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Inactivation of glucose oxidase during heat-treatment de-crystallization of honey

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Abstract

Glucose oxidase (GOD) is an enzyme which is secreted from hypopharyngeal gland of the bee into the nectar during the honey ripening process. Most honey types are supersaturated solutions of carbohydrates, as a result of which they are subject to crystallization, which is undesirable for many reasons. In order to prevent or eliminate honey crystallization, it is heat-treated and liquefied; however this results in decreased enzyme activity.

We investigated how short-time heat treatment of honey affected GOD activity and stability at temperatures ranging from 20 to 70°C using a “Melitherm” heater and in air-tight vessels in a water bath.

During honey de-crystallization at a temperature of up to 55°C, GOD remained stable, its activity varied only negligibly, while at 55–70°C it declined. Activation energy of GOD inactivation process at the heat treatment temperature ranges from 20 to 50°C and 50 to 70°C was calculated as 15 and 60.5 kcal mol⁻¹ respectively and it was found to be related to crystal melting time and temperature.

Key words: glucose oxidase, heat-treatment, honey, crystallization, HMF, lactone acidity, melting.

Introduction

The world's annual honey production totals about 1.2 million tons and accounts for up to 1% of the total sugar consumption. Economically developed countries have the highest consumption of honey. In the European Union, its annual consumption per capita varies between countries. In Italy, France, the UK it amounts to 0.4 kg, while in Germany, Austria, Portugal, Greece, and Lithuania it amounts to 1–1.8 kg (Bogdanov et al., 2008).

Honey is used for various purposes – human nutrition, treatment of digestive and skin diseases, and it has been applied in folk medicine since olden times (Jeffrey, Echazarreta, 1996; Baltuškevičius, 2003; Alvarez-Suarez et al., 2010). As a result, its quality has to be as high as possible and must meet specific requirements and standards (Bogdanov et al., 2001; LST, 2004).

Honey is composed of organic compounds, mainly reducing sugars, of which fructose accounts

for the largest share 38.4%, glucose for 30.3%, maltose for 7.3%, sucrose for 1.3% etc. (White, 2003). These compounds are suitable for the propagation of sugar-tolerant yeasts, as a result, at favourable temperatures (23–27°C), moisture content of 17.1–21.1%, and depending on the yeast count present in honey, fermentation starts, which renders honey unfit for consumption (White, Kushnir, 1992; Wang et al., 2004). In order to keep honey fit for consumption as long as possible its moisture content should be <17.1% and storage temperature below 11°C (Subramanian et al., 2007), it should be kept in a dry and dark place. However, it is not always possible to meet these storage requirements, since sometimes rainy and wet weather occurs during the honey ripening, which may result in honey moisture exceeding 18% because of incomplete honey evaporation. It is complicated to maintain optimal storage temperature because extra cooling facilities are needed,

especially for beekeepers, as well as when honey is sold in markets or shopping centres; as a result honey is often stored at a common temperature of 16–25°C. Unprocessed honey stored at 0–27°C temperature starts to form crystals due to glucose present in it, which begins crystallizing by gradually binding water molecules (White, 2000). The size of crystals, crystallization time etc. depend not only on the storage temperature but also on botanical origin of honey, and size and amount of mechanical particles present in it, and ratio of sugars (Чернигов, 1992; Subramanian et al., 2007). Biochemical parameters of honey change in relation to storage temperature, shelf life, processing, treatment, microorganism contamination (Baltuškevičius, 2003; Khan et al., 2007).

Honey consistency is changed and de-crystallized through heat treatment for the following reasons:

1. To prepare a consistency (liquid, soft, creamy etc.) attractive to a consumer.
2. To prevent or stop fermentation process of honey whose moisture content exceeds 18% by pasteurization and at the same time reduce moisture content to 17.1%.
3. To facilitate packaging of honey stored in large wholesale containers into smaller cruets 150–1500 g in volume, which is more acceptable for consumers.

Most consumers prefer liquid, freshly – looking honey, the consistency of which is achieved by heat-treatment at a temperature of 35–48.9°C (James, 1992). However, this treatment does not prevent fermentation and honey is subject to rapid crystallization. As a result, it is recommended heating honey at 63–77°C followed by cooling (Subramanian et al., 2007). During this process, yeast is killed, crystals melt and alterations occur in biochemical parameters, inactivation of enzymes (GOD, diastase, invertase) starts (White, 1966), and antioxidant activity can decline (Wang et al., 2004). An opinion prevails in literature that GOD stability in honey exposed to high temperature is lower than that of other enzymes (diastase, invertase) (White, 1966).

High value GOD activity is a very important property, because it produces gluconolactone and hydrogen peroxide by oxidizing glucose, during honey ripening process. GOD could begin to produce hydrogen peroxide repeatable in mature honey, when honey is diluted with warm water. During this action, growth of most bacteria and other pathogens may be inhibited (White, 1966; Alvarez-Suarez et al., 2010).

The parameter of diastase (α -amylase) activity is regulated by the International Honey Commission (IHC) and it should indicate whether or not honey has been exposed to high temperatures. Invertase (α -glucosidase) is an enzyme much more sensitive to heat treatment and longer storage time, as a result the Commission recommends using it to estimate the damage level of softly heat-treated honey and its freshness. Invertase activity is measured in routine assays in Germany, Belgium, Spain, Italy and Switzerland (Bogdanov et al., 2001). The activity of GOD has not been regulated in honey.

A rather grave problem resulting from honey heating is formation of hydroxymethylfurfural (HMF) during fructose breakdown process. HMF is toxic and cancerogenic (Michail et al., 2007), therefore it is mentioned in all honey standards and is used while regulating honey quality. In unheated honey HMF amounts to ~5–30 mg kg⁻¹ (Bogdanov et al., 2001), and the allowable limit laid out in Lithuania's and EU standards is up to 40 mg kg⁻¹.

In Lithuania, honey raw material is generally packed and stored in plastic food containers with a storage capacity of 30–60 kg. Before packing, honey has to be de-crystallized and strained. The most common technique used for honey liquefaction is convection method which involves placing a honey container into a thermostat heated by air or water. For a complete dissolution of crystals, such honey is heated in a pre-set, constant temperature for up to several days. Liquefaction/melting starts from the container's sides and progresses towards the centre, since honey firstly heats up at the wall sides and heat moves towards the centre. Honey that has already melted is further unnecessarily heated, which is a waste of energy, and which inactivates the above-mentioned enzymes and increases HMF content. To prevent this it is recommended heating honey by a special heater "Melitherm" (Bogdanov, 1994). The peculiarity of the melitherm system is that honey is melted only in the area where heating element comes into direct contact with honey. Heating element is adjusted to the required temperature. Contact time of heater with honey is short. Only liquefied/melted honey gets into collection vessel, which can be cooled in ice. De-crystallisation of honey using this method prevents honey from unnecessary long-term overheating.

Considerable research evidence on how heat treatment of honey can affect GOD activity has been found in literature. However, there is no research into GOD from honey temperature inactivation kinetics and heat resistance. Our study was designed to assay GOD heat resistance in honey and

to ascertain the effects of a device widely used in Europe for honey de-crystallisation on GOD activity, lactone acidity and HMF content.

Materials and methods

Samples of fresh unprocessed buckwheat honey were collected in Trakai district from 81 ha of one variety crop field, spring honey from Kaunas district, rape honey from 53 ha crop field in Kėdainiai district in 2008 season.

Honey botanical origin was determined using melissopalynological method (Louveaux et al., 1978). Pollen number in a sample was counted and expressed in percent value. If honey has more than 45% of pollen from one plant, honey is considered to be monofloral and is named after that plant species.

Each 1500 g fresh unprocessed honey sample was placed in a plastic container. The samples were kept in the original container and stored at 5°C in the dark for sixth months. After sixth months, when honey became fully crystallized, liquefaction was performed.

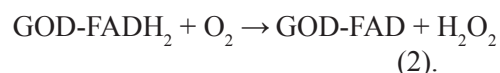
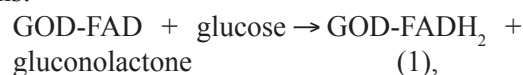
Honey processing was performed by two methods.

Using a special heater "Melitherm", with a power of 500 W, temperature adjustment range 30–85 ±5°C, made in France. Using this device buckwheat, spring, and rape honeys were heated at 40, 55 and 70°C temperatures. To make sure that the coming out liquefied honey is being dissolved in the required temperature, a control mercury thermometer was fitted very close to the heating element. A total of 5 kg honey was de-crystallized at each temperature tested. In the collection vessel, the honey was immediately cooled to 25–27°C and packed into food containers 1.5 kg per container. Each temperature treatment was replicated three times. Then the containers were placed for storage at 5°C in the dark until laboratory analyses were commenced.

The other method involved honey liquefying by convection in 1 ml vessels by heating them in a thermostatic water bath at ±0.5°C precision. 0.5 g of honey was placed per jar and time was recorded with a chronometer. De-crystallization was performed at 20, 30, 40, 50, 55, 60, 70°C temperatures for a time span of 0, 10, 20, 30, 60, 90, 120, 150, 180 minutes. 0 min is a starting time when variable temperature (transient heating) reaches the target de-crystallization temperature (isothermal heating) in a jar. For each temperature treatment, to control temperature and monitor transient heating, an electronic thermometer probe was placed in one jar with

honey, which was not being tested. The samples were cooled in ice-cold water to room temperature (20°C). Each experimental treatment was replicated twice.

Enzyme assay. Glucose oxidase (GOD) performs selective oxidation of glucose in the presence of the dissolved oxygen, according to the following reactions:



The enzymatic oxygen consumption was detected amperometrically using Clark-type membrane oxygen electrode (Clark et al., 1953). The reaction was performed in the stirred 1.4 ml PMMA cell, equipped with oxygen electrode. Both – Pt working (diameter 0.2 mm) and Ag/AgCl reference – electrodes were covered with gas-permeable 5 µm thick Teflon membrane. The controller was used to apply a voltage to the Pt electrode that is –0.6 V versus Ag/AgCl reference electrode. The resultant current proportional to the oxygen concentration in the air-saturated solution was displayed and saved with a data logger. Analytical grade, previously desiccated glucose (Lach-Ner, Czech) solutions were prepared one day before the experiment, for mutarotation complete.

The reaction mixture consisted of 3 M glucose in the 0.1 M sodium acetate buffer (pH = 5.8), 0.05 mM NaN₃ was added to suppress a catalase activity. Honey test samples were prepared by dissolving honey in the acetate buffer solution (1:1 w/v). The substrate solution was thermostated in the cell, 0.1 ml of honey solution was added, and a rate of oxygen uptake was recorded. The amount of oxygen utilized during the test corresponds exactly to the amount of glucose in µmol. The GOD activity is expressed as IU (International Units): µmol of oxygen consumed in 1 min by the enzyme contained in 1 g of honey. The honey GOD activity was calculated after equation:

$$IU / g) = \frac{[O_2] \times v \times 1.4}{I_0 \times 0.058},$$

where: [O₂] (µmol ml⁻¹) – concentration of oxygen in the buffer solution,

v (nA min⁻¹) – the rate of oxygen consumption,

1.4 (ml) – cell volume,

I₀ (nA) – blank oxygen reduction current,

0.058 g – amount of honey in the 0.1 ml of

sample.

GOD activity was measured in triplicate.

Lactones acidity, pH were determined according to International Honey Commission (IHC, 2002). The pH values were measured in 10% honey solution. The acidity of the lactones was obtained by adding an excess sodium hydroxide to the honey solution and plotting the neutralization curve of the excess sodium hydroxide by a back titration with 0.05 M hydrochloric acid.

HMF content was determined after Winkler method (IHC, 2002). The method determines the content of HMF defined as the constituents of honey which are capable of combining with barbituric acid and p-toluidine under the conditions of the test after clarifying samples of honey with Carrez solutions (IHC, 2002). Spectrophotometer ("Thermo Spectronic", model UV 300, UK) for measuring at the wavelength 550 nm was used. HMF content was determined in triplicate.

Sugars content was determined by HPLC method, according to IHC (2002).

The moisture content was determined using the refractometric method according to IHC (2002). The moisture content of honey samples was measured at 20°C temperature after waiting for 6 mi-

minutes for equilibration using a digital refractometer ("Gentaur Europe", model VHN1, Belgium), which is suitable for special honey moisture content determination with resolution 0.1%. Determinations were triplicated.

Statistical analysis. All values are expressed as the mean \pm standard deviation. Standard deviations were calculated using *MS Excel 2003*. Statistical significance was set at $p < 0.05$, according to statistical recommendations (Songailienė, Ženauskas, 1985).

Results and discussion

Three different honey types before heat treatment with "Melitherm" were tested. We investigated three different honey samples and identified buckwheat, rape and spring-multifloral pollen more than 45% of pollen from one plant, or simultaneously flowering blooms (spring-multifloral) in separate samples. Honey whose GOD activity was established to be the highest was chosen for further thermostability experiments. Table 1 reflects physico-chemical characteristics of three honey types differing in botanical origin.

Table 1. Characterization of the different honey samples (means \pm standard deviation)

Parameters	Buckwheat	Spring	Rape
GOD activity $\mu\text{mol min}^{-1} \text{g}^{-1}$	1.13 \pm 0.13	1.08 \pm 0.11	0.55 \pm 0.09
Lactone acidity mekv kg^{-1}	29.2 \pm 1.42	16.40 \pm 1.01	8.4 \pm 1.08
HMF content mg kg^{-1}	8.2 \pm 0.90	1.4 \pm 0.34	2.8 \pm 1.12
Fructose g %	39.2 \pm 1.2	35.5 \pm 0.7	38.1 \pm 1.1
Glucose g %	36.8 \pm 0.9	36.5 \pm 1.3	43.4 \pm 1.3
Sucrose + maltose g %	4.4 \pm 0.4	2.3 \pm 0.3	1.6 \pm 0.3
pH	3.6 \pm 0.04	4.5 \pm 0.03	3.7 \pm 0.03
Moisture %	18.4 \pm 0.04	17.2 \pm 0.06	16.3 \pm 0.04

The lowest GOD activity was identified in oilseed rape honey, while the highest in buckwheat honey. GOD is produced by bee hypopharyngeal gland (Ohashi et al., 1999). We practically did not find any research evidence explaining the reasons for different enzyme activity as influenced by botanical origin of honey. It is thought that the amount of enzymes in honey is influenced by nectar thickness and sugar content and by the fact how many times it is transferred from bee to bee before nectar is turned into honey (Bonveli, Torrendo, 2000). In liquid nectar, the activity of GOD results in the appearance of gluconolactone and hydrogen peroxide.

When nectar thickens and is turned into honey, the activity of GOD ceases, however, gluconolactone remains in honey (White, 1966). Lactone acidity and GOD activity in oilseed rape honey are 1.95 times lower than those in spring honey. GOD activity and lactone acidity of spring honey are 5% and 56% lower than those of buckwheat honey. Lactone acidity accounts for the largest share of the total acidity of honey (White, 2003), thus increasing its antibacterial characteristics (Ruks, 1993), and higher enzyme stability of that kind of honey.

The highest HMF content was identified in buckwheat honey. It is an indirect breakdown

product of fructose, and the content of fructose in buckwheat honey was also found to be the highest, moreover, the content of disaccharide was twice as high.

Physico-chemical changes after heat treatment of honey with a “Melitherm” device are presented in Table 2.

GOD activity after heat treatment with up to 55°C practically did not alter, while after exposure to 70°C GOD activity decreased by 8.8%, 11.5%

and 45.1% in spring, buckwheat and oilseed rape honey, respectively.

Lactone acidity with increasing treatment temperature in buckwheat honey was slightly increasing and after treatment with 70°C increased by 13%, compared with the control (without heat treatment). This suggests that even in undiluted honey when it was being liquefied, GOD was activated and produced its metabolic product.

Table 2. Characterization of buckwheat honey samples after heat-treatment using “Melitherm”

T °C	GOD $\mu\text{mol min}^{-1} \text{g}^{-1}$	Lactone acidity mekv kg^{-1}	HMF mg kg^{-1}	Moisture %
Initial	1.13 ± 0.13	29.2 ± 1.42	8.15 ± 0.90	18.4 ± 0.04
40	1.14 ± 0.08	31.2 ± 1.18	11.36 ± 1.17	16.5 ± 0.03
55	1.11 ± 0.11	30.8 ± 0.86	11.52 ± 1.09	16.6 ± 0.01
70	1.00 ± 0.12	33.4 ± 0.85	10.69 ± 2.34	16.2 ± 0.02

A slightly elevated HMF content after treatment with “Melitherm” did not exceed the allowable level specified in the international honey standard (40 mg kg^{-1}). Compared with the long-time heat treatment of honey in a water bath for 24 hours at 65°C temperature, with the initial HMF content of 9.7 and 8.78 mg kg^{-1} , which amounted to 52.7 and 48.2 mg kg^{-1} respectively after treatment, described in literature (Karabournioti, Zervalaki, 2001), we think that the HMF increase after short-time heating is negligible.

Moisture content in honey after melting, irrespective of the treatment temperature, decreased by approximately 2% and might have reached the concentration of supersaturated solution, and it would be difficult to further reduce the moisture content of such honey by a simple heat treatment.

Since GOD activity and lactone acidity were the highest in buckwheat honey we chose it for the experiments. Biochemical changes showed that when honey was shortly heated at 70°C, GOD practically did not lose its activity. As a result, we conducted another experiment which could explain GOD heat resistance in more detail.

Honey samples were heated at 20, 30, 40, 50, 55, 60, and 70°C temperatures for up to 3 hours. The changes of GOD activity in time may be described by a first order kinetic with 0.95 significance (Figure 1).

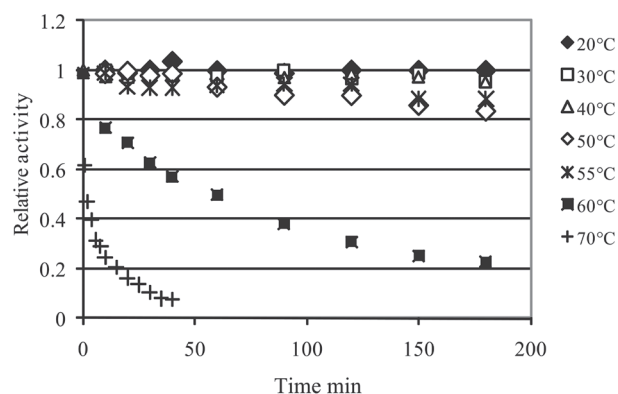


Figure 1. Effect of temperature on the activity of GOD

It is evident that honey heating at temperatures not exceeding 50°C did not exert any significant effect on GOD activity. The half-life of inactivation declines from 450 to 27 hours, when the temperature is raised by 30 degrees. However, having reached 70°C, the half-life shortens up to 5–10 min, although after 1 minute about 80% of the initial activity is still present. Such critical change in the stability is illustrated in Figure 2.

Rate constants of inactivation of GOD were plotted in Arrhenius coordinates. Results show that there are two ranges of temperature affect on enzyme activity (E_a) with different models. In the temperature ranges 20–50°C and 50–70°C, E_a of

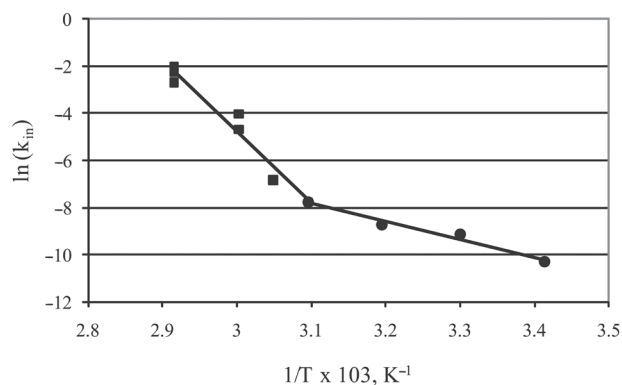


Figure 2. Arrhenius plot for honey GOD inactivation constant

inactivation process was 15 kcal mol⁻¹ and 60.5 kcal mol⁻¹, respectively. The break-point of the graph is observed at 50°C. Four times lower E_a indicates that inactivation process is slackened and is less influenced by the temperature. At this temperature honey is still crystallised, composed of oversaturated sugar solution and glucose in the form of crystal hydrates. Measurement of optical density changes of honey samples during the heating showed that honey de-crystallizes at a temperature of 55°C after several hours. When crystal-melting temperature is reached, the water bound here is released. We suggest that preservation and half-life of enzyme are directly related to sugar-water interactions. Concentration of the soluble enzyme in liquid phase of granulated honey was higher than in fully de-crystallized. Due to water extraction from sugar hydrate after melting, enzyme solution becomes more diluted, and that defines lower enzyme stability. Stabilization phenomenon may be explained as well by the fact that the inactivation of some part of enzyme molecules can be reversible and they are in balance between active and non-active form, as reported in the case of α -amylase (Tosi et al., 2008). Furthermore, thermostability of enzymes purified from honey is lower when it is heat-inactivated in a buffer solution compared to that when honey sample is heated for the same or longer time in the same temperatures (Babacan, Rand, 2007). Buckwheat honey has a rather high content of fructose (Table 1). Fructose crystallises less readily than glucose; as a result, supersaturated fructose solution sticks around glucose crystals and causes formation of fine-crystal structure (Чернигов, 1992). Sorption of the enzyme on crystals surface may take part causing enzyme stabilization. Therefore it is likely that enzyme stability in honey is influenced not only by

the concentration of sugars but also by their composition and consistence.

Conclusions

1. Short-time heat treatment of honey with a temperature up to 55°C using a “Melitherm” device did not affect GOD, while at 70°C GOD activity declined by approximately 10%.

2. HMF increase was inappreciable and did not depend on the treatment temperature.

3. Activation energy of GOD heat inactivation process in crystallized honey up to 50°C was 15 kcal mol⁻¹, and in 50–70°C it amounted to 60.5 kcal mol⁻¹.

4. Our research evidenced that using both methods honey can be heated up to 50–55°C with no damage to GOD activity.

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Gliukozės oksidazės terminė inaktyvacija medaus dekrystalizacijos metu

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Santrauka

Gliukozės oksidazė (GOD) yra išskiriama bičių pažandinių liaukų ir medaus brendimo metu patenka į nektarą. Daugelis medaus rūšių yra persotinti angliavandenių tirpalai, todėl prasideda medaus kristalizacija, kuri dėl daugelio priežasčių yra nepageidaujama. Norint jos išvengti ar panaikinti, medus yra termiškai apdorojamas ir suskystinamas, tačiau sumažėja jo fermentų aktyvumas.

Tirta, kokią įtaką trumpalaikis medaus apdorojimas turi GOD aktyvumui ir stabilumui +20–70 °C temperatūroje, naudojant dekrystalizacijai skirtą prietaisą „Melitherm“ ir hermetiškuose indeliuose vandens vonioje.

Tyrimai parodė, kad medų dekrystalizuojant temperatūroje iki +55 °C, GOD išlieka stabili, jos aktyvumas mažai kinta, o nuo +55 iki +70 °C jos aktyvumas smarkiai mažėja. GOD inaktyvacijos proceso aktyvacijos energija apdorojimo intervaluose nuo +20 iki +50 °C ir nuo +50 iki +70 °C yra atitinkamai 15 kcal mol⁻¹ ir 60,5 kcal mol⁻¹ ir susijusi su angliavandenių dekrystalizacijos laiku bei temperatūra.

Reikšminiai žodžiai: gliukozės oksidazė, dekrystalizacija, medus, kristalizacija, HMF, laktoninis rūgštingumas.