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Evolution of acid phosphatase activity of honeys from different climates

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Abstract

Acid phosphatase is an enzyme of honey whose values have been related to honey's fermentation. In this work, we have studied the evolution, throughout thirty months, of acid phosphatase activity on honey samples originating in continental and oceanic areas. We have also researched the influence of induced granulation and pH on acid phosphatase activity evolution. Acid phosphatase activity has been determined by measuring, at 400 nm, 4-nitrophenol formed after the hydrolysis, at acid pH, of 4-nitrophenylphosphate in the presence of acid phosphatase. From the start, acid phosphatase activities have been higher in honeys from oceanic climate. Similar trends of evolution of acid phosphatase activity have been found in both honeys from continental and oceanic climates. In the end of the study, 100% samples showed a decrease of acid phosphatase activity. Induced granulation does not modify the trend of acid phosphatase activity evolution, although this technological process has an influence on the time at which changes within the evolution occurs. pH of samples has demonstrated to have a strong influence on the activity of acid phosphatase, so that the higher the pH, the lower the decrease of acid phosphatase activity. In honeys originated from continental climate area, pH and acid phosphatase activity have been correlated throughout the study.

Keywords: Honey; Acid phosphatase activity; Evolution; Induced granulation

1. Introduction

Enzymes are very well known as freshness and ageing attributes of honey. Giri (1938) was the first researcher that discovered the presence of acid phosphatase on honey. This enzyme is a hydrolase that provides inorganic phosphates from organic phosphates. Acid phosphatase is mainly present in pollen, although it is also a component of nectar (White, 1979). Zalewski (1965) and Ivanov (1981) studied the storage influence on honey's acid phosphatase, observing that after 6 months the activity of this enzyme had significantly decreased. Despite the fact that acid phosphatase is one of the honey's enzymes that show lower enzymatic activities and are less resistant to both heat and storage than diastase, invertase and glucose oxidase (Ivanov, 1981; Sánchez, 1999; White, 1975, 1978, 1979; Zalewski, 1965), acid phosphatase activity determination is very interesting because its values could be related to honey's spoilage by fermentation. Giri (1938) observed that fermented honeys showed higher acid phosphatase activities than unfermented honeys. In addition, some researchers found that this enzyme could be related to the botanical origin of honey (Ivanov, 1978; Santana, 1993), thereby

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making this enzyme a suitable parameter for honey characterization. Santana (1993) and Sánchez (1999) determined acid phosphatase activiy on Galician honeys (NW Spain) from different botanical sources, observing that *Rubus* sp. honeys showed the highest acid phosphatase activities.

The vast majority of literature references about honeys' acid phosphatase are dated (i.e., more than 30 years old), spread widely over time, and published in many different languages, so all research results about this enzyme remain largely unknown. Giri (1938) determined acid phosphatase activity by means of a method based on determining inorganic phosphorous produced from β-glycerol phosphate after incubating the samples 24 h at 35 °C. Zalewski (1965) used as substrate disodium *o*-phenyl phosphate by incubating the samples 2.25 h at 37 °C. Günther and Burckhart (1967) modified Giri (1938) procedure, reducing the time of incubation to 3 h and considering as optimum, pH between 5.0 and 5.3. Matsuka and Shimizu (1971) studied acid phosphatase activity determination with and without dialyzing the samples. They found no differences between both procedures. Ivanov (1978) modified Günther and Burckhart (1967) method by using a lower concentration of substrate, acetate buffer instead of citrate buffer and with magnesium sulphate as catalyst. Afterwards, Sánchez (1999) obtained precise and accurate results modifying the quantity of sample used by Günther and Burckhart (1967).

Studies on food freshness's periods are of particular importance nowadays. The purpose of this work have been to study the evolution, over 3 years, of acid phosphatase activities on honeys originating in areas with two different climates, continental and oceanic, stored at room temperature. Influences of both induced granulation and pH on the evolution of this enzyme have also been researched.

2. Material and methods

2.1. Samples

This study has been carried out on 60 fresh and unheated honey samples 35 of which were harvested in Burgos, a Spaniard area with typical continental climate (CC), and the other 25 were harvested in Galicia, a Spaniard area with typical oceanic climate (OC).

Each sample (1 kg) was divided into two aliquots of 500 g and aseptically bottled. One of the aliquots was labelled as "A" and was directly stored. In the second one, labelled as "B", crystallisation was induced by seeding with 10% of finely crystallised honey. In order to achieve the best conditions for the induced granulation, moisture percentage of this finely crystallised honey was lower than 18.5% and glucose/water ratio was higher than 1.80 (Gonnet, 1992). Acid phosphatase activity and pH of this finely crystallised honey were analysed, as well. Both honeys were mixed avoiding air bubbles, which could spoil the *new crystallised* honey. Complete and homogeneous granulation was reached between 3 and 20 days from the seeding. The texture observed was very fine-grained in all samples.

Samples were stored in darkness at room temperature. The analyses were carried out over 3 years, each five months, at 5, 10, 15, 20, 25 and 30 months. The first five months after harvesting were necessary for collecting and selecting the samples for induced crystallization.

2.2. Methods

Acid phosphatase activity was determined according to the method of Günther and Burckhart (1967), modified by Sánchez (1999). The method is based on the reaction between disodium 4-nitrophenylphosphate and water at acid pH catalysed by acid phosphatase. The amount of 4-nitrophenol produced is measured spectrophotometrically at 400 nm, using a Kontron 922 Uvikon double beam spectrophotometer. A blank is prepared with 2 ml buffer and 10 ml NaOH 0.02 N. A calibration curve must be drawn. Acid phosphatase activity is calculated as mg P/100 g honey/24 h.

$$\text{mg P/100 g honey/24 h} = \text{mg P(std)} \times \frac{30.97 \times 5 \times 24 \times 100}{139.1 \times 1 \times 3 \times 1.25}$$

where: P = phosphorous; 30.97 = atomic weight of phosphorous; 5 = ml in which sample is dissolved; 1 = ml of honey solution for the assay; 3 = h for the assay; 139.1 = molecular weight of 4-nitrophenol; 1.25 = g sample; 100 = grams honey; 24 = h.

Statistical approaches were developed by applying Student's *t*-test, ANOVA and Kruskal-Wallis tests of STATGRAPHIC 4.0 PLUS 3 for Windows.

Taking into account the coefficient of variation of the method, a confidence interval has been calculated for each acid phosphatase activity value for a confidence level of 95%. Then, the interval of confidence is

$$x \pm Z_{\alpha/2}\frac{\sigma}{\sqrt{n}},$$

where x is the value obtained; $z_{\alpha/2} = 1.96$ is the theoretical statistical; σ is the standard deviation obtained after 10 measurements of the same sample; n is the number of measurements on the same sample, in this case, 10.

For this parameter, the confidence level for each value at a level of 95% ($\alpha = 0.05$) will be $X \pm 0.8$.

pH was determined using a pHmeter Crison micropH 2001 with an electrode Crison Ref. 104053931, according to the AOAC (2000) procedure (method 962.19).

3. Results and discussion

Fig. 1 shows the evolution of acid phosphatase activity averages. Acid phosphatase activities have been higher in honeys from OC (average 95.2 mg P/100 g honey/24 h) than in honeys from CC (average 60.3 mg P/100 g honey/24 h). In comparison with the initial values, all phosphatase activities have decreased in all samples at 30 months. The decrease of acid phosphatase activity in honeys from OC has been significantly lower (95% confidence level) than the decrease of this enzyme in honeys from CC. In general, trends of acid phosphatase activity evolution for "A" and "B" samples originated from CC and OC areas have been similar.

3.1. Acid phosphatase activity average evolution

Acid phosphatase activity average has decreased during the first months, then it has increased, but reaching lower values than those found in the beginning of the study. In all samples significant differences have been found between acid phosphatase activity averages at 5, 10, 15, 20, 25 and 30 months at the 95% confidence level ($p < 0.05$). In samples "A" from CC acid phosphatase activity average increases at 20 months. In the rest of the samples, acid phosphatase activity average increases at 25 months. At 30 months the mean value for this enzyme starts to decrease in all honeys.

With regard to the maximum percentages of decrease for all samples no significant differences have been found for CC "A" and "B" samples and for OC "A" and "B" samples.

3.2. Acid phosphatase activity evolution for individual samples

3.2.1. Honeys from continental climate (CC samples)

5–10 months: 91.4% "A" samples and 91.4% "B" samples showed a decrease of acid phosphatase activity.

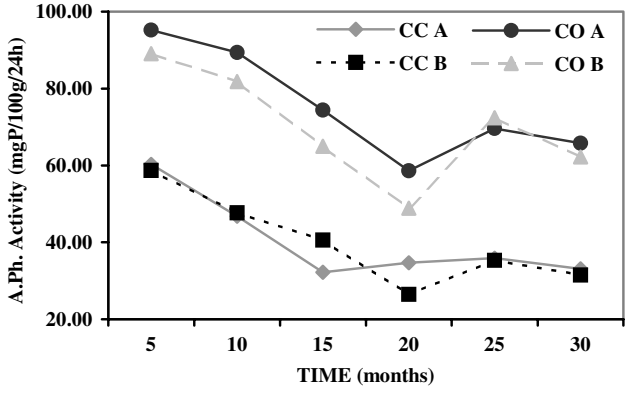

Fig. 1. Evolution of acid phosphatase activity averages for "A" and "B" samples originated from continental climate area (CC) and oceanic climate area (OC).

10–15 months: 94.3% "A" samples, and 91.4% "B" samples showed a decrease of acid phosphatase activity.

15–20 months: 34.3% "A" samples, and 97.1% "B" samples showed a decrease of acid phosphatase activity.

20–25 months: 8.6% A" samples, and 2.9% "B" samples showed a decrease of acid phosphatase activity.

25–30 months: 51.4% A" samples, and 77.1% "B" samples showed a decrease of acid phosphatase activity.

3.2.2. Honeys from oceanic climate (OC samples)

5–10 months: 60% "A" samples and 88.0% "B" samples showed a decrease of acid phosphatase activity.

10–15 months: 100% "A" samples, and 100% "B" samples showed a decrease of acid phosphatase activity.

15–20 months: 100% "A" samples, and 96.0% "B" samples showed a decrease of acid phosphatase activity.

20–25 months: 0% "A" samples, and 0% "B" samples showed a decrease of acid phosphatase activity. This enzyme increased in all samples.

25–30 months: 60.0% "A" samples, and 96.0% "B" samples showed a decrease of acid phosphatase activity.

From 25 to 30 months, the vast majority of samples from both origins showed a decrease of acid phosphatase activity.

In general, the behaviour of "B" samples, was similar of the behaviour of "A" samples, with the exception of the results of honeys from CC at 20 months, that showed a decrease of acid phosphatase activity in more samples "B" than "A", as well as the results at 25 months that showed an increase of acid phosphatase activity in more samples "B" than "A".

As it has been said, Giri (1938) demonstrated that acid phosphatase was related to honey's fermentation. This fact agrees with the results of our study. Honey's acid phosphatase activity has decreased throughout the first months of storage, but as soon as honeys has started to ferment, this enzyme has increased before in honeys "A" from Burgos (at 20 months) than in the rest of the analyzed honeys (at 25 months). After this first increase of acid phosphatase activity, this enzyme has tended to decrease in all samples. It is necessary to remember that the growth of yeasts is exponential. At first yeasts grow very fast, giving rise to an increase of acid phosphatase activity. Then, the rate at which microorganisms grow slows down, so that the logical decrease of acid phosphatase activity due to honey's aging, goes beyond the increase of this enzyme due to yeasts' growth.

If fermentation is characterized by an increase of acid phosphatase activity, it would start at 20 months in all samples from OC and in "B" samples from CC. In "A" samples from CC, acid phosphatase activity increased continuously from 15 months onward. Thus, induced granulation seemed to delay fermentation apparently, but no statistical significant differences have been found between honeys "A" and "B". We have

observed that in general, free acid, which is also a parameter related to honey fermentation, steadily increased from 20 months onward.

After studying the evolution of each sample, two main types of kinetics have been found: linear kinetic ($y = A + Bx$) and reciprocal-x kinetic ($y = A + B/x$). In the vast majority of samples, the relationships found have been significant. Coefficient of correlation (r) has ranged between 0.7351 and 0.9688. Probability (P) has ranged from 0.0001 to 0.1. With regard to honeys originated from continental climate area, 54.3% "A" samples have shown a linear kinetic whereas 31.4% have shown a reciprocal-x kinetic. 60.0% "B" samples have shown a linear kinetic whereas 28.6% have shown a reciprocal-x kinetic. In respect of honeys originated from oceanic climate area, 76.0% "A" samples have shown a linear kinetic, whereas 20.0% have shown a reciprocal-x kinetic. 32.0% "B" samples have shown a linear kinetic, whereas 28.0% have shown a reciprocal-x kinetic.

It is very interesting to highlight that a linear and significant relationship has been found between honeys' acid phosphatase activity at the beginning of the study and the activity of this enzyme at each time of analysis. This relationship is significant for both all "A" and "B" samples. Table 1 shows all coefficients of correlation, as well as the equations for the relationships at each period of analysis. After applying a t-test to each pair of results, no significant differences has been found (95% confidence level) between experimental values and those calculated with the equations. Thus, with simple equations it is possible to know at each point acid phosphatase activity if the initial phosphatase activity value is known.

Table 1
Coefficients of correlation and equations between initial acid phosphatase activity and values of this enzyme found at each period of analysis

Acid phosphatase activity	CC (5 months)		OC (5 months)	
	A	B	A	B
10 months	0.8547* $y = 9.9764 + 0.6103x$	0.9531* $y = 9.1267 + 0.6571x$	0.9593* $y = -16.8923 + 1.1167x$	0.9665* $y = 2.9508 + 0.8812x$
15 months	0.8430* $y = 1.3344 + 0.5116x$	0.8690* $y = 12.2887 + 0.4830x$	0.9667* $y = -8.0929 + 0.8668x$	0.7642* $y = 5.2170 + 0.6709x$
20 months	0.8281* $y = 8.7083 + 0.4307x$	0.7394* $y = 1.8920 + 0.4198x$	0.9134* $y = -18.5326 + 0.8101x$	0.9359* $y = -9.1076 + 0.6513x$
25 months	0.8075* $y = 9.0397 + 0.4457x$	0.6854* $y = 13.7355 + 0.3672x$	0.9343* $y = -13.1192 + 0.8692x$	0.9066* $y = -1.7253 + 0.8321x$
30 months	0.7376* $y = 9.9939 + 0.3824x$	0.7441* $y = 10.6707 + 0.3557x$	0.9088* $y = -10.7393 + 0.8045x$	0.9238* $y = -4.6615 + 0.750x$

y, acid phosphatase activity at each time; x, acid phosphatase activity at 5 months.
* Significant correlation at 99% ($P < 0.01$).

Table 2
Coefficients of correlation and equations between initial pH and percentages of variation of acid phosphatase activity observed in the different analysis that have been carried out

% Variation	CC		OC	
A.Ph.	pH A	pH B	pH A	pH B
10 months	0.2962* $y = 33.364 + 12.887x$	0.0035 $y = 15.823 + 0.150x$	−0.6549*** $y = 93.86 - 20.614x$	−0.1052 $y = 18.126 - 2.505x$
15 months	−0.3017* $y = 115.136 - 16.585x$	−0.1549 $y = 57.201 - 7.104x$	−0.3823* $y = 56.46 - 8.136x$	−0.1444 $y = 52.070 - 6.115x$
20 months	−0.2527 $y = 81.398 - 9.939x$	−0.0401 $y = 63.067 - 2.279x$	−0.5723*** $y = 125.813 - 20.462x$	−0.5357*** $y = 102.503 - 13.618x$
25 months	−0.3385** $y = 96.319 - 14.016x$	−0.2295 $y = 90.771 - 13.138x$	−0.4432** $y = 93.564 - 15.664x$	−0.5303*** $y = 116.405 - 23.539x$
30 months	−0.3066* $y = 101.327 - 14.185x$	−0.2251 $y = 86.251 - 10.330x$	−0.3452* $y = 77.559 - 10.967x$	−0.5382*** $y = 97.345 - 16.045x$

y, acid phosphatase activity at each time, starting at 5 months; x, pH at 5 months.
* Significant correlation at 90%.
** Significant correlation at 95%.
*** Significant correlation at 99%.

Table 3
Coefficients of correlation and equations between pH and acid phosphatase activity found at each period of analysis

	CC		OC	
	A	B	A	B
5 months	0.4210^{**} $y = 3.8097 + 0.0050x$	0.3954^{**} $y = 3.8219 + 0.0047x$	0.1222 $y = 4.0561 + 0.0015x$	0.1547 $y = 3.9881 + 0.0018x$
10 months	0.3026^{*} $y = 3.9163 + 0.0043x$	0.4856^{***} $y = 3.7389 + 0.0071x$	0.1859 $y = 4.0325 + 0.0015x$	0.1988 $y = 3.9630 + 0.0020x$
15 months	0.6413^{***} $y = 3.7678 + 0.0106x$	0.6523^{***} $y = 3.5629 + 0.0128x$	0.1798 $y = 4.0284 + 0.0020x$	0.1501 $y = 3.9654 + 0.0016x$
20 months	0.6709^{***} $y = 3.5978 + 0.0130x$	0.4402^{***} $y = 3.8526 + 0.0080x$	0.3188 $y = 3.916 + 0.0033x$	0.2691 $y = 3.8902 + 0.0034x$
25 months	0.6531^{***} $y = 3.6052 + 0.0112x$	0.7218^{***} $y = 3.4446 + 0.0141x$	0.2472 $y = 3.8963 + 0.0022x$	0.2735 $y = 3.8459 + 0.0024x$
30 months	0.7682^{***} $y = 3.496 + 0.0132x$	0.7262^{***} $y = 3.4261 + 0.0150x$	0.2882 $y = 3.8412 + 0.0027x$	0.2800 $y = 3.7863 + 0.0028x$

y, pH at each time; x, acid phosphatase activity at each time.
* Significant correlation at 90%.
** Significant correlation at 95%.
*** Significant correlation at 99%.

3.2.3. Influence of pH

In all samples of this study pH ranged from 3.6 to 4.9. There have neither been found significant differences between pH of honeys from CC and pH of honeys from OC, nor between pH of honeys "A" and "B" (at 95% confidence level). Honeys' pHs have been lower than the optimum pH for acid phosphatase activity (4.5–6.5). However, we have found that honeys with higher acid phosphatase activities are not those samples with higher pH.

In "A" samples a relationship has been found between the initial value of pH and some of the different percentages of acid phosphatase activity variation throughout time. However, in the vast majority of "B" samples this relationship has not been found. In all samples in which this relationship has been significant, it has been demonstrated that the higher pH, the lower the decrease of acid phosphatase activity, except at 5 months in "A" samples from CC. These results agree with those of Sánchez (1999), who observed in honeys from Galicia an inverse relationship between initial pH of samples and the percentage of decrease of acid phosphatase activity. Table 2 shows all coefficients of correlation, as well as the equations for the linear relationships found for each group of samples.

A linear and significant relationship has been found between acid phosphatase activity and pH values throughout the study for both "A" and "B" samples originated from CC. This relationship has not been found for honeys originated from oceanic climate area. Table 3 shows all equations and coefficients of correlation in respect to this relationship.

In "A" samples, acid phosphatase activity has steadily decreased throughout 15 months in honeys from continental climate and throughout 20 months in honeys from oceanic climate. Then, it has increased probably due to yeasts' growth. Eventually, acid phosphatase activity has decreased in honeys from oceanic climate, whereas it has kept pretty constant in honeys from continental climate.

Induced granulation hardly modifies the trend of acid phosphatase activity evolution.

pH of samples has demonstrated to have a strong influence on the activity of acid phosphatase, so that the higher the pH, the lower the decrease of acid phosphatase activity. In honeys from continental climate, pH and acid phosphatase activity are correlated throughout the study.

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References

AOAC (2000). *Official methods of analysis of AOAC international* (17th ed.). Arlington, Virginia, USA.

Giri, K. V. (1938). The chemical composition and enzyme content of indian honey. *Madras Agricultural Journal, XXVI*(2), 68–72.

Gonnet, M. (1992). Cristalización dirigida de mieles. *Vida Apícol., 51*, 46–55.

Günther, F., & Burckhart, O. (1967). Bestimmung der Sauren Gesamt-phosphatase in Honig. *Deutsche Lebensmittel-Rundschau, 63*(2), 41–44.

Ivanov, T. (1978). Study of invertase, acid and alkaline phosphatase and esterase activities in bee honey. *Zhivonovud Nauk., 15*(3), 103–112.

Ivanov, T. (1981). Glucose oxidase, catalase and proteolytic enzyme activity and enzyme inactivation in heated and preserved honey. *Zhivonovud Nauk., 18*(6), 119–125.

Matsuka, M., & Shimizu, Y. (1971). Enzyme activities in domestic and imported honey samples. *Tamagawa Daigaku Nogakubu Kenyu Hokoku, 11*, 73–77.

Sánchez, M. P. (1999). Evolución de la actividad de las principales enzimas de la miel "Producto galego de calidade-mel de Galicia". Tesis Doctoral. Universidad de Santiago de Compostela, Facultad de Farmacia.

Santana, F. J. (1993). Diastasa, invertasa, β-glucosidasa y fosfatasa en la miel. *Tesina de Licenciatura*. Facultad de Farmacia, Universidad de Santiago de Compostela.

White, J. W. Jr., (1975). La miel. In C. C. Dadant (Ed.), *La colmena y la abeja melífera* (pp. 655–682). Montevideo, Uruguay: Hemisferio Sur.

White, J. W. Jr., (1978). Honey. *Advances in food research* (vol. 24, pp. 288–354). New York, San Francisco, London: Board Academic Press.

White, J. W. Jr., (1979). Composition of honey. In E. Crane (Ed.), *Honey: A comprehensive survey* (pp. 157–206). London: Heinemann.

Zalewski, W. (1965). Fosfatazy w miodach. *Pszczel. Zeszyty Nauk., 9*(1/2), 1–34.