STANDARDIZATION, CERTIFICATION, QUALITY AND SAFETY

INVESTIGATION OF KINETIC PARAMETERS OF THE DIETARY SUPPLEMENT "AMIL-ING"

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Abstract: Enzyme inhibitors are widely used in experimental studies in various spheres to evaluate the mechanism of catalytic effect of enzymes, determine the nature of protein reactive groups, to identify the role of various enzymatic processes in metabolism. But inhibitors are not stable and thus, they need to be stabilized through immobilization on matrixes. The study of immobilization using the infrared spectroscopy ensures to prove the interaction between the inhibitor and polysaccharidic matrix. The results of infrared spectroscopy showed that the linking between the matrix and the inhibitor occurs by formation of intramolecular covalent linkings, electrostatic correlation between the charged groups of agar and inhibitor. The derived comparison curve VVOP shows the reduction in the intensity of the immobilized inhibitor (dietar y supplement) in the area of 3400 cm^{-1}, that is consistent with the valent variations of the free group -OH that indicates on strengthening of the immobilized specimen hydrogen binding. Comparative study of pH-optimum of pancreatic α-amylase, native and immobilized inhibitor made it possible to conclude that pH-optimum of pancreatic α-amylase ispH 6.0, that of the native inhibitor α-amylase equals to 5.5, and the pH-optimum in the immobilized inhibitor considerably varies from 5.0 to 6.8 at the physiologic temperature (37 ± 1)ºC. Linearization methods of Michaelis-Menten equation by Lineweaver-Burk and Hanes were used to determine kinetic parameters of the dietary supplement inhibition. The kinetics of enzyme inhibition was assessed using the immobilized form of the inhibitor that resulted in the enzyme activity decrease at zero variations K_m at the decreasing V_{max} values which makes it possible to classify the inhibition to the linear uncompetitive type (catalyzed inhibition).

Keywords: pancreatic amylase inhibitor, hydrolysis kinetics, kinetic parameters of pancreatic amylase inhibition

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INTRODUCTION

As the endocrine system disease in developed countries, including the Ukraine, where the patient population is over 3% of locals, the high incidence of diabetes mellitus with severe morbidities is ranked as the civil country disease that requires comprehensive therapeutical and prevention measures to manage it. Use of dietary supplements is considered one of major measures to prevent the diabetes milletus in developed countries that contain pancreatic amylase inhibitors. In turns, these are able to lower the level of blood glucose due to the ability to suppress the segregation of starch and starch-like alimentary polysaccharides. The use of herbal α-amylase inhibitors does not cause "habituation effect" since the plant source contains contributory biologically active substances wholesome for health.

Today, scientists believe that inhibitors play a vital role in the functions of main biochemical mechanisms that specify and regulate physiological status of the cell, its reactions and interactions with neighboring cells and environmental factors. The complexity of physiological effects caused by inhibitors offer wide opportunities to produce inhibitor-based multi-functional dietary supplements.

Enzyme inhibitors are widely used in experimental tests in biochemistry, physiology, cytology, and genetics to study mechanisms of enzyme catalytic action, to specify the nature of protein functional groups, to define the role of various metabolic processes of enzymes. Recently, enzyme inhibitors are used in medicine as pharmaceutical agents and dietary supplements. The concern in amylase inhibitors is nonrandom. First of all, it is associated with the ability of efficient suppression of hydrolitic processes of alimentary polysaccharide disintegration to reduce the level of blood glucose with diabetes mellitus, obesity, hyperlipidemia and other abnormalities associated with carbohydrate metabolism disorder. Amylolytic enzyme inhibitors are able to specifically slow the reaction behavior in the human body based on the catalysis of

glycosidic linkage disruption in such substrates as starch and amyloid polysaccharides and oligo-saccharides.

Substitution therapy based on antidiabetic insulin still remains the most effective method to manage the diabetes mellitus by medication administration.

The advantage of amylolytic enzyme inhibitors is that they are not involved in the obvious stimulating effect on insulin secretion and administration of amylase inhibitors in parallel with the alimentary therapy does not result in hypoglycaemia development. Amylase inhibitors act in the intestinal lumen. It means they have peripheral mechanism of action that does not result in depletion of β-cells of the pancreas and degeneration of insula and in the diabetes enhancement.

Inhibitors of α-amylase of plant origin are responsible to maintain the activity of their amylase at the certain level, control the negative impact in the complicated glycometabolizm system and also, they decrease the level of blood glucose and insulin injection in healthy people and diabetic patients.

Amylolytic enzyme inhibitors are prevalent in the plant kingdom [1]. In certain cases, the plant-based inhibitors excel the animal and microbial analogs, they are of less toxicity, less allergenic capacity, they contain contributory biologically active component of polysaccharidic, lipidic, pigmental and other origin to be wholesome for health. Grain crops are known for the considerable content of amylolytic enzyme inhibitors [2, 3]. However, the inhibitors are not reported to have high pH values and thermal stability. This significatly reduces their effect on the human body. Various methods are used to stabilize and concentrate inhibitors as follow:

– Physisorption methods on matrixes of natural origin;
– Microencapsulation methods: complexation due to electrostatic interaction (between proteins and polysaccharides) or common coacervation; membraneless osmosis or complex coacervation;
– Method of sedimentation in the isoelectric point may be used for biologically active substances (BAS) of protein origin, though this method results in considerable drop or total loss of the biological activity of BAS.

The purpose of this study is to determine kinetic properties of the biologically active additive based on the pancreatic amylase inhibitor isolated from the oat dust.

OBJECTS AND METHODS OF STUDY

Biotechnology to obtain "Amil-ing" BAA consists of the following:

The oat dust was delipidated in the Soxhlet's extraction apparatus using ten volumes of petroleum-ether. The pancreatic α-amylase inhibitor was extracted from the oat dust 0.15 M NaCl in 0.10 M hydrocarbonate buffer (pH 9.2) at 13.4ºC (30 min, RH 7.2). The extract was heated up to 70ºC for 10 minutes for amylase inactivation that may be extracted from the oat dust. The extract was heated up to 70ºC for 10 minutes. The sediment was decanted by centrifuging at the rate of 3000 rpm for 20 minutes [6].

Water-insoluble polyelectrolyte complexs were obtained by adding to the oat dust extract containing the inhibitor so that the protein content in the mixture was stable within 0 up to 1.1%.

Ammonium sulfate precipitation with the degree of saturation within 40 and 75% was conducted at 4ºC. The deposit generated was dissolved in the distilled water, and the protein suspension was put into the porous membrane and dialyzed against 500 cm^3 of the distilled water for 3 days. The speciment obtained was then centrifugated at the rate of 3000 rpm (for 30 min) and the inhiiting activity was established [6].

4B sepharose was used as a sorbent. The sorbent was activated using the benzoquinone synthesized as per the guidelines of the pre-purified hydroquinone [7–10].

The affine sorbent "pancreatic α-amylase-sepharose 4B" was obtained by covalent binding of the activated carrier with the α-amylase of animal origin as follows: 3 cm^3 of 0.1 M hydrocarbonate buffer of pH 8 was added to 3 cm^3 of gel. The binding reaction occurred at 4ºC within 24 hours. The gel obtained was washed with the distilled water using the glass filter, and then, in the column (1x15 cm) in the sequence as follows: 1 M KCl in 0.1 M Na-acetate buffer, pH 4 for 24 hours; 1 M KCl in 0.1 M Na-bicarbonate buffer, pH 8.5 for 24 hours and distilled water until zero adsorption at 280 nm [6].

The protein solution was passed through the column (1x15 cm) with the bio-specific sorbent "pancreatic α-amylase-sepharose 4B" at a rate of 15 cm^3/min. As soon as the sorbent is saturated which is monitored by the inhibiting activity in the filtrate related to the pancreatic α-amylase, the gel was washed with 0.05 M tris/HCl buffer, pH 8. In the column, the sorbent was washed with 1 M solution of NaCl, 8 M urea in 0.05 M tris/HCl buffer, pH 8, in turn. The inhibitor was desorbed using 10^{-3} M solution of HCl. The active fraction was neutralized with 1 M solution of NaOH and lyophilized [6].

The inhibiting activity (IA) was specified by the rate of enzymatic activity suppression of α-amylase of animal origin and expressed in inhibitory units (IU). The activity of pancreatic amylase was expressed by the content of starch broken with 1 g of enzyme for 1 min.

The amylase activity was specified as follows: 10 cm^3 of 1% starch solution in 0.5 n acetate buffer pH 4.7, thermostated at 37°C was added into the tube containing 5 ml of enzyme solution or 2…10 mg of immobilized specimen in 5 cm^3 of water upon 5-min incubation at 37°C. The reference solution was 10 cm^3 of 1% starch solution in 5 cm^3 of water. In 10 minutes of incubation with the starch, 0.5 cm^3 of the incubated mixture was collected and put in the idione solution of 50 cm^3 in volume (0.0025% iodine solution). The iodine solution is stained in blue when mixed with the reference solution, and in violet color of varying intensity – when mixed with the test solution depending on the volume of starch not reacted.

The optical dense of the reference and test solutions was measured at 590 nm. Amylolytic activity was calculated by the formula:

$$AA = \frac{(D_к - D_o)\cdot 100}{D_к \cdot 10 \cdot н},$$

where $D_к$ is the optical dense of the reference solution; *Do* is the optical dense of the test solution; *100* is the volume of starch taken as the substrate, in kg; for testing; *10* is the incubation time; *н* is the weigh of specimen, in g.

RESULTS AND DISCUSSION

Among natural polymer carriers, agar – polysaccharide, consisting of agarose and agaropectin isolated from cellular membranes of certain algae, is often used for immobilization. It is reported to have adequate physical integrity that may be intensified by mixing with specific regaents. Agar matrix-based hydroxy groups allow immobilization of most biologically active substances (BAS) by both chemically and by sorption. Dextrane and agarose derivats form the relatively heavy gel. Dextrane derivates are mainly used as porous polymers – sephadex with various binding rate. Today, the affine chromatography is the major procedure to isolate hydrolyzing enzyme inhibitors. Nevertheless, the use of affine chromatography to isolate proteic biologically active substances to obtain biologically active additives (BAA) is not economicaly and technologically feasible. Currently, the methods are specially considered to obtain BAA with valuable BAS along with concomitant herbal source components that stabilize the basic BAS and have the biological activity. This is why, the urgent is the search of methods for BAA containing basic BAS as concentration along with other source components.

Even the minor volume of polyelectrolyte (10%) available resulted in the partial protein sedimentation; the dependence was extreme to the peak extent corresponding to 40% of the sedimented inhibitor during the polyelectrolyte concentration in the mixture $8x10^{-3}$%. The aggregated sediment that occurred during the greatest inhibitor sedimentation, contained the protein and sedimented at the ratio 0.52: 1.00 mg/mg or 40:1 mol/mol that indicates that under these conditions, only the minor portion of the polyelectrolyte forms the water-insoluble complex with the protein. The isolation of the sedimented complex even from such low concentration systems confirms towards its electroneutrality. Since the flocculation process is suppressed in presence of 6 M urea, we may suggest that flocculus develop due to hydrogen bonds between protein molecules and polysaccharide.

Consolidation of the protein globule of the inhibitor by intra-molectular covalent linking and binding with the carrier, inclusion of the carrier in concentrated gels, limit conformational motility of the BAS polypeptide chain. All this results in the higher BAS resistance to denaturation. Carrier-based immobilization with charged groups or buffer properties that ensure the best local pH value in the BAS micro-environment, prevents the protein globule unfolding that depends on its ionization modification. The consistency of immobilized BAS also increases due to prevention of unfavorable dissociation and association processes.

The process of inhibitor complexing with anionic polysaccharide agar in the acidic area of pH scale mainly occurs due to electrostatic interaction between charged groups of agar and protein (inhibitor) and hydrogen linking, and due to hydrogen linking and poor hydrophobic interactions in case if it is higher than the pI (isoelectric point) of complexing.

The inhibitor infra-red spectrum is known to have the characteristic strip of absorption 1168 cm^{-1} induced by skeletal vibrations. The absorption peak in the area of 1616 cm^{-1} is identified by binding vibrations in β-conformations, and in 2314 cm^{-1} area – by symmetric vibrations of methyl groups.The absorption in 3305 cm^{1} area occurs due to amine group vibrations associated with hydrogen linkings, and the separate peak in 3428 cm^{-1} area evidences on vibrations of free amine group. Absorption bands within 2840 ... 2900 cm^{-1} speak for valent vibrations of CH and CH_2 groups.

In the agar spectrum, within 700 ... 900 cm^{-1} area, the absorption bands are seen typical for sugar spectrum that contain galactose chains, also the absorption bands of groups -S_2-O- groups are available (strong absorption band with two peaks 1260 and 1230 cm^{-1}), induced by valent asymmetric vibrations of O = S = O groups.

An intensive wide band with the peak absorption is seen at 3400 cm^{-1} in the spectrum of immobilized inhibitor specimen that is shifted to the low-frequency area as compared with that of free OH-groups. This speaks for involvement of hydroxyls in the hydrogen linking system. Absence of absorption band at 3650 cm^{-1} indicates that almost all hydroxyls groups are involved in the hydrogen bond. We used the method of differential infrared spectroscopy (Fig. 1, 2) to compare and assess test specimens of free inhibitor and "Amil ing" BAA.

The differential infra-red spectrum of comparison (Fig. 1) is characterized by the intensive absorption within 670 ... 1225 cm^{-1} area for the free inhibitor which is due to amine groups available in the protein molecule. The reduction in the value of relative optical density (RODV) for immobilized specimens in this area may be explained by the shielding effect of the matrix (agar).

The derived comparison curve for the specimen RODV is specified by the reduction in absorption intensity of the immobilized inhibitor in 3400 cm^{-1} area which is consistent with the valent vibrations of the free OH-group. This is the evidence that the hydrogen links of the immobilized specimen are intensified.

The differential infra-red spectrum of comparison of the immobilized inhibitor specimen and matrix is also known for higher values of RODV in the hydrogen link absorption area (3000 cm^{-1}) which may be due to hydrogen link formation between the inhibitor and the matrix. Considerable absorption in the area 1230 ... 1260 cm^{-1} for the agar indicates on availability of the sulfonate group in it. The comparison of RODV absorption band for carbonyl groups (1648 ... 1690 cm^{-1}) indicates the absorption shielding in this group resulting from the agar-based inhibitor immobilization.

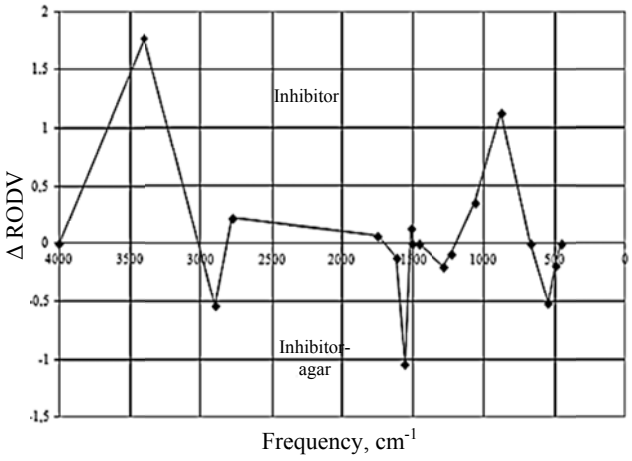

Fig. 1. Differential infra-red spectrum for "Amil-ing" BAA (inhibitor-agar) against the free inhibitor.

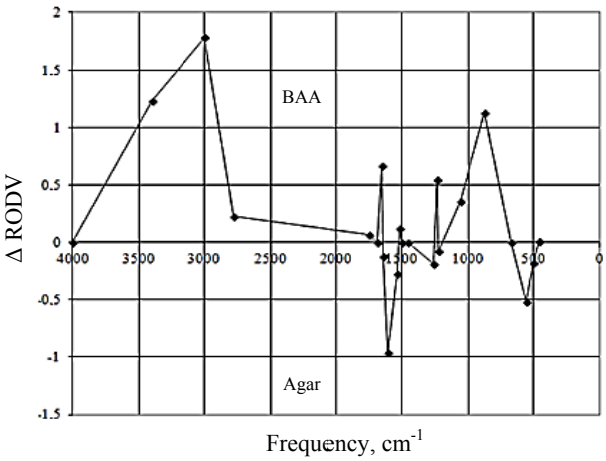

Fig. 2. Differential infra-red spectrum for BAA against the agar.

The differential infra-red spectrum of comparison of the immobilized inhibitor specimen and matrix is also known for higher values of RODV in the hydrogen link absorption area (3000 cm^{-1}) which may be due to hydrogen link formation between the inhibitor and the matrix. Considerable absorption in the area 1230 ... 1260 cm^{-1} for the agar indicates on availability of the sulfonate group in it. The comparison of RODV absorption band for carbonyl groups (1648 ... 1690 cm^{-1}) indicates the absorption shielding in this group resulting from the agar-based inhibitor immobilization.

A rise of relative absorption intensity (up to 44%) is reported in the differential spectrum of the protein within the "protein-agar" system in the area 1610 cm^{-1}, this is consistent with presence in the COOH-group system, as well as (up to 59%) in the are of absorption band 1540 cm^{-1} (Amide II) of the differential agar spectrum within the system "protein-agar" and corresponds to availability in the NH-group system.

Density and reliability of hydrogen link network were evaluated using the characteristic absorption band of 3400 cm^{-1} that is consistent with the valent vibrations of OH groups. For this purpose, the half-width of the band was identified as per wavenumbers of the tested spectrum region and the relative intensity of the band as per RODV (Table 1).

It is seen in data given in Table 1 that the reduction in the half-width of the absorption band is noted consistent with the valent vibrations of OH-groups when comparing specimens of agar and BAA. This is indicative of increase in the number of OH-group involved in strong hydrogen bonds.

Table 1. Characteristics of hydrogen links in “Amil ing” BAA and its main components

Specimen	RODV of 3400 cm^{-1} band	Half-width of band, cm^{-1}
Inhibitor	1.28	400
Agar	1.37	680
“Amil-ing” BAA	1.49	650

Thus, the test results evidence that when the agar-based inhibitor is immobilized, hydrogen linkings form between the inhibitor and the matrix.

The results of colorimetric studies confirm that, in all cases of hydration of initial components, their mechanic mixtures and immobilized samples, exothermic effects occur. It is seen from the data obtained that thermal effect values of physical mixtures obtained through testing exceed the theoretically calculated by the sum of thermal effects of their component hydration.

To forecase the inhibitor action and inhibitor-based BAA andthe development of diabetic food product process, one should be aware of factors and methods that affect the inhibitor activity. Whereby, the pH value and the temperature of the environment where the inhibitor acts are of high importance. Enzyme inhibitors are used to slow down or neutralize the activity of relevant enzymes and thus, the pH value and the best termperature value for the inhibitor are main parameters.

The comparative study of pH-optimum of pancreatic α-amylase, pancreatic amylase inhibitor from the oat dust and oat dust-based BAA resulted in the conclusion that pH-optimum of pancreatic α-amylase is pH 6.0, α-amylase inhibitor from the oat dust is 5.5, and the pH-optimum of BAA is more expanded and is within pH 5.0 to 6.8 at the physiological temperature (37 ± 1)ºC. The inhibitory activity of BAA is reduced for 3.5% within this pH range.

The quantitative evaluation of the inhibitor reactivity and namely, the determination of the response kinetic parameters is the vital element of the enzyme assay. Kinetic characteristics of enzyme reaction to inhibition are based on principles of mechanic interaction between inhibitors and enzymes [4, 5, 12].

By the results of kinetic studies the best effective process conditions are determined, the affinity degree of the substrate and inhibitor to the enzyme is assessed, the origin of enzymatic process is specified, and so forth. Involvement in mechanism of enzymatic reactions of intermediate compounds

$E + S \xleftrightarrow{K_m} ES \xrightarrow{K_{кат}} E + P$ results in the following dependence of the fixed reaction rate on the substrate concentration (Michaelis equation):

$$v = \frac{-dS}{dt} = -d\left[S = \frac{V_{\max}[S]}{K_m + [S]}\right],$$

where V_{max} and K_m have the effective value, in most cases, since they include constant rates of elementary chemical acts of multiphase enzymatic process.

Ten sets of tests were performed to study the amylase inhibition mechanism. The first set of tests was conducted to identify the amylolytic activity of pancreatic amylase. During further sets, the amylase activity was identified by varying the volume of amylase inhibitor of plant origin 0.1, 0.3, 0.5, 0.7, 0.9, 1.1, 1.3, 1.5, 1.7, and 1.9 g/dm^3 (dry substances). The concentration of the pancreatic amylase was 0.01%. The reaction mix to determine kinetic parameteres of pancreatic amylase inibition process included pancreatic amylase solutions, solutions of its inhibitor and 0.1% of starch solution.

The data collected were reviewed by linearization methods of Michaelis-Menten equation by Lineweaver-Burk and Hanes [10, 13]. The inhibition constant value (K_i) was calculated by Dickson and Webb methods [10, 13].

Calculations made by Lineweaver-Burk and Hanes indicate on the linear type of experimental dependence proved by high linear correlation values.

Within the range of inhibitor concentrations 50 mg…3.2 g/dm^3, decrease is reported as V_{max} the concentration of inhibitor increases with no considerable changes in values K_m as compared with the intact enzyme (with the pancreatic amylase inhibitor absent).

The value K_m remains almost constant to the entire range of immobilized inhibitor concentrations; it is approximately 1.5 ... 1.6 times less than those for both intact enzyme and non-competetive inhibition by ingibitor purification.

When comparing results of kinetic studies of intact and immobilized inhibitors, the increase K_m for the immobilized inhibitor was reported against that for the intact inhibitor. Discrepancies in these values calculated by using the data of the Lineweaver-Burk plot are considered statistically unauthentic due to the great value variations; the statistical significance is seen in data obtained based on Hanes plot (value t equals to 3.487 for 1990.1–424.4 = 1565.7 that gives $0.002 < p < 0.01$ at n = 8, the value t = 3.391 for 678.4–445 = 233.3 that also gives $0.002 < p < 0.01$ at n = 11); differences are significant in case of data comparison obtained by Webb's method (t = 7.844 for 1360.4–403.8 = 956.6 that gives $p < 0.001$ at n = 48).

Since the use of immobilized inhibiting agents refers to the area of hetcrogenic processes (inhibition), the CV growth may be caused by:

– Diffusion diseases; due to that the molecular mass of the inhibitor is 25.1 kDa, and that of agar is in several orders greater, the protein binding with such a carrier should inevitably result in diffusion complications;

– Steric (spatial) restrictions; due to abrupt increase in the molecular mass of the product of inhibitor and agar interaction, its binding with the amylase may become complicated.

In addition, the following potentials should be also considered:

– Conformational changes in the inhibitor molecule resulting from its covalent immobilization of the agar; they may well impact the inhibitor binding with the enzyme;

– Electrostatic effect of the agaropectin sulphogroup;

– Impact of these groups on the pH value in the inhibitor and enzyme micro-environment varies the rate and strength of interaction in between.

The reduction in Michaelis constant is deemed as the increase in interaction between the enzyme and substrate (stabilization of enzyme-substrate complex). Thus, the review of experimental data for the pancreatic amylase inhibition by the immobilized inhibitor using mathematical analysis of kinetic studies showed no variations at reduced Vmax values that allow us consider inhibitions to the linear inhibition of non-competitive type (catalyzed inhibition).

The decrease in the enzymatic activity featured by no variations K_m at reducing V_{max} values allows us consider the inhibition process to non-competitive type (catalyzed inhibition). At such, it's quite valid to use Dixon and Webb plots to define the inhibition constant values. Baseline values to graph the Dixon plot are given in Table 2.

Statistic and kinetic paraeters of reaction of the starch hydrolysis with the pancreatic amylase over the inhibitor calculated based on the Dixon diagram as shown in the Table 3.

The Webb plot helps to perform the statistic evaluation of parameters obtained, since not only $tg\alpha = K_i$ is defined by calculations, but also its mean square deviation (S_a). Same refers to evaluation of the initial response rate since the section on the Y-axis ("*b*") is equal to one as the expression $v_0/(v_0–v_i)$ when the enzyme is saturated with the inhibitor, that is, at $v_i \rightarrow 0$ against conditions $[I] \rightarrow \infty$ and, accordingly, $1/[I] \rightarrow 0$ turns into v_0/v_0, and "S_b" is its mean square deviation value. In addition, we may state based on the Webb plot on the extent to which the inhibition constant values correlate in between as in $c = –K_i$.

Table 2. Kinetic parameters of the pancreatic amylase starch hydrolysis over the inhibitor

[I], g/dm^3	1/V`$_{max}$	
	As per Lineweaver-Burk plot data (value *b*)	As per Hanes plot data (value *a*)
0	$2.5679 \cdot 10^{-3}$	$2.5781 \cdot 10^{-3}$
$5 \cdot 10^{-2}$	$2.8763 \cdot 10^{-3}$	$2.8649 \cdot 10^{-3}$
$1 \cdot 10^{-2}$	$3.1988 \cdot 10^{-3}$	$3.3180 \cdot 10^{-3}$
$2 \cdot 10^{-2}$	$3.9772 \cdot 10^{-3}$	$4.0261 \cdot 10^{-3}$
$4 \cdot 10^{-2}$	$4.6375 \cdot 10^{-3}$	$4.9322 \cdot 10^{-3}$
$8 \cdot 10^{-2}$	$6.9369 \cdot 10^{-3}$	$6.7864 \cdot 10^{-3}$
1.6	$1.0419 \cdot 10^{-2}$	$1.0039 \cdot 10^{-2}$
3.2	$1.8181 \cdot 10^{-2}$	$1.9768 \cdot 10^{-2}$

Table 3. Statistical and kinetic parameters of pancreatic amylase starch hydrolisis over the inhibitor calculated based on the Dixon plot

As per Lineweaver-Burk plot data	As per Hanes plot data
$a = 4.8313 \cdot 10^{-3}$	$a = 5.2127 \cdot 10^{-3}$
$b = 2.7646 \cdot 10^{-3}$	$b = 2.6517 \cdot 10^{-3}$
$c = -5.7221 \cdot 10^{-1}$	$c = -5.0870 \cdot 10^{-1}$
$S_a = \pm 6.5854 \cdot 10^{-5}$	$S_a = \pm 1.5709 \cdot 10^{-4}$
$S_b = \pm 8.6028 \cdot 10^{-5}$	$S_b = \pm 2.0521 \cdot 10^{-4}$
$r = 9.9944 \cdot 10^{-1}$	$r = 9.9729 \cdot 10^{-1}$
$n = 8$	$n = 8$
$K_i = 5.7221 \cdot 10^{-1}$ g/dm^3	$K_i = 5.0870 \cdot 10^{-1}$ g/dm^3
V_{max}=361.7216 A.U./mg	V_{max} = 377.1098 A.U/mg

As per Keleti and with regard to data in Lineweaver-Burk plot (Fig. 3), the calculation formula appears as follows:

$$K_i = \frac{tg\alpha \cdot I}{tg\alpha' - tg\alpha} = \frac{\Delta Y \cdot I}{\Delta Y' - \Delta Y},$$

where the character " ' " refers to the relevant parameter for inhibition response.

In view of the physical significance of parameters given, the folowing transformations are made with regard to the Hanes plot:

$$K_i = \frac{\Delta Y \cdot I}{\Delta Y' - \Delta Y} = \frac{tg\alpha \cdot I}{tg\alpha - tg\alpha'}$$

When evaluating K_m the inhibited enzyme by Lineweaver-Burk (Fig. 3) and Hanes (Fig. 4) at 1.6 g/dm^3 inhibitor concentration, 7 points were used to consider when calculating the value of mean square deviation and standard mean square error. Results of statistic constant determinations are shown in Table 4.

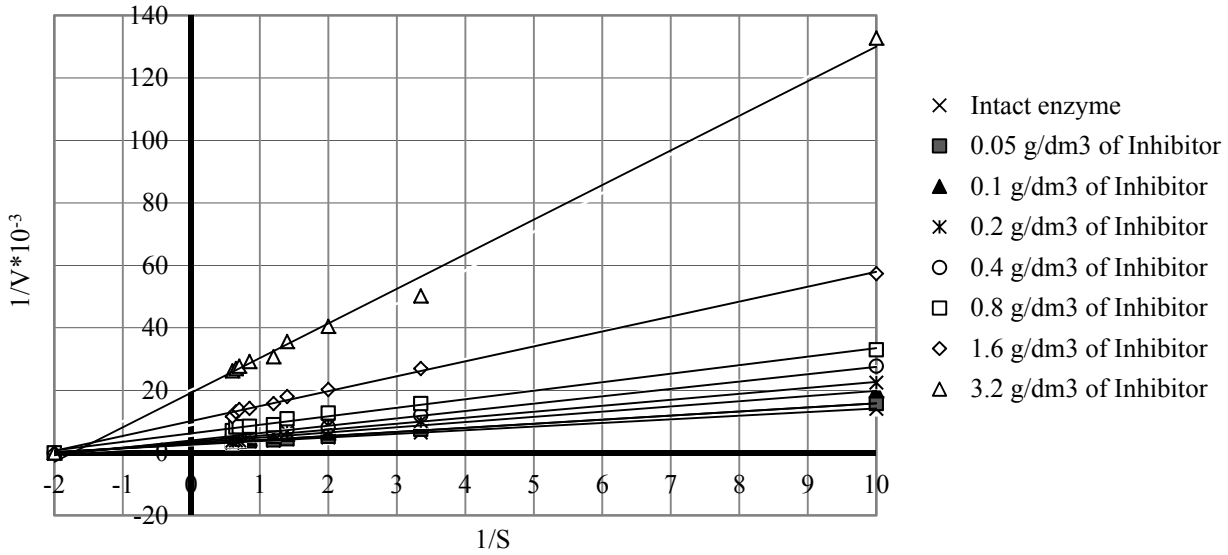

Fig. 4. Inhibition plot by Lineweaver-Burk.

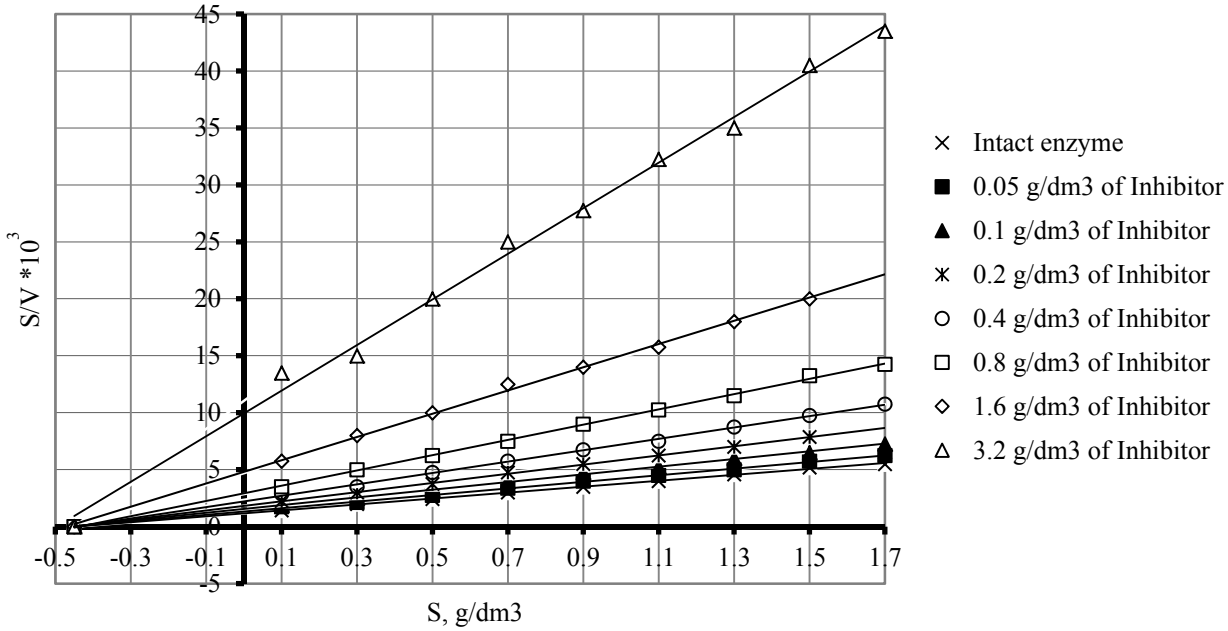

Fig. 3. Inhibition plot by Hanes.

Table 4. Statistical constants to calculate the kinetic parameters by Lineweaver-Burk and Hanes

By Lineweaver-Burk	By Hanes
$\overline{X} = 4.8677 \cdot 10^{-1}$	$\overline{X} = 4.63669 \cdot 10^{-1}$
$G_X = \pm 7.1463 \cdot 10^{-2}$	$G_X = \pm 3.6471 \cdot 10^{-2}$
$S_X = \pm 9.0758 \cdot 10^{-3}$	$S_X = \pm 4.6318 \cdot 10^{-3}$
$t = 53.6333$	$t = 100.1053$
$p < 0.001$	$p < 0.001$
$n = 7$	$n = 7$

Thus, the value K_m by Lineweaver-Burk for the enzyme over the inhibitor K_m = 486.8 ± 9.1 mg/dm^3, and by Hanes, it is K_m = 463.7 ± 4.6 mg/dm^3.

Webb plot data review is shown in Table 5.

Thus, as per the Webb plot, K_i = 403.8 ± 3.4 mg/dm^3. This method results in the reduced values of peak rate for non-inhibited enzyme equal to (92 ± 3)% of theoretical value. The process data of calculation results by Lineweaver-Burk and Hanes plots are shown in Table 6.

As it is seen in data given calculations by K_m/V_{max} give less absolute values K_i than those by $1/V_{max}$. Still, in view of errors when finding these values, the results are, in fact, similar. The analysis results ontained are summarized in Table 7.

CONCLUSIONS AND RECOMMENDATIONS

Therefore, we can state on the linear non-competetive enzyme inhibition upon processing of experimental data obtained upon inhibition of the pancreatic amylase by the proteic inhibitor based on methods of mathematical analysis above of kinetic studies.

Table 5. Statistical calculation constants K_i and V_{max} by the Webb plot

For K_i	For V_{max}
$\overline{X} = 4.0377 \cdot 10^{-1}$	$\overline{X} = 9.2006 \cdot 10^{-1}$
$G_X = \pm 2.6708 \cdot 10^{-2}$	$G_X = \pm 2.3464 \cdot 10^{-1}$
$S_X = \pm 3.3919 \cdot 10^{-3}$	$S_X = \pm 2.9799 \cdot 10^{-2}$
$t = 119.0391$	$t = 30.8754$
$p < 0.001$	$p < 0.001$
$n = 9$	$n = 9$

Table 6. Statistical constant values processed for starch hydrolysis over the maylase inhibitor by Lineweaver-Burk and Hanes methods

For Lineweaver-Burk plot	
As per tg α (K_m/V_{max})	As per Δ У ($1/V_{max}$)
$\overline{X} = 4.1921 \cdot 10^{-1}$	$\overline{X} = 4.5772 \cdot 10^{-1}$
$G_X = \pm 1.2919 \cdot 10^{-1}$	$G_X = \pm 6.2762 \cdot 10^{-2}$
$S_X = \pm 4.8833 \cdot 10^{-2}$	$S_X = \pm 2.3722 \cdot 10^{-2}$
$t = 8.5845$	$t = 19.2953$
$p < 0.001$	$p < 0.001$
$n = 7$	$n = 7$
For Hanes plot	
As per Δ У (K_m/V_{max})	As per tg α ($1/V_{max}$)
$\overline{X} = 4.2436 \cdot 10^{-1}$	$\overline{X} = 4.4498 \cdot 10^{-1}$
$G_X = \pm 1.0203 \cdot 10^{-1}$	$G_X = \pm 7.3228 \cdot 10^{-2}$
$S_X = \pm 3.8563 \cdot 10^{-2}$	$S_X = \pm 2.7678 \cdot 10^{-2}$
$t = 11.0044$	$t = 16.0773$
$p < 0.001$	$p < 0.001$
$n = 7$	$n = 7$

Table 7. Kinetic parameters of the hydrolysis response by the pancreatic amylase starch over the inhibitor based on the oat dust

Parameter to be defined	Lineweaver-Burk plot X ± m n = 7	Hanes plot X ± m n = 7	Computational approach X ± m; n = 7				Dixon plot		Plot by Webb X ± m n = 9
			As per Lineweaver-Burk plot data		As per Hanes plot data		$1/V_{max}$ by Lineweaver-Burk plot	$1/V_{max}$ by Hanes plot	
			tg α K_m/V_{max}	ΔY $1/V_{max}$	ΔY K_m/V_{max}	tg α $1/V_{max}$			
Michaelis constant (a)	486.8 ± 9.1(c)	463.7 ± 4.6 (c)	–	-	–	–	–	–	–
Maximum reaction rate (в)	–	–	–	–	–	–	361.7	377.1	92 ± 3 (d)
Inhibition constant (a)	–	–	419.2 ± 48.8	457.7 ± 23.7	424.4 ± 38.6	445.0 ± 27.7	572.2	508.7	403.8 ± 3.4

Note. a – mg/dm^3; в – A.U./mg; consistent with V_{max} of the intact enzyme; c – statistically significant difference, t = 2.267; 0.02 <p <0.05; d – here, results are given as percentage. Anywhere, apart in case "c", p < 0.001.

REFERENCES

1. Kabachnyy P.I. *Perspektivy sozdaniya lekarstvennykh sredstv gipoglikemicheskogo deystviya na osnove prirodnykh ingibitorov amiloliticheskikh fermentov: Lekarstvennye sredstva. Ekonomika, tekhnologiya i perspektivy polucheniya. Obzor* [Perspectives to create medicinal products of glycemic action based on natural inhibitors of amylolytic enzymes: Medicinal products. Economics, technology and perspectives of production. Review]. Moscow: VNIISENTI of Minmedprom of the USSR, 1990, ed. 1. 23 p.
2. Krusir G.V. and Kushnir N.A. Prevalence of amylase inhibitors in crops. *Zernovi produkty i kombikormy* [Cereal products and combined feed], 2008, no. 4, pp. 15–20. (In Ukrainian).

3. Sidenius U., Olsen K., Svensson B., and Christensen U. Stopped-flow kinetic studies of the reaction of barley α-amylase/subtilisin inhibitor and the high pI barley α-amylase. *FEBS Letters*, 1995, March 20, vol. 361, iss. 2–3, pp. 250–254. DOI: 10.1016/0014-5793(95)00187-E.
4. Keleti T. *Osnovy fermentativnoy kinetiki* [Fundamentals of enzyme kinetics]. Moscow: Mir Publ., 1990. 350 p.
5. Dzyuba N.A. and Zemlyakova O.V. Features of immobilization α-amylase on protein's and polysaccharide's matrixes. *Bulletin of the National Technical University "Kharkiv Polytechnic Institute": Mechanical-technological systems and complexes*, 2015, no. 52 (1161), pp. 108–112. (In Ukrainian).
6. Krusir G.V. and Kushnir N.A. *Sposib soderzhaniya ingibitora amilazy* [A method of producing an inhibitor of amylase]. Patent Ukraine, no. 35845, 2008.
7. Agromonov A. E. and Shabarov Yu. S. *Laboratornye raboty v organicheskom praktikume* [Laboratory works in the organic practice]. Moscow: Khimiya Publ., 1974. 376 p.
8. Perrin D.D., Armarego W.L.F., and Perrin D.R. *Purification of Laboratory chemicals*, Oxford: Pergamon Press, 1966. 181 p.
9. Hartree E.F. Determination of protein: A modification of the lowry method that gives a linear photometric response. *Analytical Biochemistry*. 1972, August, vol. 48, iss. 2, pp. 422–427.
10. Krusir G.V. and Kushnir N.A. Biochemical characterization an inhibitor of α-amylase from boroshenets of oats. *Proceedings of Odessa National Academy of Food Technologies*, 2008, iss. 34, vol. 1, pp. 252–257. (In Ukrainian).
11. Kornish–Bouden E. *Osnovy fermentativnoy kinetiki* [Fundamentals of enzyme kinetics]. Moscow: Mir Publ., 1979. 280 p.
12. Dzyuba N. Identification of complexforming amylase – glutin by infrared spectroscopy. *Commodities and markets*, 2015, no. 2 (20), pp. 33–40. (In Ukrainian).
13. Murray R.K., Granner D.K., Mayes P.A., Rodwell V.W. *Harper's Biochemistry*. Norwalk, San Mateo, 1988. (Russ. ed.: Marri R., Grenner D., Meyes P., Roduell V. *Biokhimiya cheloveka*: in 2 t. Tom 2. Moscow: Mir Publ., 2004. 414 p.).

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