.

A turbidity test was done with 20% honey solutions in the liquid medium (see above). The incubation was carried out for 16 hours at 37 C and the turbidity was determined at 540 nm. The inhibitory effect due to sugar osmosis was about 20%. *Staphylococcus aureus* ATCC 6538P was used for both antimicrobial tests.

The lysozyme activity was determined under the same conditions as used for the turbidity test with a DIFCO lysozyme test.

For measurement of H_2O_2 production 1 ml 20% honey solutions in the above phosphate buffer were incubated for 1 hour at 37 C without shaking. The determination of the H_2O_2 formed was done essentially as described (12).

For structural analysis of the inhibines, the honey was extracted with diethyl ether. In a typical experiment 0.5 kg of honey was extracted portionwise with 2 litres of diethyl ether. After evaporation of the solvent 0.2 to 0.4 ml of a brownish mixture was left, which was used for subsequent analysis. This extract was analyzed by tlc on silica gel (13) and polyamide (14) plates and the chromatograms were examined under UV light after spraying with specific flavonoid reagents (see material-section) and compared with the flavonoid standards. The identification of antimicrobial

substances directly on the tlc plates was done by bioautography (15), using *Bacillus subtilis* ATCC 6633 as a test strain. Pinobembrine was identified by comparison of tlc behaviour, coloration on the tlc plates and spectroscopic analysis (16) of the suspected substance with genuine pinocembrine. For spectroscopic analysis, the fractions, showing antimicrobial activity on the bioautography tests, were eluted from the tlc plate with methanol.

Results

Antimicrobial tests

In **Fig. 1 (a)** the antibacterial activity (as tested with the agar diffusion test) was correlated to the H_2O_2 production capacity of the same honey samples. There was a significant correlation ($p = 0.001$) between the two parameters. In agreement with this finding, a treatment with catalase, as to destroy all H_2O_2, caused a complete loss of antibacterial activity. On the other hand, there was no correlation between the antibacterial potency as tested with the turbidity test and the H_2O_2 production capacity of the honey **(Fig. 1 B.)**. In agreement with this, only very elevated H_2O_2 concentrations, which were produced by glucose oxidase standards, have but a small effect on bacterial growth **(Fig. 2)**. The very small concentrations of H_2O_2, found in honey solutions under the same incubating conditions **(Fig. 2)**, would not be able to influence the growth of Staphylococcus aureus. In agreement with this finding, a treatment of the honey solutions with catalase, as to distroy all H_2O_2 present, did not have an influence on the antibacterial activity.

Effects of heat and storage on the honey inhibines. **Tab. 1.** A summarizes the results on the effects of heat on the two types of inhibines. While the H_2O_2 production capacity of honey is severely damaged, especially that of the light honeys, the antibacterial activity of the "non H_2O_2"-inhibines remains virtually unchanged.

After storage of honey samples for three and six months at room teperature, there is a considerable loss of the H_2O_2 production capacity of honey, and practically no change in

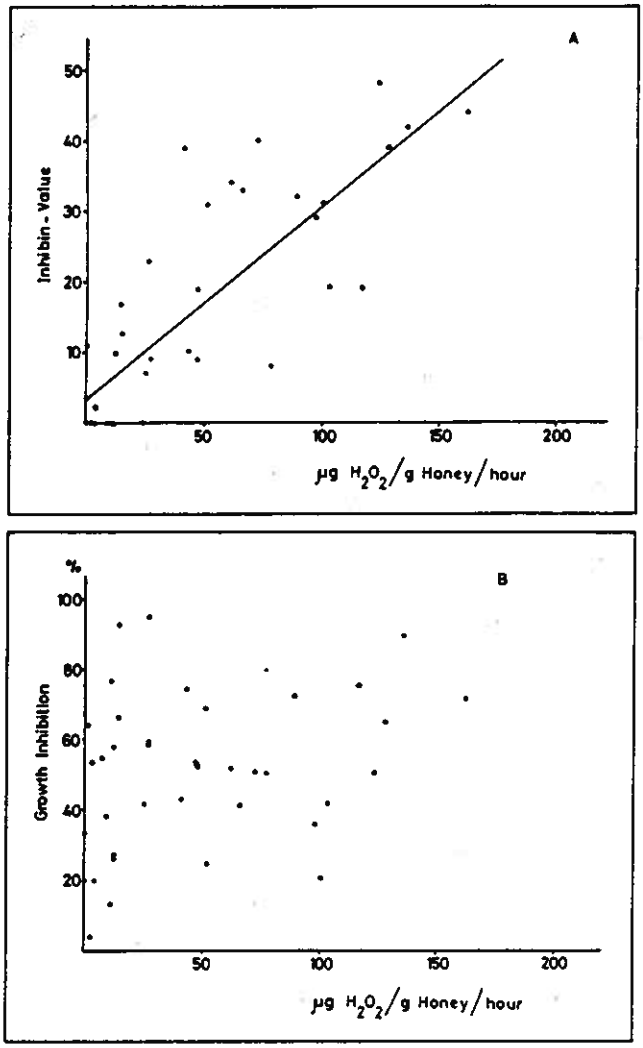

Fig. 1 Correlation between antibacterial action and H_2O_2 production capacity

Antimicrobial activity tested with agar diffusion test (A) and turbidometric test (B). with *Staphylococcus aureus*. 37 commercial honey samples were analyzed (11 honey-dew honey samples, the rest were of floral origin).

Tab. 1 Effects of heat and storage on honey inhibines

Values are means ± SEM. Fresh honeys of honey-dew or floral origin were used.

A. Heat: The honey samples were heated for 15 minutes at 70°C.

	% of initial activity		
	H_2O_2-production	other inhibines	n
light honey	8 ± 1	86 ± 4	3
dark honey	78 ± 3	94 ± 1	4

B. Storage. The honey samples (8 fresh honeys of swiss origin and 4 commercial honeys of foreign origin) were stored in the light (open on shelves) or in the dark at room temperature (20–25°C) for 3 and 6 months.

	% of initial activity				
	H_2O_2-production		other inhibines		
	light	dark	light	dark	n
3 months					
light honey	62 ± 3	85 ± 4	94 ± 1	100 ± 1	7
dark honey	76 ± 3	84 ± 4	104 ± 1	104 ± 1	5
6 months					
light honey	47 ± 4	73 ± 2	100 ± 2	107 ± 1	7
dark honey	67 ± 3	76 ± 3	101 ± 2	101 ± 1	5

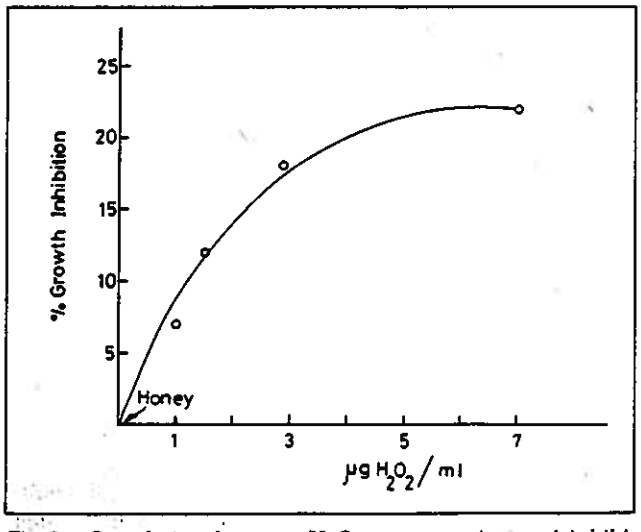

Fig. 2 Correlation between H_2O_2 concentration and inhibition of growth of Staphylococcus aureus

The different levels of H_2O_2 were achieved by incubations with different concentrations of glucose oxidase standards or with honey solutions. The H_2O_2 concentration was measured after the end of the turbidometic tests. The values are means of two independent experiments.

the antibacterial activity of the other inhibines **(Fig.2 B)**. There was a significant ($p = 0.05$) light dependence decrease of the H_2O_2 production capacity of honey, especially of light honey.

Chemical nature of the heat-stable inhibines. in 10 fresh honeys, all having a considerable "non-H_2O_2"-activity, we could not detect any lysogenic activity. Under the same conditions, addition of endogenous lysozyme caused the expected lysis of Micrococcus lysodecticus. We thus conclude, that under our experimental conditions no lysozyme activity is present in honey.

We then tested the antibacterial activity of the honey ether extracts by bioautography. Having found antimicrobial activity of different tlc fractions we proceeded to identify their chemical structure (see methods section). In 11 out of 12 honey samples examined (6 honey-dew honeys and 6 honeys of different floral origin) we could prove the presence of the flavonone pinocembrine.

Discussion

There can be several explanations for the different behaviour of *Staphylococcus aureus* in the two different antimicrobial tests. The antibacterial agents, which are active under the conditions of the turbidity test might not be able to diffuse into the agar under these experimental conditions. The aeration of the agar and the liquid beeing different, this might influence the physiological condition of *Staphylococcus aureus* as to make it less H_2O_2 sensitive in the liquid medium. The catalase activity of the bacteria can also depress the H_2O_2 concentration in the liquid medium much more than under the conditions of the agar test, where much higher concentrations of H_2O_2 can be built in the agar wells. Whatever the reason for this different behaviour of the two antibacterial tests might be, this can be used to distinguish between two "inhibine systems" in honey: the H_2O_2 system and a "non-H_2O_2" system. Our results, concerning the heat- and light sensitivity of the honey H_2O_2 system have been confirmed many times (see introduction).

There is one report in the literature (10), that lysozyme is the heat stable honey inhibin. Under our experimental conditions, we could not detect any lysogenic activity in honey, even in very fresh samples.

The antimicrobial substances, which we analyzed by tlc-bioautography, belong mostly to the group of the flavonoids, which are solid and relatively stable substances and thus have nothing in common with the volatile antimicrobial substances, reported in other publications. Work in our laboratory is in progress to identify them. Pinocembrine, the antimicrobial substance, identified in our work is also one of the main antibacterial substances of propolis (14, 17). Propolis is the resinious material collected by the bees from different plant sources. Bees use it in the hive for closing crevices, to repair combs and to attach loose parts. Because of its antisceptic effect, it is also thought to play an important role for the bee-hive hygiene. The pattern of the thin layer chromatograms of honey extracts under UV light, after spraying with specific flavonoid spray reagents, was amazingly similar to that of propolis ethanol extracts, analyzed in the same way. The principal flavonoids of propolis have their origin in the poplar buds (14) and some of them are antimicrobially active (17). The presence of flavonoids in honey might come through direct mixing of propolis raw products into honey by the bees, or might be the result of secondary diffusion in the bee-hive, where propolis is very abundant.

As to the relative role of the different honey inhibines, we think, that the heat-stable ones have a major importance in honey antimicrobial action. The H_2O_2 content of honey is insignificant and H_2O_2 can be produced only after an aerobial incubation of diluted honey solutions (3). The heat stable inhibines, on the other hand, can theoretically exert their antibacterial action in undiluted honey, and also are relatively insensitive to heat and light influences.

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