

ENZYME PRODUCTION OF ACID IN HONEY

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The acidity of honey is one of its more important properties. It contributes to the flavor complex and the amounts present appear to be a function of floral type.

In the older procedure for determining the acids of honey, alkali is added to the sample until the pink color of an indicator is maintained for ten seconds (1). It was necessary to specify it this way because the indicator color faded rather rapidly, indicating that the solution became more acid. This phenomenon had not received detailed attention and had been explained as possibly due to acids produced from sugars under the very slightly alkaline conditions (2). An English investigator, Cocker, in 1951 (3) noted the fall in pH of neutralized honeys and when he found it did not occur with a heated honey solution, ascribed it to an acid producing enzyme.

At the time we tried to repeat his work but found the pH to drift in both heated and unheated honey. This, of course, ruled out enzymic action as the immediate cause of the drop in pH. (4).

The first slide ----- S L I D E 1 ----- shows that the boiled sample required slightly less alkali to neutralize than did the unboiled sample. This is probably due to a loss of volatile acids in the boiling of the sample. Both samples were let stand several hours; the pH dropped at about the same rate. After 3 hours they had

dropped to 5.2. They they were again titrated. The unboiled sample required 0.25 ml more alkali than did the boiled one. Thus, the experiment shows that the drop in pH of neutralized honey solutions is not enzymic, and it also shows that there is a possibility of acid production due to enzymic action, but it cannot be shown simply by pH fall of neutralized honey solutions, as Cocker attempted to do.

Several years later we became interested in isolating and identifying the acids of honey. During isolation of the acids by ion-exchange methods, a fraction was obtained that reacted on titration just as does honey, with a drift in pH after neutralization. This effect is commonly ascribed to lactones, which are internal ester compounds, as shown in the next slide.

----- S L I D E 2 -----

They exist in an equilibrium between the two forms. The relative amounts depending on the pH of the solution.

Lactones are commonly titrated by adding an excess of alkali, which forces the reaction to the left. Then the excess alkali is neutralized by added acid.

Next, this procedure was applied to honey. It was found that by adding a known excess of alkali to a neutralized honey solution and backtitrating with acid, a reproducible, stable end-point was attained.

It was further found that the brief period of strong alkalinity required in the procedure did not produce <sup>significant amounts of</sup> acids from the reducing sugars

of honey, though if the alkaline solution were allowed to stand, sugar decomposition did take place. Accordingly, the procedure for determining acidity in honey was revised (4) and is now being used in our analytical honey survey. It has been found that all honey contains lactone material, in an amount averaging about a third of the acidity determined by the older methods.

In one part of the analytical survey of American honey referred to, we are studying the effect of storage on honey composition. This is being done by deep-freezing a part of each sample when received and then analyzing the frozen sample side-by-side with another part which had been stored at room temperature. When the acidity of a number of such samples was determined, it was noted that in many cases, the acidity of the samples kept at room temperature was greater. The amounts of the changes were not large, so the differences were examined statistically. The next slide shows:

----- S L I D E 3 -----

that of 13 samples, 8 showed differences giving "t" tests exceeding critical values at the 1% probability level, with 3 more exceeding the 5% level. Only two were not significant. The differences shown had developed in 8 to 10 months. These data do not eliminate the possibility that the increase in acidity is due to fermentation of the sample, though none showed any visible signs of spoilage.

Next, a portion of sample 104 was diluted and divided into several portions. Half was boiled for 5 minutes, then each was further divided

and incubated for 4 hours at 35°C, with and without oxygen flowing through. An antiseptic, phenyl mercuric acetate, was present at 5 ppm. The samples were run in triplicate, and titrated by the new procedure. Results are shown in the next slide.

----- S L I D E 4 -----

The enzymic nature of the reaction is easily evident, and the participation of atmospheric oxygen is also apparent. There is still a possibility that the difference between the boiled and un-boiled samples might be due to microbiological action, so the experiment was repeated using 40 ppm of the antiseptic, with the same results, as may be seen in the next slide.

----- S L I D E 5 -----

As a further demonstration that the effect is not caused by microbiological action during the incubation period, a honey sample was selected that did not show a significant change of acidity on storage. It was incubated as previously described, with 40 ppm of phenyl mercuric acetate, and the results shown on the next slide were obtained.

----- S L I D E 6 -----

No significant difference in acidity of incubated boiled and unboiled samples was obtained.

As is well known, enzyme activities of honeys vary widely. We are determining diastase (amylase) activity of many of our samples, and have found a rough correlation between the diastase number of the sample and the apparent acid-producing enzyme activity. In a group of 12 samples upon which we have both values, the relationship, in the next slide, appear.

-----S L I D E 7 -----

We do not know what compound is produced by the enzyme. It is tempting to relate the enzyme to one reported in 1941 by ~~Ba~~auhe (5) in Munich to occur in the pharyngeal glands of the honey bee. Although she did not prove it, she had some indication that the enzyme produced gluconic acid from glucose. Such enzymes are well known to occur in molds; in fact they are in commercial production for use <sup>in the food industry</sup> as agents for oxygen or glucose removal ~~by the food industry~~. These enzymes produce glucono-lactone directly by dehydrogenation of glucose. The lactone then equilibrates to the acid as shown on an earlier slide.

We have not done any further work to isolate the enzyme or to identify its products, though we do hope to be able to fit it into our program.

Acknowledgement

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