Journal of Research in Pharmacy
Research Article
www.jrespharm.com

# Effect of different additives on amylase activity

**Safija HERENDA [1] *, Nataša ĐORĐEVIĆ [2], Selma FETAHOVIĆ [1], Tamara KLISARA [3], Sabina PREVLJAK [4], Edhem HASKOVIĆ [3]**

[1]Department of Chemistry, Faculty of Science, University of Sarajevo, Sarajevo, Bosnia and Herzegovina.
[2]Department of Biomedical Sciences, State University of Novi Pazar, Novi Pazar, Srbija.
[3]Department of Biology, Faculty of Science, University of Sarajevo, Sarajevo, Bosnia and Herzegovina.
[4]Clinical Center of the University of Sarajevo, Organizational unit clinic for radiology, Sarajevo, Bosnia and Herzegovina.

* Corresponding Author. E-mail: safija@pmf.unsa.ba (S.H.); Tel. +387-33 279 995.

Received: 2 February 2024 / Revised: 15 March 2024 / Accepted: 16 March 2024

**ABSTRACT**: Enzymes are catalysts of biological origin, and according to their chemical composition, they are simple or complex proteins. There are several theories about the enzyme's mechanism of action. Today, the Michaelis-Menten theory is generally accepted. According to this theory, during enzymatic reactions, an intermediate compound is created between the enzyme and the substrate. After the formation of this complex, the enzyme catalyzes a chemical reaction that changes the substrate into another molecule, which we call the product. The product is then separated and released from the active site of the enzyme, which is then ready to bind the next substrate molecule. Enzyme activity can be affected by different molecules. The purpose of this study is to use the spectrophotometric approach to determine whether sodium benzoate and ascorbic acid (vitamin C) serve as activators or inhibitors of enzymatic reactions. The obtained results show that both additives bind to the enzyme-substrate complex, causing non-competitive inhibition.

**KEYWORDS**: Enzymes; additives; ascorbic acid; sodium benzoate; inhibition.

## 1. INTRODUCTION

Enzymes catalyze chemical reactions, and this is their biological function. In order to effectively catalyze the transformation of a substrate molecule into a product, the arrangement of chemically reactive groups within the active site must change in terms of spatial orientation, bond strength and bond angle, as well as changes in electronic character. To effect these changes in the structure of the active site the overall conformation of the enzyme molecule must be adjusted, causing changes not only in the active site, but also in the allosteric binding pockets [1]. Salivary amylase (HSA) (Figure 1), the most abundant enzyme in human saliva, initiates the digestion of complex carbohydrates in the human oral cavity, where starch is partially digested into oligosaccharides, maltose and glucose [2].

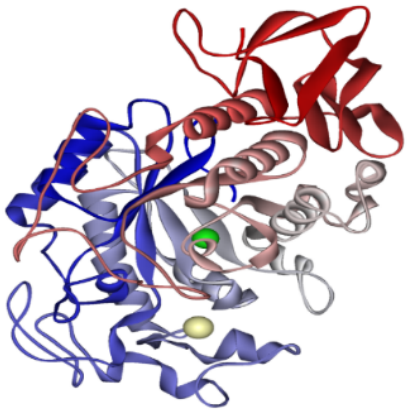

**Figure 1.** Human salivary amylase: the calcium ion is marked with a white ball, while the chloride ion is marked with a green ball

**How to cite this article:** Herenda S, Dordevic N, Fetahovic S, Klisara T, Prevljak S, Haskovic E. Effect of different additives on amylase activity. J Res Pharm. 2025; 29(1): 346-351.


https://dx.doi.org/
ISSN: 2630-6344

The process is then completed by pancreatic α-amylase. There are five isoenzymes of α-amylase in humans. Three isoforms of salivary α-amylase and two isoforms of pancreatic α-amylase are classified as two different isoenzyme families. The three-dimensional structure of α-amylase from human pancreas and saliva and from porcine pancreas was determined by crystallography [3]. All these enzymes are structurally very closely related. Because of its importance in some metabolic disorders, including diabetes and obesity, pancreatic α-amylase has been more extensively studied than salivary α-amylase. As a consequence, a number of pancreatic α-amylase inhibitors are available on the market, such as acarbose, voglibose and miglitol [4]. α-amylase inhibitors help prevent and treat metabolic syndromes such as type 2 diabetes and obesity, in which they control the rise in blood glucose levels by delaying and blocking postprandial carbohydrate digestion and absorption [5]. It has been found that there are different types of molecules that possess inhibitors of α-amylase activity. Among these molecules are additives, flavonoids, polyphenols, condensed tannins, hydrolyzable tannins, terpenes, and cinnamic acid derivatives [6]. Additives are substances with a known chemical structure, that are not consumed on their own, nor are they a typical food ingredient, and as a rule, they have no nutritional value. They are added to food (but also to medicines) during production, processing, storage or packaging, in order to improve their physical and organoleptic properties. Since they are not a natural ingredient in food or medicine, they carry certain health risks. The use of additives in the food and pharmaceutical industry must be under strict legal control and subject to constant checks. From a legislative point of view, any food additive must have a useful and acceptable function or property for its use to be justified. An acceptable function of additives is considered to be the improvement of some of the quality attributes, extension of the shelf life, improvement of texture and organoleptic properties, increase of nutritional value, creation and improvement of functional properties, facilitation of processing and increase of consumer acceptability. If additives are introduced into the body in quantities that are not allowed, then they are harmful and cause diseases. With longer exposure, the risk increases. Many additives are not harmful such as ascorbic acid, sodium carbonate, glycine or citric acid. However, there is a considerable number of additives that have no place in food, and more often those that are obtained synthetically and have no nutritional value [7].

Sodium benzoate (according to the European nomenclature E211) is a salt of benzoic acid that is most commonly produced by the reaction of sodium hydroxide with benzoic acid. It is well soluble in water, tasteless and odourless, and is used as a preservative in food in strictly defined doses due to its antifungal and antibacterial properties.

The permissible limit for its consumption is 0-5 mg/kg of body weight. Sodium benzoate is considered safe for human health if consumed in amounts less than 5 mg/kg of body weight per day. Sodium benzoate does not accumulate in the body. Benzoate is conjugated with glycine to form hippurate in the liver and kidney in a reaction that occurs in the mitochondrial matrix [8].

Upon entering the matrix, the compound is converted to benzoyl-coenzyme A (CoA) (ligase) and then to hippurate (glycine *N*-acyltransferase), which leaves the mitochondria. It is excreted primarily through the urinary system. Some studies indicate that not only is sodium benzoate an excellent preservative, but it may also have potential therapeutic uses in the treatment of diseases such as major depressive disorder (MDD), schizophrenia, autism spectrum disorder (ASD), and neurodegenerative diseases. Ascorbic acid is a derivative of glucose that many plants and animals produce, while humans have to get it through their diet. Ascorbic acid, also known as vitamin C, has previously been reported to inhibit the activity of pancreatic α-amylase, the primary digestive enzyme for starch. The main implication of such inhibition is a slowed rate of digestion of starch into glucose, thereby reducing postprandial hyperglycemia [9]. Considering that starch is the primary source of energy in the human diet, slow starch digestion and glucose absorption can serve as an effective way to prevent and treat hyperglycemia and related metabolic diseases.

Accordingly, some antidiabetic drugs, such as acarbose, have been used to slow starch digestion through inhibition of digestive enzyme activity [10]. However, some drugs are reported to have certain side effects, such as: hepatotoxicity, gastrointestinal disorders and diarrhea. As a group of dietary compounds with beneficial health effects, phenolic compounds have been proposed as natural inhibitors of human digestive enzymes, including α-amylase, α-glucosidase, lipase, etc. One of the known compounds is ascorbic acid, which has shown the ability to inhibit starch digestive enzymes. The basic mechanism could be the presence of hydroxyl groups in ascorbic acid molecules, which can be crucial in binding to pancreatic α-amylase and thus leading to inhibitory activity. When the concentration of ascorbic acid increases, more ascorbic acid molecules contribute more hydroxyl groups to form hydrogen bonds with amino acid residues

Journal of Research in Pharmacy
Research Article

at α-amylase binding sites, which may explain the dose-dependent inhibitory effect [9]. In this work, the influence of sodium benzoate and ascorbic acid additives on amylase enzyme activity was investigated.

## 2. RESULTS AND DISCUSSION

Amylase is the enzyme responsible for the hydrolysis of starch into simpler sugars, and inhibition of amylase activity can have significant implications for controlling blood sugar levels and managing conditions such as diabetes. This study investigates the inhibitory effects of sodium benzoate and ascorbic acid on amylase activity and specifically focuses on the type of inhibition observed. The inhibitory effect of sodium benzoate on amylase activity is a process in which sodium benzoate, as an inhibitor, reduces the rate of the reaction catalyzed by amylase, i.e. the hydrolysis of starch into simpler sugars. Sodium benzoate is a common food additive and preservative used to extend the shelf life of various food products. After oral and dermal ingestion, the benzoate is metabolized in the liver by conjugation with glycine, resulting in the formation of hippuric acid [11,12]. In humans, after oral doses of up to 160 mg/kg body weight, 75-100% of the administered dose is excreted as hippuric acid within 6 hours after application, and the rest within 2-3 days. [13-15]. Based on the Lineweaver-Burk diagram, we determined the type of inhibition and concluded that it is a non-competitive type of inhibition where the maximum reaction rate decreases ($V_{max}$) with the addition of an inhibitor while the value of the Michaelis-Menten constant ($K_m$) does not change. Figure 2, Table 1.

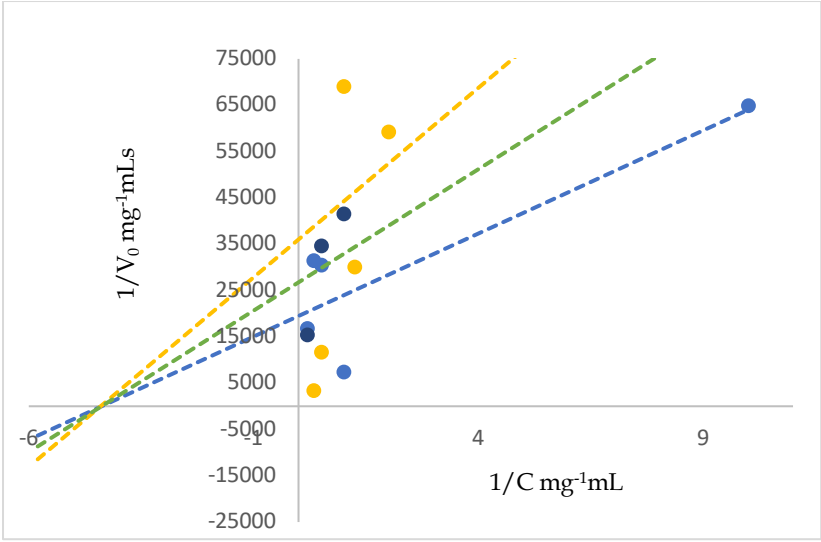


**Figure 2.** Lineweaver-Burk diagram for determining $V_{max}$ and $K_m$ without and with the presence of different concentrations of sodium benzoate; blue line 0 mg/mL; green line 0.02 mg/mL; yellow line 0.033 mg/mL

**Table 1.** Values of $K_m$ and $V_{max}$ without the presence and with the presence of different concentrations of sodium benzoate

| [sodium benzoate] (mg/ml) | $V_{max}$ x10$^{-5}$ (mg·mLs$^{-1}$) | $K_m$ (mg/mL) |
|---|---|---|
| 0 | 5.132 | 0.22 |
| 0.02 | 3.738 | 0.22 |
| 0.033 | 2.777 | 0.22 |

Non-competitive inhibition is a type of enzyme inhibition that occurs when an inhibitor molecule binds to an enzyme-substrate complex and thus prevents the reaction from proceeding in a normal manner. This type of inhibition is characterized by the fact that it is related to the presence of the substrate and does not affect the affinity of the enzyme for the substrate itself. In the absence of an inhibitor, the enzyme binds to the substrate and forms an enzyme-substrate complex, which then catalyzes the reaction and converts the

Journal of Research in Pharmacy
Research Article

substrate into a product, reflecting the maximum speed at which the enzyme can convert the substrate into a product under optimal conditions.

In non-competitive inhibition, the inhibitor specifically binds to the enzyme-substrate complex, changing its conformation. As a result, the enzyme can no longer catalyze the reaction at the same rate as in the absence of inhibition. This lowers Vmax because it reduces the number of functional enzyme sites needed to catalyze the reaction. The Michaelis-Menten constant ($K_m$), which reflects the affinity of the enzyme for the substrate, remains unchanged in uncompetitive inhibition because the enzyme can still bind the substrate with the same affinity, although it cannot catalyze the reaction at the same rate as in the absence of inhibition.

The inhibitory effect of ascorbic acid (vitamin C) on amylase activity can be studied to understand how this vitamin can affect amylase-catalyzed starch hydrolysis. Ascorbic acid is an essential vitamin present in many foods and plays an important role in various biological processes. Based on the presented figure 3, we can conclude that in this case it is also about non-competitive inhibition. Table 2 shows the values of Vmax and Km, which also indicate that it is a non-competitive type of inhibition at concentrations of ascorbic acid of 0.01 mg/ml and 0.02 mg/ml.

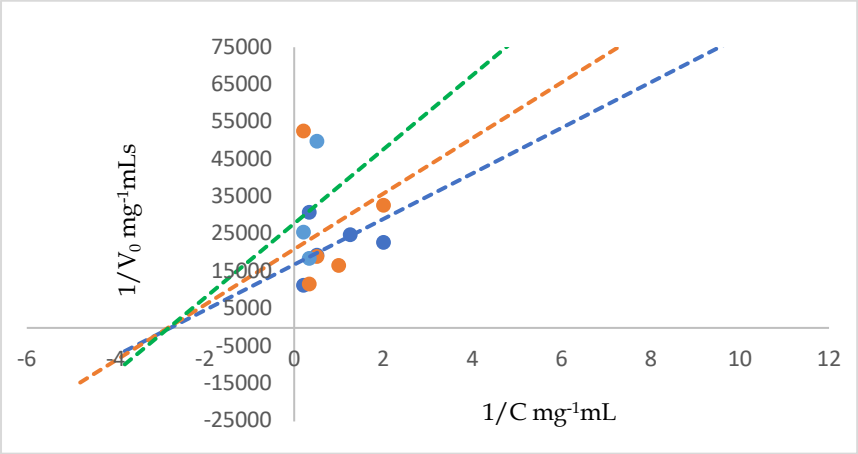


**Figure 3.** Lineweaver-Burk diagram for determining Vmax and Km without and with the presence of different concentrations of ascorbic acid blue line 0 mg/mL; orange line 0.01 mg/mL; green line 0.02 mg/mL

**Table 2.** Values of $K_m$ and $V_{max}$ without and with the presence of different concentrations of ascorbic acid

| [ascorbic acid] (mg/ml) | $V_{max}$ x10$^{-5}$ (mg·mLs$^{-1}$) | $K_m$ (mg/mL) |
|---|---|---|
| 0 | 5.892 | 0.35 |
| 0.01 | 4.753 | 0.35 |
| 0.02 | 3.585 | 0.35 |

As mentioned earlier, the inhibitory effect of ascorbic acid on starch digestion is primarily due to the inhibition of α-amylase. The underlying mechanism could be a consequence of the hydroxyl groups present in ascorbic acid molecules, which may be crucial in binding to pancreatic α-amylase, leading to inhibitory activity. When the concentration of ascorbic acid increases, multiple molecules of ascorbic acid may contribute more hydroxyl groups in the formation of hydrogen bonds with amino acid residues in α-amylase binding sites, which may explain the dose-dependent inhibitory effect. In many studies, different methods are used to determine the effect of ascorbic acid (AsA) on enzyme activity. These methods are primarily based on the determination of dehydroascorbic acid (DHAsA) as a product of AsA oxidation by oxygen or hydrogen peroxide in the presence of ascorbate oxidase and peroxidase [16-19]. Shekhovtsova et al. determined the influence of ascorbic acid on peroxidase and proved that consequently the duration of the induction period on kinetic curves in the presence of AsA may change depending on the pH of the reaction mixture. The reason for this is either a partial inhibition of the enzyme by DHAsA, or a lack of hydrogen peroxide for the oxidation of the main substrate as a result of its partial expenditure to oxidize AsA. [20]. In a study conducted by Tang et al, they also demonstrated the inhibitory effect of ascorbic acid on peroxidase

(HRP). They found that AsA has a serious inhibition effect on the oxidation reaction of 1,5-bis(p-hydroxybenzaldene)-thiocarbohydrazone (BHBTZ) with $H_2O_2$ under the catalysis of HRP [21].

The inhibitory effect of ascorbic acid on β-amylase was proven in the work of authors Rowe and Weill. Evidence is presented to demonstrate that the inhibitory effect of ascorbic acid on β-amylase is due to the formation of an inactive cuprous enzyme. Cuprous ion has been shown to give approximately the same amount of inactivation as cupric ion plus ascorbic acid [22].

## 3. CONCLUSION

The results of this study show that sodium benzoate and ascorbic acid are effective inhibitors of amylase activity, showing non-competitive inhibition. These inhibitors bind to the enzyme-substrate complex and not to the free enzyme or free substrate. As a result, they change the conformation of the enzyme complex and reduce the maximum reaction rate (Vmax), while not affecting the affinity of the enzyme to the substrate (Km). The discovery that sodium benzoate and ascorbic acid act as noncompetitive amylase inhibitors may have significant implications in the context of blood sugar control and the treatment of conditions such as diabetes. These inhibitors may serve as a basis for the development of new therapies or nutritional interventions aimed at controlling the absorption of carbohydrates and regulating blood sugar. Further studies are needed to elucidate the precise molecular mechanisms involved in this noncompetitive inhibition and to explore the potential therapeutic applications of these inhibitors.

## 4. MATERIALS AND METHODS

Material: Starch p.a. Sigma-Aldrich; Amylase enzyme 30 U/mg, Sigma-Aldrich; Phosphate buffer, $KH_2PO_4$ and $Na_2HPO_4$, Fisher Chemical (Wien, Austria); Sodium benzoate ($C_6H_5COONa$), p.a, Sigma Aldrich, Vitamin C, p.a, Sigma Aldrich.

Method: The test of amylase inhibition was carried out by the spectrophotometric method, where the obtained absorbances, using kinetic laws, were translated into appropriate velocities. The Michaelis-Menten equation was used to determine the effect of additives on amylase activity. The measurement was performed at 595 nm, and the reaction mixture (total volume 300 µL) consisted of phosphate buffer pH 7.00 (100 mM), 50 µL enzyme solution, different concentrations of starch from 0.1 mg/mL to 5.0 mg/mL, different concentrations of sodium benzoate 0.02 mg/mL and 0.033 mg/mL, as well as different concentrations of ascorbic acid 0.01 mg/mL and 0.02 mg/mL.



**Acknowledgements:** This study and the project was supported by the Federal Ministry of Education and Science, contract number 05-35-2538-1/23.

**Author contributions:** Concept – S.H., E.H.; Design – S.F., T.K.; Supervision – S.P.; Resources – N.Đ.; Materials – N.Đ.; Data Collection and/or Processing – S.F., T.K.; Analysis and/or Interpretation – S.H., N.Đ., S.P., E.H.; Literature Search – S.F., T.K.; Writing – S.H.; Critical Reviews – S.H., N.Đ., S.P., E.H.

**Conflict of interest statement:** "The authors declared no conflict of interest" in the manuscript.

## REFERENCES

**[1]** Bisswanger H. Enzyme Kinetics. Principles and Methods. 2nd Ed., WILEY-VCH Verlag GmbH & Co. KGaA; 2008.
**[2]** Boehlke C, Zierau O, Hannig C. Salivary amylase—the enzyme of unspecialized euryphagous animals. Arch Oral Biol. 2015; 60(8): 1162–1176. https://doi.org/10.1016/j.archoralbio.2015.05.008.
**[3]** Brayer GD, Luo Y, Withers SG. The structure of human pancreatic α-amylase at 1.8 ˚A resolution and comparisons with related enzymes. Protein Sci. 1995; 4(9): 1730–1742. https://doi.org/10.1002/pro.5560040908.
**[4]** Fujisawa T, Ikegami H, Inoue K, Kawabata Y, Ogihara T. Effect of two α-glucosidase inhibitors, voglibose and acarbose, on postprandial hyperglycemia correlates with subjective abdominal symptoms. Metabolism. 2005; 54(3): 387–390. https://doi.org/10.1016/j.metabol.2004.10.004.
**[5]** Jayaraj S, Suresh S, Kadeppagari RK. Amylase inhibitors and their biomedical applications. Starch. 2013; 65(7-8): 535–542. https://doi.org/10.1002/star.201200194.
**[6]** Zajácz A, Gyémánt G, Vittori N, Kandra L. Aleppo tannin: structural analysis and salivary amylase inhibition. Carbohydr Res. 2007;342(5):717-723. https://doi.org/10.1016/j.carres.2006.12.016.
**[7]** Luo H, Sun X, Xu L, He W, Liang X. A review on stress determination and control in metal-based additive manufacturing. Theor Appl Mech Lett. 2023; 13(1): 100396. https://doi.org/10.1016/j.taml.2022.100396.

**[8]** Lennerz B, Vafai SB, Delaney NF, Clish CB, Deik AA, Pierce KA, Ludwig DS, Mootha VK. Effects of sodium benzoate, a widely used food preservative, on glucose homeostasis and metabolic profiles in humans. Mol Genet Metab. 2015; 114(1): 73–79. https://doi.org/10.1016/j.ymgme.2014.11.010.
**[9]** Guo J, Gutierrez A, Tan L, Kong L. Inhibitory Effect of ascorbic acid on in vitro enzymatic digestion of raw and cooked starches. Front Nutr. 2021; 8: 758367. https://doi.org/10.3389/fnut.2021.758367.
**[10]** Clissold SP, Edwards C. Acarbose. A preliminary review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential. Drugs. 1988; 35(3): 214–243. https://doi.org/10.2165/00003495-198835030-00003.
**[11]** Feldmann RJ, Maibach HI. Absorption of some organic compounds through the skin in man. J Invest Dermatol. 1970; 54(5): 399-404. https://doi.org/10.1111/1523-1747.ep12259184.
**[12]** Feillet F, Leonard JV. Alternative pathway therapy for urea cycle disorders. J Inherit Meta Dis. 1998; 21(1): 101-111. https://doi.org/10.1023/a:1005365825875.
**[13]** Kubota K, Horai Y, Kushida K, Ishizaki T. Determination of benzoic acid and hippuric acid in human plasma and urine by high performance liquid chromatography. J Chromatog. 1988; 425(1): 67-75. https://doi.org/10.1016/0378-4347(88)80007-0.
**[14]** Fujii T, Omori T, Tagucji T, Ogata M. Urinary excretion of hippuric acid after administration of sodium benzoate (biological monitoring 1). J Food Hyg. 1991; 32(3): 177-182. https://doi.org/10.1007/BF01805533.
**[15]** Kubota K. Ishizaki T. Dosedependent pharmacokinetics of benzoic acid following oral administration of sodium benzoate to humans. Eur J Clin Pharmacol. 1991; 41(4): 363-368. https://doi.org/10.1007/BF00314969.
**[16]** Matos R, Angelli M, Lago C, Angnes L. Flow injection analysis-amperometric determination of ascorbic and uric acids in urine using arrays of gold microelectrodes modified by electrodeposition of palladium. Anal Chim Acta. 2000; 404: 151-157. https://doi.org/10.1016/S0003-2670(99)00674-1.
**[17]** Akylmaz E, Dinskaya E. A new enzyme electrode based on ascorbate oxidase immobilized in gelatin for specific determination of L-ascorbic acid. Talanta. 1990; 50:87-93. https://doi.org/10.1016/s0039-9140(99)00107-1.
**[18]** Zhu M, Huang X, Li J, Shen H. Peroxidase-based spectrophotometric methods for the determination of ascorbic acid, norepinephrine, epinephrine, dopamine and levodopa. Anal Chim Acta. 1997; 357:261-267. https://doi.org/10.1016/S0003-2670(97)00561-8.
**[19]** Volotovsky V, Kim N. Ascorbic acid determination with an ion-sensitive field effect transistor-based peroxidase biosensor. Anal Chim Acta. 1998; 359(1-2):143-148. https://doi.org/10.1016/S0003-2670(97)00653-3.
**[20]** Shekhovtsova TN, Muginova SV, Luchinina JA, Galimova AZ. Enzymatic methods in food analysis: determination of ascorbic acid. Anal Chim Acta. 2006;573-574:125-132. https://doi.org/10.1016/j.aca.2006.05.015.
**[21]** Tang B, Wang Y, Du M, Ge JC, Chen ZZ. Catalytic spectrophotometric determination of ascorbic acid in tea drink with 1,5-bis(p-hydroxybenzaldene)thiocarbohydrazone as the substrate for horseradish peroxidase. J Agric Food Chem. 2003; 51(15): 4198−4201. https://doi.org/10.1021/jf030012e.
**[22]** Rowe AW, Weill CE. The inhibition of β-amylase by ascorbic acid. Biochim Biophys Acta.1962; 65(2): 245-251. https://doi.org/10.1016/0006-3002(62)91043-0.