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GLYCOSIDASE INHIBITORS - A FUNCTIONAL FOOD ADDITIVE

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Abstract

Glycosidase inhibitors attract scientific and practical interest being glycomodificators during carbohydrate digestion. It is very important developing antidiabetic preparations that will suppress activities of enzymes involved in carbohydrate metabolism. The goal of this research is to determine regularities of amylase inhibitor production by actinomycetes during the starch bioconversion and to develop food additives with the separated inhibitor to be used in diabetic products.

Hydrolyzed corn starch was used as a raw material. The following devices were applied: shaking incubator Multitron; baromembrane unit Sartorius, cartridge filters with molecular weight cut-off of 100 and 15 kDa for inhibitor separation, extraction and purification. The inhibitor content was evaluated by UK-spectral analysis. The separated inhibitor action was tested on rats. A sugar level in blood under the forced carbohydrate feeding without the inhibitor and with it was determined. The producers screening helped to select actinomycetes *Streptomyces* capable to synthesize direct-pancreatic α-amylase inhibitors.

The producers *Streptomyces lucensis* and *Streptomyces violaceus* showed the highest activity among the selected strains in respect of this enzyme. These strains produce inhibitors with different effect on glycosidase depending on the chemical nature of the carbohydrate substrate. It was established that a glucoamylase inhibitor is synthesised in the simple sugars medium (glucose or sucrose). During the complex carbohydrate fermentation (hydrolysed starch, dextrins) α-amylase is synthesised. Nutrient solution formulations for different amylase inhibitor efficient biosynthesis were developed (pancreatic, bacterial, human amylases). Method to separate a target product was created, a stable α-amylase inhibitor formulation with the pancreatic amylases activity of (700000 ± 1000) IU/g was derived. The inhibitor oral intake by test animals after forced carbohydrate feeding lowers their blood glucose level by 40-60% without their behaviour and overall health modifications. Food additives with the separated glycosidase inhibitor were developed.

The experiment results can serve as a scientific foundation that will help to develop a carbohydrate-based food additives technology for dietetic foods and will permit to expand the preventive medicine range for "glyco-modulation".

***Key words**: Glycosidase inhibitor, Diabetes, Functional products.*

1. Introduction

Glycosidase inhibitors attract interest as glycomodifiers during carbohydrate hydrolysis in the gastrointestinal tract. It is important for solving nutrition problem for people with diabetes mellitus and coexistent diseases. It is known that diabetes comes third by fatal cases after cardiovascular and oncological diseases. One of the main reasons of the pathogenic pathway is the unbalanced carbohydrate nutrition. Various methods are developed to prevent carbohydrate metabolism disorder: use of sugar substitutes and sweeteners in food, physical exercises together with diet, insulin therapy.

Glycosidase inhibitors are an alternative to medical preparations and have fundamentally different influence mechanisms on the blood sugar level. They act directly on enzymes on whose functions depends sugar absorption by humans. There are very effective glycosidase inhibitor-based antidiabetic medications produced around the world. They are mainly microbiological synthesis products. The following preparations are known: Glucobay, Miglitol, Emiglitate, Voglibose ("Bayer AG", Germany), Tendamistate ("Hoechst", Germany), trestatin and valienamin based hypoglycemic agents ("Roche Japan", Japan) [1, 2]. Single-unit infor-

mation on glycosidase inhibitors introduced into the human diet with high-calorie food products is found out [3]. The most widespread producers of glycosidase inhibitors are *Streptomyces* spp. actinomycetes [1].

The goal of this investigation is to determine regularity of amylase inhibitor production by actinomycetes during the corn starch bioconversion. The practical interest is to develop food additives with the separated inhibitor for the use in diabetic products.

2. Materials and Methods

Streptomyces lucensis S65 and *Streptomyces violaceus* S20 strains from VNIIPAKK's collection were used in the work. Hydrolyzed corn starch was used as the raw material.

Hydrolyzed starch sugar content was estimated by the dextrose equivalent (DE) value which defines total glucose and maltose content in terms of dry substances. The fermentation was carried out by batch method using Multitron shaking incubator (INFOS, Switzerland). The nutrient medium volume was (100±5) cm^3, the temperature was (30 ± 1) °C, durability of the vegetative seed mycelium growth stage was 48 h, of the fermentation stage - 96 h, mixing regime was 180 - 220 rotations/min.

Soya flour, yeast extract and ammonium salts were used as the nitrogen sources. The sources for micro and macro elements were salts of hydrochloric and sulphuric acids.

The glycosidase inhibitor was separated from the liquid culture by baromembrane methods. Microfiltration was performed using polyethersulphone cartridge filters ("Sartorius", Germany) with the pore size of 0.45/0.20 μm ("Sartobran P"). Polysulphon-base hollow fiber membrane modules separating substances with the molecular mass of 15 kDa were used for ultrafiltration. The baromembrane filtration was carried out under the pressure of 0.2 MPa and the temperature of 25 °C. Spectrophotometry method was used to determine the inhibitor activity [4]. The quantity of the inhibitor able to suppress the pancreatic a-amylase activity by 50% under 37° C and pH = 7 during 10 min. of the soluble potato starch hydrolysis (1% solution) was taken as a unit of the inhibitor activity (IU). Pig pancreatic α-amylase whose action specificity is similar to human pancreatic α-amylase was used.

Gel filtration was carries out at h : d = 40 column with G-15 Sephadex. Distilled water was the eluent. Elution velocity was 10 cm^3 ($h \cdot cm^2$). The inhibitor was spread on the column at 200 IU for 1 cm^3 of the Sephadex with 1 mg of Blue Dextran, the volume of the eluate fraction was 10 cm^3.

The inhibitor composition was studied by the infrared spectroscopy: resolution - 4,000, enhancement - 8.0, mirror velocity - 0.6329; diaphragm - 100.00; detector - DTGS KBr, beam splitter - KBr. Groups were identified by characteristic frequency value.

The inhibitor action of the isolated preparation was studied on laboratory outbreed white male rats with the wei6ght of 210 - 290 g. The scheme of the experiment was the following: after a day of hunger, a soluble starch suspension in the dose of 3.0 g/(kg of a rat weight) was administrated to the animals via an oro-pharyngeal tube, and then the blood sugar curve was determined. The blood was collected from the tail vein with an interval of 30, 60, 90, 120 and 180 minutes. The blood was also collected just before the starch suspension was introduced orally to determine the background glucose content. Seven days after the non-responsiveness to carbohydrate loading was determined, the same animals were used to study the glycosidase inhibitor action. It was introduced orally in the dose of 0.5 g/(kg of the rat weight) together with the starch suspension in the above concentration. The inhibitor activity was evaluated by the difference between glucose quantity before and after the inhibitor was introduced into the animals' system taking the background into account.

3. Results and Discussion

Screening of cultures from the VNIIPAKK collection allowed choosing two producers capable to synthesize pancreatic α-amylases directly at the soluble potato starch bioconversion. Pancreatic α-amylase is one of the main glycosidases at the first stage of carbohydrate hydrolysis in the body of an animal. Recurrent selection resulted in the choice of the *Streptomyces lucensis* S65 and *Streptomyces violaceus* S20 strains, which are more active towards the enzyme.

As a part of the study it is found that the producers' growth and development depend on the chemical nature of the nitrogen source and its content in the nutrient medium. For example, glucose favored intensive biomass accumulation and activation of the producers' enzymatic system. Actinomycetes biosynthetic activity depended on the chemical nature of the nitrogen substrate at the fermentation stage. There was no biosynthesis of the pancreatic α-amylase inhibitor at the individual sugar fermentation (glucose, maltose, dextrins). However it became active when both of them were in the nutrient medium. Biosynthetic activity of the studied strains depends on the carbohydrate concentration. It is established that when the amount of glucose is more than 1 g/dm^3 and that of maltose is more than 6 g/dm^3 in the medium, the pancreatic α-amylase inhibitor biosynthesis decreases. The elevated dextrin amount (up to more than 90 g/dm^3) inhibits physiological and biochemical development of actinomycetes. The biomass level was only (2.7 ± 0.3) g/dm^3.

The intensive inhibitor biosynthesis of pancreatic α-amylase by *S. lucensis* S65 and *S. violaceus* S20 strains took place at the hydrolyzed corn starch fermentation. Hydrolyzed corn starch DE was determined for each strain. It was 20 - 25% for *S. lucensis* S65 and 32 - 35% for *S. violaceus* S20. Glucose, maltose and dextrin correlation in the hydrolyzed starch was 1 : 8 : 30 - for *S. lucensis* S65 and 1 : 6 : 14 - for *S. violaceus* S20. The obtained results indicate that the presence of the oligosaccharides in the medium is desired for the productive biosynthesis of the inhibitor. These substances are probably involved in chemical processes of the biomass accumulation and used by streptomycetes as a structural element for synthesis of their own amylase inhibitor [5].

The chemical nature of the carbohydrate substrate and its concentration in the nutrient medium influence the specific activity of the synthesized inhibitors. For example, polysaccharides favour biosynthesis of the substances which influence the α-amylase, glucoamylase and dextrins activity [1]. When disaccharides and monosaccharides are present in the liquid culture, activity inhibitors against maltase, saccharase and isomaltase accumulate. For example, acarbose inhibits saccharase activity from *Saccharomyces cerevisiae*, maltase activity from *Aspergillus awamori*, but does not suppress dextranase activity from *Aspergillus insuetus* [6].

Dextrins and maltose forming a part of the hydrolyzed corn starch are specific substrates for α-amylase, glucoamylase, dextranase and maltase. According to the research results, the strains produce inhibitors with various specific actions on glycosidase at fermentation of individual sugars and hydrolyzed starch. These results are shown in Table 1.

Replacing hydrolyzed starch by individual carbohydrates (dextrins, maltose or saccharose), the inhibitor active to glucoamylase (*Aspergillus awamori*) and not active to α-amylase synthesizes. Studying the dynamics of the hydrolyzed starch fermentation with the use of actinomycetes strains, it was found, that the maximum inhibitor activity occurs on the 72 - 96th day for *S. lucensis* S65, and on 96 - 120th day for *S. violaceus* S20. Data, existing for other producers, show that the intensive biosynthesis of amylase inhibitors takes place after 72 - 96 h of fermentation [7].

Depending on the medium composition, actinomycetes synthesize amylase inhibitors with protein, saccharide or glycopeptide nature. *Streptomyces* spp. representatives are the most spread pseudo saccharide producers, with sugar derivatives in the structure [8]. While the corn starch fermentation by the streptomycetes under study, the reducing substances content was at the same level during the whole process. The research results allowed making a supposition that synthesized amylase inhibitors belong to saccharides or glycopeptides group. To establish the development stage, when actinomycetes synthesize glycosidase inhibitor, the dynamics of amylolytic and inhibitory activities related to pancreatic α-amylase in the liquid culture was studied. It is established that the producer synthesizes amylase as early as the logarithmic growth phase. By the third fermentation day, the amylase biosynthesis practically stopped and inhibitor accumulation activated. Actinomycete was in the stationary development phase. In this case, this refers to specific process direction. It is obvious that the carbohydrate concentration in the nutrient medium is the factor that limits synthesis of

Table 1. Carbohydrate substrate influence the glycosidase inhibitor biosynthesis

Strain	**Carbohydrate substrate**	**Inhibitor activity, IU/cm^3 acting on**		
		α-amylase pig pancreatic	α-amylase *Bacillus subtilis*	glucoamylase *Aspergillus awamori*
S. lucensis S65	Hydrolyzed corn starch	3700 ± 50	400 ± 15	1120 ± 30
	Soluble potato starch	1700 ± 50	240 ± 20	1300 ± 50
	Glucose	none	none	660 ± 50
	Maltose	none	none	2000 ± 100
	Dextrins	none	none	1100 ± 80
	Saccharose	none	none	250 ± 80
S. violaceus S20	Hydrolyzed corn starch	3500 ± 20	350 ± 30	1100 ± 30
	Hydrolyzed corn starch	2500 ± 90	130 ± 10	1200 ± 50
	Glucose	none	none	500 ± 12
	Maltose	none	none	1500 ± 10
	Dextrins	none	none	800 ± 10
	Saccharose	none	none	300 ± 12

glycosidase inhibitors. Its amount is 20 - 40 g/m^3 and it is 2 - 6 times less than the amount needed to synthesize amylases efficiently. The metabolic processes of *S. lucensis* S65 and *S. violaceus* S20 might be directed to balance the cell carbohydrate exchange.

It is noted that these are dextrins and maltose, whose concentration decreases more intensively in the liquid culture. They may contribute not only to the accumulation of biomass, but also to the formation of the carbohydrate skeleton of the glycosidase inhibitor. To accumulate the inhibitor, *S. lucensis* S65 strain needs more carbohydrates than *S. violaceus* S20.

Nutrient media compositions were developed as a part of study to perform biosynthesis of the amylase inhibitor. Protein containing compounds are mainly used as a nitrogen source for glycosidase inhibitor biosynthesis. It is known that corn flour, yeast extract, soy flour and fish flour were introduced in the nutrient medium composition [7, 9]. Albuminous nitrogen together with medium carbohydrates might induce the inhibitor biosynthesis. The above hypothesis was confirmed by the research results. It is found, that the pancreatic α-amylase inhibitor is synthesized at the *S. lucensis* S65 and *S. violaceus* S20 cultivation in the soy flour medium. Replaced by NH_4NO_3 or NH_4Cl, amylases were synthesized by actinomycetes and not by their inhibitor. It is possible to suppose that the inhibitor synthesis in the presence of the organic nitrogen is a feedback to the chemical nature change of sources of nitrogen and carbohydrate nutrition. When soy flour was substituted by yeast extract or peptone, streptomycetes synthesized a hypo active inhibitor. The results are shown in Table 2.

Table 2. Influence of the organic nitrogen source on the activity level of the pancreatic α-amylase inhibitor

Nitrogen source name	Nitrogen source concentration in nutrient medium, g/dm^3	Inhibitor activity, IU/cm^3	
		S. lucensis S65	*S. violaceus* S20
Soya flour	1.0 2.5 5.0 10.0	1500 ± 30 3200 ± 40 3700 ± 50 3700 ± 50	1100 ± 50 3100 ± 50 3500 ± 20 3500 ± 20
Yeast extract	1.0 2.5 5.0 10.0	300 ± 10 540 ± 20 740 ± 10 616 ± 20	220 ± 40 600 ± 20 700 ± 30 580 ± 30
Dry fermentative peptone for bacterial purposes	1.0 2.5 5.0 10.0	480 ± 20 500 ± 10 680 ± 30 530 ± 10	170 ± 20 525 ± 10 710 ± 30 590 ± 30

Complex influence of sodium (or potassium) and calcium ions in the composition of hydrochloric and carbonic acids correspondingly, had a more effective influence on the efficiency of the target metabolite biosynthesis at the stage of the seed material preparation stage. Sodium (or potassium) and magnesium introduced in the medium composition were more effective at the fermentation stage. They may favour permeability increase of streptomycetes cell wall. It is confirmed by the fact that mass concentration of reducing substances and protein increased in the liquid culture. Calcium and magnesium ions may function as activity stabilizers of the enzymatic producer system.

Cultivation of the studied actinomycetes, within 5 days of the use of the developed medium composition, resulted in the inhibitor activity in the liquid culture of (3700 ± 50) IU/cm^3 for *S. lucensis* S65 and of (3500 ± 20) IU/cm^3 for *S. violaceus* S20.

Taking the obtained data into account, there were investigated conditions that allow to separate glycosidase inhibitors using the membrane technology. The ultrafiltration helped to remove microorganism cells and proteins from native solutions. Activated charcoal powder was applied to reduce the content of colored substances. The solution was clarified 2.5 folds at the temperature of 50 ^{0}C and the process lasted 60 min. The clarified ultrafiltrate passed concentration under vacuum and was dried at the temperature of (55 ± 5) ^{0}C. The temperature increase led to caramelization. The inhibitor preparation was produced in the quantity of 7 - 12 g for 1 dm^3 of the liquid culture. According to the published data, the quantity of the inhibitor needed to create a drug formulation of 1 - 29 g/dm^3 [1]. A method for target product separation was developed. A stable inhibitor preparation with the pancreatic α-amylase activity of (700000 ± 1000) IU/g was obtained. The separated inhibitor preparations are 85 - 88% of carbohydrate substances. High-purity preparations with the activity of (4500 ± 20) IU/cm^3 are produced by gel filtration method. An active peak was observed at repeated gel filtration. The carbohydrate content in the lyophilized preparations was (98 ± 1)%.

As the result of the infrared analysis, high-purity preparations peaks were obtained, corresponding to the wave numbers of 3550 sm^{-1}, 3010 sm^{-1}, 2900 sm^{-1}, 2500 sm^{-1}, 1600 sm^{-1}, 1250 sm^{-1}, 1150 sm^{-1}, 1050 sm^{-1}, 950 sm^{-1}, 850 sm^{-1} и 520 sm^{-1}. They are illustrative of α-1,2-glycosidase, α-1,4- glycosidase and double bond, aldehyde, hydroxyl, imine groups.

The investigation of the inhibitor biochemical properties showed their similarity to the pig pancreatic α-amylase, α-amylase from *Aspergillus niger*, α-amylase *Bacillus subtillis*, glucoamylase from *Aspergillus niger* and to α-amylase from the human blood. The inhibition constant numbers were 6,5·10^{-5}, 1,1·10^{-6}, 3,4·10^{-6},

$2,5 \cdot 10^{-6}$ and $1,7 \cdot 10^{-6}$ mol/dm^3 correspondingly. It became possible to perform tests of the inhibitors with hypoglycemic activity in vivo due to the obtained results. In the male rats experiments the starch was introduced in their diet as the carbohydrate load. The pure glucose was used to perform a standard glucose tolerance test. The starch was chosen because the inhibitor has the carbohydrate nature and can serve as substrate for glycosidase in the gastrointestinal tract of animals; it is known from the inhibitor high dosages [4]. The starch as a substrate is competitive for the inhibitor as opposed to the glucose. As the result a number of peculiarities of changes in blood glucose level of the experimental animals at the oral starch administration in comparison with the glucose are discovered. First and foremost, the increase of their blood glucose level was less evident. Secondly, the maximum increase of the glucose concentration in comparison with the standard glucose tolerance test was marked 1.5 h after the starch was administrated. Its level decreased up to the background values after 3 h at the end of the test. The return to the initial level normally occurs after 2 h. The curve changes of the glucose tolerance test in the rats had such character and dynamics at the starch oral administration because of the starch digesting. The experiments on male rats with the glycosidase inhibitor introduced into their diet together with the starch showed the decrease of the blood glucose level by 40 - 60%. The glucose level fluctuations were less evident than in the cases, when only the starch was used. The results are shown in Figure 1.

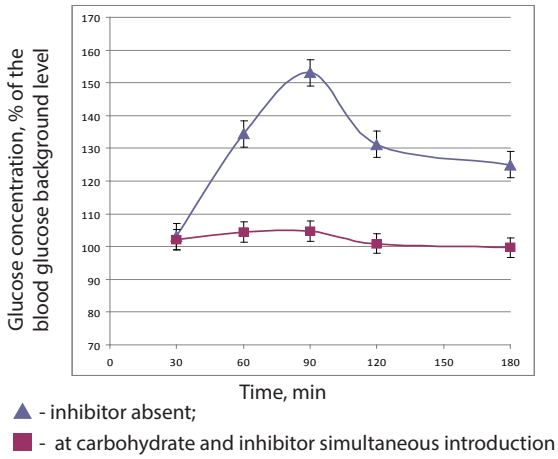

Figure 1. Dependence of the blood glucose level in rats when the starch is used as a carbohydrate load

Food additives with separated glycosidase inhibitors are developed on the base of the obtained data. The additive preparations were produced to be used in bread baking. They contain potassium citrate and maltodextrin beside glycosidase inhibitors. Potassium citrate regulates food system acidity. Maltodextrin makes the test more fluent and easy. The additives tested at the bread baking showed that they can be introduced into the wheat bread formulations to decrease the glycemic index. Their glucose content was 1.1-1.16 folds less in comparison with the bread without the additive.

4. Conclusions

- The efficiency of the glycosidase inhibitor biosynthesis depends not only on the polymerization level but on the saccharide structure and concentration.

- Studied strains synthesize activity inhibitors of the enzymes which have specific substrates or their hydrolysis products in the liquid culture.

- Carbohydrate structure and concentration in the liquid culture influence the nature and specificity of the synthesized inhibitors.

- Synthesized inhibitors are carbohydrate substances by their nature.

- It is possible to create a scientific base to develop a technology for food additives of the carbohydrate nature for dietary purposes. It will allow expanding the range of the medications for prevention and treatment of the glycomodulation.

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