EFFECT OF OLIGOSACCHARIDES ON SALIVARY α-AMYLASE IN VITRO

Ivica Dimov
Medical University Plovdiv, Bulgaria, ivicadimov@gmail.com
Anelia Bivolarska
Medical University Plovdiv, Bulgaria, anellena@abv.bg
Iliya Iliev
Plovdiv University "Paisii Hilendarski", Bulgaria, ilievini@abv.bg

Abstract: Amylases are a part of the group of glycosyl hydrolases enzymes, that catalyze the breakdown of complex carbohydrates. The three main classes are α-, β- and ɣ-amylases. The main function of all amylases is to degrade starch, glycogen and other sugars. All α-amylases are metalloenzymes requiring Ca^{2+} activity. In animals and humans, α-amylases occur in the pancreas, saliva, liver, serum, urine, etc. Salivary and pancreatic amylases are the main ones. They have 97% protein chain homology with each other. The secreted salivary α-amylase initiates the carbohydrates digestion in the mouth.
The concept of prebiotics was introduced in 1995 by Gibson and Roberfroid, as a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves the host's health. Later it became clear, that prebiotics have beneficial effects beyond the gastrointestinal tract. The purpose of the present experiment is to investigate the effect of oligosaccharides on the enzymatic activity of salivary α-amylase, since enzyme inhibitors are used as second line drugs in the therapy of type 2 diabetes.
For this purpose, the α-amylase activity was determined by the Bernfeled method (1955) with some modifications. The reaction time was 10 minutes. Ten measurements for each group have been made. Groups were separareted as it follows: control, 1% lactulose, 2% lactulose, 1% fructooligosaccharide (FOS), 2% FOS, 1% inulin and 2% inulin. Statistical analyses were performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA), descriptive statistical tests including mean, standard deviation were performed. The groups were compared using Student's *t* test. Differences with $p < 0.05$ were considered statistically significant.
Statistically significant decrease in the α-amylase activity was found when the control group (at 0 minute) was compared with FOS 1%, FOS 2%, 1% inulin and 2% inulin groups ($p<0.05$). Statistically decreased activity was registered in the presence of all oligosaccharides ($p<0.05$) on the 10^{th} minute. At the results reported on the 10^{th} minute, statistically decreased activity was found only between FOS 1% and FOS 2% ($p=0.001$). Between other groups were found no statistically significant differences ($p>0.05$). Lowering of the postprandial glucose level was observed in an inhibition of pancreatic α-amylase. Increased levels of salivary α-amylase are found in patients with diabetes. Our results indicate that the salivary α-amylase activity decreases significantly in the presence of oligosaccharides after 10 minutes, which could be a prerequisite for a potential inhibitory effect and lowering of glucose concentration. From the present experiment we can't conclude the mechanism of inhibition, but it is known that lowering the pH levels in the oral cavity leads to a decrease in the activity of salivary α-amylase. Prebiotics can optimize the oral microflora and, hence, lower the local pH in the oral cavity, reducing the activity of α-amylase. This could give us a possible explanation for the effect of oligosaccharides on salivary α-amylase.
Keywords: oligosaccharides, α-amylase, prebiotics, lactulose, FOS, inulin

1. INTRODUCTION

Amylases are a part of the group of glycosyl hydrolases enzymes, that catalyze the breakdown of complex carbohydrates. Fifty-seven families of glycosyl hydrolases have been classified on the basis of their function and specificity. The three main classes are α-, β- and ɣ-amylase. α-amylase (1,4-alpha-D-glucan glucanohydrolase) is found in animals, plants, fungi and bacteria and is an endohydrolase enzyme, which hydrolyzes internal α-1,4-glycosidic bonds in glucose homopolysaccharides, such as amylose and glycogen, leading to the release of products such as maltodextrin, maltose and glucose. β-amylase is found in plant seeds, bacteria, and fungi and ɣ-amylase is found in yeast and fungi. The main function of all of the amylases is to degrade starch and sugars; however, there is a difference in their structure and mode of action. α-amylase cleaves randomly along the starch chain and because of this non-specificity, digestion is more rapid than other amylases. All α-amylases are metalloenzymes requiring Ca^{2+} activity. In animals and humans, α-amylases occur in the pancreas, saliva, liver, serum, urine, etc. Salivary and pancreatic amylases are the main ones. They have 97% protein chain homology with each other (Nater & Rohleder, 2009; Azzopardi et al, 2016).

The secreted salivary α-amylase initiates carbohydrates digestion in the mouth. Salivary α-amylase accounts for 40% to 50% of the total salivary gland-produced protein, most of the enzyme being synthesized in the parotid gland. It consists of two families of isoenzymes, one of which is glycosylated and the other contains no carbohydrates. The molecular weight of the glycosylated isoform is about 57 KDa and the weight of the non-glycosylated isoform is about 54 KDa. Pancreatic α-amylase acts in the small intestine. It breaks down glycogen into disaccharides and trisaccharides. These oligosaccharides are further degraded by an α-1,4-glucosidase (maltase), which, as an exoenzyme, hydrolytically cleaving one glucose residue from the chain, breaking α-1,4-bonds and a debranching enzyme (amylo- α- 1,6- glucosidase) that removes branching points (hydrolytically breaks α-1,6- and 1,4-bonds). The enzymatic hydrolysis of the bonds in the internal regions of the glycosidic chain is random, but the bonds at both ends of the chain are resistant to the action of α-amylase (Nater & Rohleder, 2009).

Enzyme contains three domains termed A, B and C. The active site is located in the A domain; calcium binds to the B domain and may stabilize the active site. The C domain is a globular domain with unknown function. Some amylases including human pancreatic amylase are allosterically activated by chloride, which modulates the pH (Williams, 2019).

The concept of prebiotics was introduced in 1995 by Gibson and Roberfroid, as a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves the host's health. They are non-digestible and stimulate the development and the metabolism of beneficial microbes in the large intestine (Wu at al, 2017). All prebiotics are short-chain oligosaccharides with a low degree of polymerization, composed of 3 to 10 sugar monomers (Gibson & Roberfroid, 1995). The monosaccharide composition, the glycosidic bond and the degree of polymerization are important for their prebiotic properties. Glucose, galactose, fructose and xylose are the most common oligosaccharide monomers (Barreteau et al, 2006).

The purpose of the present experiment is to investigate the effect of oligosaccharides on the enzymatic activity of salivary α-amylase, since enzyme inhibitors are used as second line drugs in the therapy of type 2 diabetes.

2. MATERIALS AND METHODS

The α-amylase activity was determined by the Bernfeled method (Bernfeled, 1955) with some modifications. The substrate breaks down, releasing reducing sugars (maltose). The degree of enzymatic hydrolysis was determined spectrophotometrically using a solution of 3,5-dinitrosalicylic acid (DNS).

The potential inhibitory effect of different oligosaccharides on the salivary α-amylase in vitro has been established using the following reagents:

- 50mM HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) activated with 50mM NaCl, pH 6.9
- substrate - 1% starch solution dissolved in HEPES buffer
- enzyme solution - in HEPES buffer (Sigma Aldrich, USA)

The reaction mixture contained 450 µl of substrate (1% starch), 50 µl of oligosaccharides in various concentrations and 100 µl salivary α-amylase. Ten measurements for each group have been made. Groups were separareted as it follows: control, 1% lactulose, 2% lactulose, 1% fructooligosaccharide (FOS), 2% FOS, 1% inulin and 2% inulin. The reaction time was 10 minutes at 37°C. The reaction was started by adding 100 µl of enzyme solution. In order to stop the reaction, 100 µl from the reaction mixture was taken and mixed with 100 µl of a 1% solution of 3,5-DNS . Then was boiled for 10 minutes and cooled down on ice. 1 ml of distilled water was added to each sample and stirred with Vortex. In the control group, instead of an oligosaccharide, 50 µl of HEPES buffer was added to the reaction mixture. Measuring of the absorbance at $\lambda = 540$ nm against standard curve for glucose was performed. The measured amount of glucose released (mg/ml) during the enzyme reaction was replaced in equation for calculation the activity of α-amylase in U/ml. The absorption was measured on a DU 800 spectrophotometer (Beckman Couter ®, Brea, CA, USA). The equation for the calculation of the enzyme activity is:

$A = (\text{glucose (mg/ml)} \times 6 \times 1000 \times P) / (180 \times 10)$

Where:

P- dilution factor of the enzyme;

6 - ratio between enzyme and substrate in the reaction mixture;

1000 - constant;

180- molecular weight of glucose;

10- reaction time in minutes.

Statistical analyses were performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA), descriptive statistical tests including mean, standard deviation was performed. The groups were compared using Student's *t* test. Differences with $p < 0.05$ were considered statistically significant.

3. RESULTS

Descriptive statistics of the obtained results is presented in Figure 1. Values are expressed as mean ± SD.
Statistically significant decrease in α-amylase activity was found when control group (at 0 minute) was compared with FOS 1%, FOS 2%, 1% inulin and 2% inulin groups ($p<0.05$). Statistically decreased activity was registered in the presence of all oligosaccharides ($p<0.05$) on the 10th minute (Table 1). At the results reported on the 10th minute, statistically decreased activity was found only between FOS 1% and FOS 2% ($p=0.001$). No statistically significant differences were found between other groups ($p>0.05$).

Fig. 1. Descriptive statistics- mean ± SD (n=10)

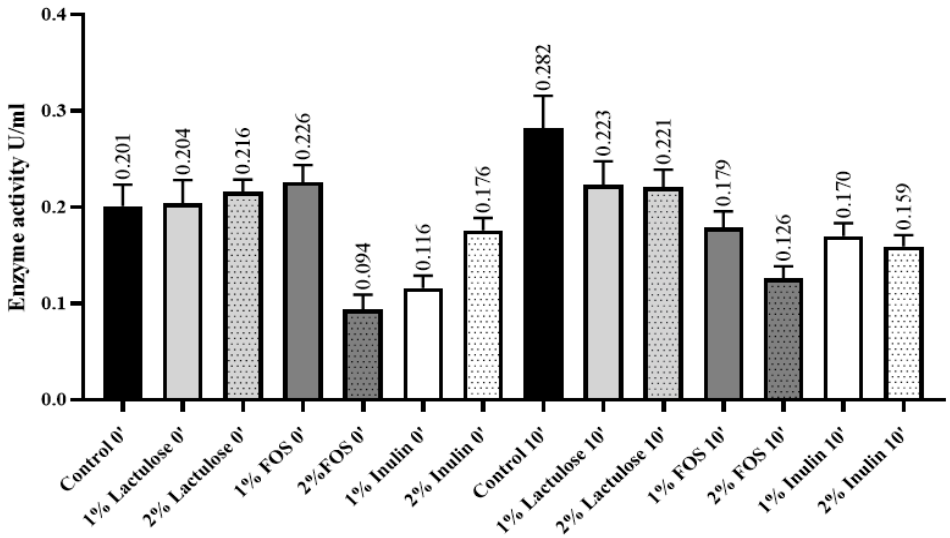

Table 1. Comparison between control (0 minute) and other samples at 0 minute and control (10th minute) and other samples at 10th minute, performed by Student's t test.

Groups	***p*** *(control 0' and other samples at 0')*	***p*** *(contol 10' and other samples at 10')*
1% Lactulose	0.776	0.001*
2% Lactulose	0.081	0.001*
1% FOS	0.013*	0.001*
2% FOS	0.001*	0.001*
1% Inulin	0.001*	0.001*
2% Inulin	0.006*	0.001*

*statistically significant difference

4. DISCUSSIONS

Diabetes is a disease, which is associated with changes in the composition of the intestinal microflora. Furthermore, the amount of bacteria that are essential for maintaining the integrity of the gut is considerably lower in individuals with diabetes than in healthy individuals. Patients with diabetes have different intestinal microflora compared with healthy individuals, as well as changes in the intestinal permeability, inflammation and insulin resistance (Murri et al, 2013). The metabolic function of the intestinal microflora is important for the control of the blood glucose level. Consequently, its modulation draws interest in the study and application of probiotics and prebiotics. In addition to reducing dysbiosis in the gut's microbial environment, prebiotics also improve glucose tolerance through mechanisms that probably involve increased production and secretion of glucagon-like peptide 1 (GLP-1), and hence activation of insulin secretion. Lowering postprandial glucose level was observed in inhibition of pancreatic α-amylase (Singla, Singh, & Dubey, 2016). Increased levels of salivary α-amylase are found in patients with diabetes (Abd-Elraheem, El Saeed, & Mansour, 2017). Our results (Table 1) indicate that salivary α-amylase activity decreases significantly in the presence of oligosaccharides after 10 minutes, which could be a prerequisite for a potential inhibitory effect and lowering of glucose concentration.

From the present experiment we can't conclude the mechanism of inhibition, but it's known that lowering pH levels in the oral cavity leads to a decrease in the activity of the salivary α-amylase (Freitas & Le Feunteun, 2019). Microbial fermentation and hydrolysis reactions transform prebiotics to short chain fatty acids (SCFAs), thereby lowering the pH of the intestinal luminal content (Whisner & Castillo, 2018). The definition and scope of prebiotics was assessed by the International Scientific Association for Probiotics and Prebiotics (Gibson et al, 2017) and updated the definition of a prebiotic: a substrate that is selectively utilized by the host's microorganisms conferring a health benefit. This definition expands the concept of prebiotics to possibly include non-carbohydrate substances, applications to body sites other than the gastrointestinal tract. However, prebiotics have now been proposed for use in other applications such as pH regulation in the vagina, infection control on the skin and to optimise healthy oral microflora (Tester & Al-Ghazzewi, 2018), hence, lower the local pH in the oral cavity, reducing the activity of α-amylase. This could give us a possible explanation for the effect of oligosaccharides on salivary α-amylase.

5. CONCLUSIONS

The present study shows us reduced activity of salivary α-amylase in the presence of lactulose, FOS and inulin. This gives us a prerequisite for continuing our scientific research towards studying the effects of oligosaccharides on other enzymes involved in carbohydrate metabolism, as well as on other metabolic pathways.

REFERENCES

Abd-Elraheem, S. E., El Saeed, A. M., & Mansour, H. H. (2017). Salivary changes in type 2 diabetic patients. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews, 11* (2), 637-641. doi:10.1016/j.dsx.2017.04.018

Azzopardi, E., Lloyd, C., Teixeira, S. R., Conlan, R. S., & Whitaker, I. S. (2016). Clinical applications of amylase: Novel perspectives. *Surgery, 160* (1), 26–37

Barreteau, H., Delattre, C., & Michaud, P. (2006). Production of Oligosaccharides as Promising New Food Additive Generation. *Food Technology and Biotechnology, 44* (3), 323-333

Bernfeld, P. (1955) Amylase α and β. *Methods in Enzymology 1*, 149-158. doi:10.1016/0076-6879(55)01021-5

Freitas, D., & Le Feunteun, S. (2019) Inhibitory effect of black tea, lemon juice, and other beverages on salivary and pancreatic amylases: what impact on bread starch digestion? A dynamic in vitro study. *Food Chemistry 297*, doi:10.1016/j.foodchem.2019.05.159

Gibson, G.R., & Roberfroid, M. (1995). Dietary Modulation of the Human Colonic Microbiota: Introducing the Concept of Prebiotics. *The Journal of Nutrition, 125* (6), 1401–1412

Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., Scott, K., Stanton, C., Swanson, K. S., Cani, P. D., Verbeke, K., & Reid, G. (2017). The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nature Reviews Gastroenterology and Hepatology 14*, 491–502 doi:10.1038/nrgastro.2017.75

Murri, M., Leiva, I., Gomez-Zumaquero, J. M., Tinahones, T. J., Cardona, F., Soriguer, F., & Queipo-Ortuño, M. I. (2013). Gut microbiota in children with type 1 diabetes differs from that in healthy children: a case-control study. *BMC Medicine 11* (46). doi: 10.1186 / 1741-7015-11-46

Nater, U. M., & Rohleder, N. (2009). Salivary alpha-amylase as a non-invasive biomarker for the sympathetic nervous system: Current state of research. *Psychoneuroendocrinology, 34* (4), 486-496

Singla, R. K., Singh, R., & Dubey, A. K. (2016). Important aspects of post-prandial antidiabetic drug, Acarbose. *Current Topics in Medicinal Chemistry 16* (23), 2625-2633. doi: 10.2174/1568026616666160414123500

Tester, R., & Al-Ghazzewi, F. (2018). Role of prebiotics and probiotics in oral health. *Nutrition & Food Science 48* (1), 16-29. doi:10.1108/NFS-03-2017-0056

Whisner, C. M., & Castillo, L. F. (2018). Prebiotics, Bone and Mineral Metabolism. *Calcified Tissue International 102* (4), 443–479 doi:10.1007/s00223-017-0339-3

Williams, J.A. (2019). Amylase. *Pancreapedia: Exocrine Pancreas Knowledge Base*, doi: 10.3998/panc.2019.02

Wu, Y., Pan, L., Shang, Q. H., Ma, X. K, Long, S. F., Xu, Y. T., Piao, X. S. (2017). Effects of isomalto-oligosaccharides as potential prebiotics on performance, immune function and gut microbiota in weaned pigs. *Animal Feed Science and Technology, 230,* 126-135 doi: 10.1016/j.anifeedsci.2017.05.013