Enzymatic activities in honey

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Abstract

Honey is often mentioned in the context of enzyme content due to their impact on organoleptic properties and the effect of such honey on human health. The aim and task of the paper was to collect expert and scientific information describing enzymes in honey and to compile them in an overview and clarify their functions. There are various types of enzymes present in honey, of which the most important are diastase (α-, β-, γ-amylase), invertase, acid phosphatase, catalase, gluco-oxidase and others. Diastase activity is key in quality analysis, determination of freshness, conditions of production and storage of honey. Diastase is most often analysed in honey. Diastase values decrease if falsified by adding inverted sugar, hydrolysed starch or high-fructose syrup (HFCS). Invertase is an enzyme which hydrolyses sucrose to glucose and fructose, and originates from nectar and bee enzymes. Invertase is considered responsible for chemical reactions during nectar maturation and honey production. Acid phosphatase is mainly found in pollen, but is also an integral part of nectar. It is predominant in fermented honey. Its activity depends on the pH – the higher the pH, the greater the activity of the acid phosphatase. Catalase degrades hydrogen peroxide and reduces the bactericidal effect of honey, and gluco-oxidase originates from the bee, leading to the oxidation of glucose in immature honey. Honey also contains a variety of proteolytic enzymes such as trypsin, chymotrypsin, elastase and others. Enzymes have several important functions, namely the preservation of organoleptic properties and the freshness of honey, preserving the consistency of honey. In addition, enzymes also give honey antimicrobial, anti-inflammatory and antioxidant properties.

Biologically active ingredients, enzymes, honey

Introduction

Enzymes catalyse a large number of reactions that occur in honey during ripening or the process of converting nectar into honey. The enzymes are in their composition or pure proteins or contain a protein fraction. A non-protein chemical compound related to a protein (amino acid) is called a cofactor. Protein components are also called apoenzymes. These two components when combined (holoenzymes) become effective and can perform their function. The entire peptide chain is not required for the catalytic activity of the enzyme. A part of the enzyme molecule that directly participates in the binding of the substrate is called an active centre. It is composed of a small number of functional groups and, if it is a proteidase, the cofactor or coenzyme is included in the active centre (Jašić 2009). The International Union of Biochemists and Microbiologists has published six complete editions of its enzyme nomenclature. In 1950, it was observed that there was a lack of leading authorities in the enzyme nomenclature, although a fairly large number of enzymes were known, and their rapid application began. The designation of enzymes was individualised by those who discovered them, so that each enzyme had several different names. This is why the General Assembly of the International Association of Biochemists established an international enzyme nomenclature in 1955. This step was taken in association with the IUPAC. For this reason, a quality nomenclature has been established to identify enzymes. Enzymes are classified according to their catalytic reactions. A number of systems that have been defined by the Enzyme Commission are used. It consists of four digits that identify the enzyme. The first digit identifies the class of enzymes. The second digit identifies under the class if the enzyme is not present in the first main class. The third digit identifies the enzyme

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class. The fourth digit identifies specific enzymes and is unique for each known enzymatic reaction (Jašić 2009). Enzymes are divided into free and complex enzymes according to their structure, and into endogenous and exogenous enzymes according to their mode of action. Enzymes of the hydrolysis class (lipase, invertase, tannase, chlorophyllase, amylase and cellulase) and oxidoreductase (peroxidase, tyrosinase, catalase, ascorbinase) play an extremely important role in the storage and processing of fruits and vegetables. Enzymes have several important functions, the most important of which are the preservation of organoleptic properties, the freshness of honey and the consistency of honey. Enzymes give honey its antimicrobial, anti-inflammatory and antioxidant properties. A high content of free sugars, oxidative enzymes and low pH make up between environments in which the development and action of most microorganisms is drastically reduced.

Enzymes in honey

Honey contains the following types of enzymes: invertase, diastase (amylase), glucoxidase, catalase, acid phosphatase, peroxidase, polyphenol-oxidase, esterase, inulin and proteolytic enzymes. Some enzymes originate from the bees which they add during the processing of nectar, while others come from pollen, nectar or even from fungi and bacteria present in honey (White et al. 1964).

Part of the enzyme originates from the bees, most often from the honey bee bladder. The bladder, in addition to the storage of nectar, also plays a role in the secretion of enzymes and the initiation of the fermentation of honey. When the bees bring this substance to the litter, it already contains an enzyme. The activity of certain enzymes, or their content in honey, indicates the process of product formation, and also its method and conditions of storage such as: temperature, thermal treatment for different purposes (viscosity reduction, prevention of crystallisation, destruction of certain microorganisms). The temperature greatly influences the decrease in the concentration of diastase and the increase in the concentration of hydroxymethylfurfural (HMF). Hydrogen peroxide forms a compound that plays a role in the antimicrobial effect of honey by the action of glucose oxidase. The activity of gluco-oxidase is adversely affected by light and heat. Since the only difference in the chemical composition between patted honey is the content of enzymes and non-peroxide substances (phenol, flavonoids, other organic compounds), which do not exist in artificial honey, these compounds account for the greater antimicrobial activity of natural honey. During storage, the values of negative optical activity are increased and acidity is low, probably due to enzymatic acid production. Honey storage was followed by a decrease in enzyme activity. The degree of inactivation of the enzyme is influenced by many factors, though the temperature and length of storage are of paramount importance. The activity of the enzyme decreases further with higher water content and lower acidity values (pH). Therefore, the changes are different for each type of honey. The greatest reduction in enzymes during storage at a temperature of 15 – 25 °C is observed in diastase, catalase and acid phosphatase, while invertase and esterase are more stable. For certain types of honey, the inactivation is different even if one of the same herbs is produced. (Plate IV, Fig. 1).

The honey bladder is a temporary warehouse for sweet foods (mostly nectar) with a volume of more than 50 mm^3. No digestion takes place in it as it does not excrete digestive juices. During the collection of nectar, part of the food passes through the honey honey in the middle intestine for the needs of the organism, while the rest refers to the hive for further processing into the final product – honey (Apiculture 2017). The honey bladder contents are handed over to young bees with cranks (8 to 10 bees). In this way, the content of the bladder from one trip is divided among the bee society between 50-fold bee cave. Each bee's radicle receives the content contained in its honey bladder to blend it with its enzymes, and alternately throws it out of the bladder into the space between the rilci and

the lower part of the head and drains back into the honey bladder. When swallowing and pulling into the honey bladder, the content of the honey muscle is mixed with secretions from the flesh (lactic) gland. In this process, the nectar undergoes great chemical changes induced by enzymes (Beekeeping 2010). Enzymes are extremely important components of honey since their activity is considered an indicator of the quality, degree of warming and durability and preservation of honey. Enzymes, together with proteins, give rise to specific characteristics that cannot be produced or replaced artificially.

Table 1. The most important enzymes present in honey (Bihać Veterinary Institute, Faculty of Food and Biotechnology, Zagreb 2013)

Name	Enzyme function
Invertase	Draws sucrose on glucose and fructose (invert sugar) with the formation of smaller amounts of complex sugars
Diastase	Stratifies starch into other carbohydrates
Glucose oxidase	The oxidative reaction translates glucose into glucagonton
Acid phosphatase	Hydrolyses phosphate esters
Protease	Hydrolyses proteins and polypeptides into less peptides
Esterase	Hydrolyses ester bonds
β-glucosidase	Translation of β-glucans into oligosaccharides and glucose

Invertase

Invertase is an enzyme derived from the hypopharyngeal glands in the bee and catalyses the most important reaction in the conversion of nectar into honey which is the hydrolysis of sucrose to glucose and fructose. The content of invertase varies depending on the origin of the honey, the age of the bees, the condition of the hive and the environmental impact. Honey production is a demanding process in which, among other things, care must be taken of the temperature. The changes are ongoing from the moment the nectar is taken from the flower and continue even after the shaking of the ripe honey from the clogged cells of the honeycomb. As the collectors of bees enter the hive when they return from the pasture, they bring the nectar to slaughter house bees that continue to ripen the honey. In this process, the water percentage is reduced to between 15 and 20 percent. The action of invertase can be slowed down if grazing is very intense, so house bees are simply "clogged" with newly created nectar. This can happen in the case of acacia, but converting sucrose into free sugars will only be prolonged for several weeks if the honey is kept at a temperature of 24 – 27 ºC. The mutual ratio of various types of sugar in honey depends on the source, i.e. on the flower grazing, and, to some extent, the enzyme itself. The mature honey usually has no more than 2 % sucrose because the enzyme invertase disposes of sucrose on simple sugars. The enzyme invertase continues its activity in the honey after feeding, if healing of the honey or some other procedure was not destroyed, so by standing between the dawn, i.e. has less and less sucrose (Persano 1995).

The activity of invertase is one of the indicators of the temperature regime during both the course of production and also storage. The activity of invertase is represented as "the number of invertases" or "invertase units". The number of invertases is the amount of sucrose in grams which hydrolyses in 100 grams of honey (1IN = 7.344732 $IU \cdot kg^{-1}$). According to the European Commission, the invertase number should not exceed 10 IN. The invertase unit is a micromolar substrate that hydrolyses for one minute (Serra 2000).

Diastase

Diastase is an enzyme that consists of α-amylase in honey which degrades starch to dextrins and β-amylase that breaks it onto maltose.

The properties of this enzyme have been thoroughly studied, though its role in the ripening of honey has not been clarified. Its activity is one of the main parameters in determining the intensity of warming of honey during processing and storage. Most European regulations, as well as the Bosnian-Herzegovinian Ordinance on methods for controlling honey and other bee products (Official Gazette 37/09), prescribe the determination of the activity of diastase as one of the parameters of honey quality assessment and it is determined for this purpose in the routine quality control of honey, as well as in numerous pieces of scientific research. Like other enzymes and diastases, it is sensitive to heat, and since it is easily measurable, it serves to determine the degree to which honey is damaged by warming. According to the existing data on honey storage at 30 ºC for 200 days, one half of the diastase is destroyed. This means that, in addition to warming, the length of storage of honey at higher temperatures can affect the content of diastase (Babacan et al. 2005).

The content of diastase varies depending on the origin of the honey, for example citrus and white clover have a low content of diastase, while honey from buckwheat has the highest value of diastase. Other factors such as the pH value, amount of nectar and bee nutrition are also affected by the diastase content. Optimal diastase activity can be achieved by controlling the pH value, with the optimum pH being within a range of 4.6 – 5. Diastase is inactive at a pH of less than 3.9, and can be stable at a pH of 7 – 8. If the honey is combined with food containing a lot of starch diastase, it can be inactivated by the addition of ingredients that increase acidity (Esti et al. 1996). According to the rules of the International Commission for Honey, and according to the rulebook on methods of controlling honey and other bee products, the activity of diastase is measured according to Shade and Phadebas.

The principle of the Shade method is based on the hydrolysis of a 1% starch solution with an enzyme of 1 g of honey for one hour at 40 °C (MFA BiH 2009). According to Phadebas, a unit of diastase activity, the Gothe unit, represents the amount of enzyme which converts 0.01 g of starch into the final product (dextrin and maltose) in one hour at 40 °C. The honey policy prescribes the activity of a diastase (according to Schade) of at least 8, i.e. for holes with a poor diastase of at least 3 units, provided that the proportion of HMF (hydroxymethylfurfural) should not exceed 15 $mg \cdot kg^{-1}$ (MVP BiH 2009).

Gluco-oxygenase

Glucose oxidase is an enzyme whose origin in the honey comes from the bee. There is evidence that there are two variants of this enzyme coming from different bee parts which explains different sensitivity of glucose oxidises to temperature and light. Glucose oxidase can originate from fungus, for example Aspergillus niger, and can reach honey. As an invert and diastase, this enzyme plays a role in ripening honey in the honeycomb. Glucose oxidase is most prominent in dilute and immature honey, and when the content of batches is 25 - 30 %. Glucose oxidase causes an oxidative reaction to convert glucose to gluconolactone which passes into gluconic acid and hydrogen peroxide.

Gluconic acid is the main acid in honey and in most cases contributes to the acidity of honey. Hydrogen peroxide is quickly discharged into water and oxygen, and its production and decomposition continue until the nectar turns into honey. Hardening of long honey increases the concentration of hydrogen peroxide. Hydrogen peroxide gives honey an antimicrobial effect. Glucose oxidase is active in nectar, but becomes inactive in the middle. However, it can be reactivated by diluting honey. It is sensitive to temperature and light. The glucose oxidase activity is measured in grams and represents the amount of glucose that is hydrolysed in 100 grams of honey in one hour at 40 °C (Mahmoud 2006).

Catalase

Catalase performs the degradation of hydrogen peroxide into water and oxygen. Its presence in honey is caused by the amount of pollen in honey which is the main source of

the enzyme. The amount of hydrogen peroxide in honey is conditioned by the concentration of the enzymes glucose oxidase and catalase. The higher the concentration of catalase, the lower the concentration of hydrogen peroxide in the honey, and vice versa. The higher the concentration of glucose oxidase, the higher the concentration of hydrogen peroxide in the honeycomb. Due to this, the amount of catalase and glucose oxidase directly influences the antibacterial potential of honey. Catalase is constructed from four subunits (tetramers), each subunit containing one molecule. It is present in almost all organisms, including microorganisms, plants, animals and humans. This enzyme is widespread in humans, especially in liver cells, the peroxisomes and red blood cells (White and Sar 1963). The basic role of this enzyme is the degradation of hydrogen peroxide into oxygen and water.

Quantitative determination of catalase activity is based on the reaction of hydrogen peroxide which remains undesirable after catalase activity with potassium permanganate with free oxygen education.

Beta-glucosidase

Beta-glucosidase, beta-glucosidase, beta-glucosidase, beta-glucosidyl glucohydrolase, arbutinase, amygdalinase, p-nitrophenyl beta-glucosidase, praimereadidase, amygdalase, linamarase, salicylinase, beta-glucosidase, beta-1,6-glucosidase is an enzyme with the systemic name beta-D-glucoside glucohydrolase. The enzyme glucosidase is ubiquitous and occurs in all living entities and is responsible for the hydrolysis of short-chain oligosaccharides and cellulose. This enzyme catalyses the next chemical reaction of terminal non-reducing beta-D-glucosyl residues with the separation of beta-D-glucose. This enzyme has wide specificity for beta-D-glucosides (Chinchetru et al. 1989).

Acid phosphatase

Acid phosphatase, or its concentration, is associated with the fermentation of honey. It is found both in pollen and in nectar. Proven fermented honey contains a higher concentration of acid phosphatase than unfermented honey. Seawater honey has a higher content of acid phosphatase than continental honey. The pH value of honey has been shown to have an effect on the acid phosphatase content, i.e. the higher the pH, the higher the acid phosphatase content. The optimal pH value is between 5.0 and 5.6.

Phosphatase is an enzyme that removes the phosphate group from its substrate by hydrolysis of monoesters of phosphoric acid in a phosphate ion and a free hydroxyl group (Alonso Torre 2006).

This reaction is directly opposed to phosphorylase and kinase reactions which add phosphate groups to their substrates using energy molecules such as ATP. The phosphatase present in many organisms is alkaline phosphatase. Protein phosphorylation is an extremely common and important form of reversible protein posttranslational modification (PTM). It is believed that up to 30% of all proteins are under phosphorylation. Protein kinases (PK) are phosphorylation effects and catalyse the transfer of γ-phosphate from ATP to specific amino acids on proteins (Zhang 2002).

Polyphenol oxidase

Polyphenol oxidase belongs to the tyrosinases (monophenol monooxygenase, polyphenol oxidase, catechol oxidase, oxygen oxide reductase). It contains copper as a prosthetic group and is known under other names such as, for example, catechol oxidase, o-diphenolase, phenolase and polyphenol oxidase (PPO). Tyrosinase is a widely represented enzyme that plays a key role in melanin biosynthesis and other polyphenolic compounds. This enzyme catalyses the ortho-hydroxylation of monophenol to o-diphenol (Jasic 2009).

Esterase

Esterase is a hydrolysis enzyme that separates esters into acids and alcohols in a chemical reaction with water – hydrolysis. There is a wide range of different esterases that differ from one another in terms of the specificity of the substrate, protein structure and biological function.

Proteases

Proteases come from bees and are also known as peptidases or proteins – all of them are enzymes that catalyse proteolysis, that is they catabolise the degradation of proteins, the hydrolysis of peptide bonds that link amino acids in the polypeptide chain. Proteases are involved in the degradation of long protein chains into shorter fragments, separation of peptide bonds in the chain of amino acid residues.

Proteolytic enzymes in very small quantities and not sufficiently well investigated in honey are elastase, trypsin and chymotrypsin. Elastas elastic elastin, in elastic fibres, together with collagen, which determines the mechanical properties of connective tissue. The neutrophil form clears the outer membranes of protein A (OMPA) of E. coli and other Gram-negative bacteria. Elastase also plays an important role in the immune response, since Shigella clears the virulence factor. This is achieved by breaking peptide bonds in the target protein. Specific peptide bonds are decomposed on the carboxyl side of small hydrophobic amino acids such as glycine, alanine and valine. Trypsin cleaves the peptide chain mainly on the carboxyl side of the amino acids lysine or arginine, except when it is followed by a proline. It is used in numerous biotechnological processes. Such processes are commonly referred to as trypsins or proteolysis trypsinisation, and proteins treated with trypsin are said to be trypsinised. Chymotrypsin is formed by the action of trypsin on trypsinogen A and B. It dissolves peptide bonds in denatured proteins.

Conclusions

Honey contains enzymes which, among other things, are responsible for the conversion of nectar and medullary into the ultimate product of honey. Enzymes are complex proteins that are the catalysts of almost all biochemical reactions. Their origin can be from bees that are brought into honey, pollen, nectar and some microorganisms during the processing of nectar. Enzymatic reactions in nectar and during the period of honey maturation contribute to the unique character and functionality of honey which cannot be artificially produced or replaced. Honey contains diastase, invertase, glucoxidase, catalase, acid phosphatase, peroxidase, polyphenol oxidase, esterase, inulin and proteolytic enzymes. Diastase activity is one of the main parameters in determining the intensity of warming of honey during processing and storage. Invertase plays a role in biochemical processes in nectar processing, as well as changes to carbohydrates during the storage of honey. Honey has been proven to have an antimicrobial activity and can even be used for wound and burn treatment. This was originally thought to be due to high osmotic pressure in the honey. It was later found that these properties are derived from glucose oxidase converting glucose into gluconolactone which converts into gluconic acid and hydrogen peroxide with antimicrobial activity. Catalytic activity increases during fermentation. Honey also contains enzymes that hydrolyse phosphate-phosphatase esters (acid phosphatase). All enzymes are almost lost at high temperatures. The analysis of enzymes in honey is carried out because of these properties, on the basis of which the method of storage of certain types of honey can be determined.

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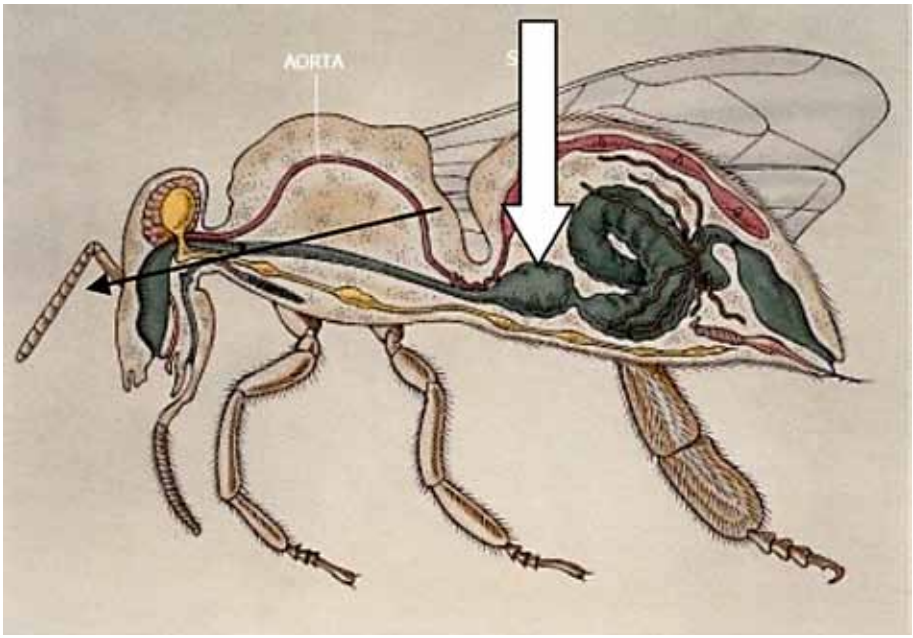

Fig. 1. Honey bladder bees (Apiculture 2017)