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Isolation, physico-chemical characterization and biological activity of α-amylase from the culture liquid of the fungus *Aspergillus oryzae for using in the enzyme-linked* immunosorbent assay

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Abstract

The article describes the development of an effective method for purifying the α-amylase enzyme from the culture liquid (CL) of the fungus Aspergillus oryzae, by using an affinity sorbent, and the development of optimal conditions for sorption and desorption of proteolytic enzymes from CL on protein-impregnated Sorsilene. The purification of α-amylase from the CL of the fungus Aspergillus oryzae was studied by salting, ion exchange, and gel chromatography with and without removal of proteinases. It was established that in the process of isolating α-amylase from the CL of the fungus Aspergillus oryzae, the preliminary removal of proteinase by its biospecific sorption contributes to an increase in the efficiency of enzyme purification. Some physicochemical characteristics and biological activity of the obtained highly purified enzyme have been investigated. It has been shown that the preliminary removal of proteinases contained in CL on biospecific sorbents contributes to an increase in the degree of purification of α-amylase. An ELISA was developed to detect the bacterial antigen, a plant virus, and a pathogenic fungus, in particular, diphtheria toxin, the tobacco mosaic virus TS-TMV, and the protein antigen of the fungus P. varioti, using α-amylase as an enzyme label. The developed method has a number of advantages in comparison with the known ELISA methods, where horseradish peroxidase and phospholipase A2 enzymes were used.

Keywords: α - amylase, *Aspergillus oryzae*, culture liquid - CL, salting, ion exchange chromatography, gel chromatography, biospecific sorbent, impregnated BSA, antibody conjugates, metal enzyme, “sandwich” ELISA.

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1. Introduction

Amylolytic enzymes are widely used in industry, scientific research, medicine, enzyme diagnostics and other fields, and many works devoted to the approaches to produce them [1-7]. In this regard, the need for especially pure preparations of this enzyme has increased in recent years. The source of highly purified amylases is usually objects of plant origin, but currently, microbial amylases are increasingly preferred due to their rather high stability [8-11]. Therefore, there is a need to develop effective ways to purify amylases from sources of microorganisms including fungi *Aspergillus oryzae* [8, 12-14]. Unfortunately, the CL of the fungus Aspergillus oryzae, along with the main enzyme α - amylase, also contains a set of hydrolytic enzymes that are active under CL storage conditions and, especially, during the isolation of the main enzyme. As a result of the action of these proteinases, the amylolytic activity of CL is significantly reduced during isolation and storage [15, 16].

Separation of proteinases from the main enzyme is quite a difficult task, since the values of molecular weights and isoelectric points of amylases and proteinases present in CL are close, and they cannot be separated sufficiently by gel filtration and ion exchange chromatography [16-18]. In addition, due to the high affinity of proteinases to amylases as a protein substrate, complexes of the proteinase-amylase type are formed. Therefore, in this work, we set out to obtain a preparation of α - amylase free from proteinases using the CL of the fungus *Aspergillus oryzae,* and use it for testing bacterial, viral and fungal antigens by ELISA method.

2. Materials and Methods

Diphtheria toxin and diagnostic diphtheria antitoxin purified by enzymolysis and specific sorption were obtained from the scientific and practical association "Biomed" (Russia). The purified preparation of the tobacco mosaic virus TSh-TMV and antibodies were obtained from the Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan and the Department of Microbiology and Biotechnology at the National University of Uzbekistan. The protein antigen of the *P. varioti* fungus, isolated from the CL by precipitation and purified by gel filtration, and antibodies to it were obtained from the Scientific Center of Medical Mycology and Protozoal Diseases of the Ministry of Health of Uzbekistan.

A culture liquid (CL) of the fungus *Aspergillus Oryzae* obtained at the Department of Microbiology and Biotechnology of the National University of the Republic of Uzbekistan named after M. Ulugbek was used in the work. Polyethylene terephthalate impregnated with bovine serum albumin was used as a sorbent for the removal of proteinases.

Removal of proteinases by biospecific chromatography. As a sorbent for removal of proteinases, polyethylene terephthalate impregnated with BSA was used. 1 g of sorbent was suspended in a 5-fold volume of 1% BSA in 0.05 M universal buffer (H_3PO_4-H_3BO_4-CH_3COOH-NaOH), pH 3.5. The mixture was stirred and kept for 10-12 hours at 6-10°C. Then, it was washed with the same buffer containing 20% ethanol. After that, concentrated CL of Aspergillus oryzae fungus was added to the sorbent, mixed, and kept for 20-30 min at 5-10°C. After incubation, the mixture was centrifuged and the supernatant was separated.

Precipitation. Further, supernatant subjected to $(NH_4)_2SO_4$ precipitation and active fraction, which precipitated from 35 to 80% $(NH_4)_2SO_4$, was dialyzed against 0.05 M Tris-HCl buffer at pH 8.3 for 24 h.

Ion exchange chromatography. Obtained precipitate after dialysis was subjected to ion-exchange chromatography on a column (2.5x20cm) packed with DEAE DE-52. Proteins were eluted using a gradient of increasing concentration of NaCI in 0.05 M Tris-HCl buffer at pH 8.3. The E280 value was determined on a Uvicord instrument (LKB, Sweden). Fractions with a volume of 10 ml were collected on an Ultropak fraction collector (LKB, Sweden). Fractions with the highest activity were pooled, dialyzed against distilled water, and lyophilized.

Gel chromatography. Active fraction was subjected to gel chromatography on a column (2.5x100cm) with AcA-54 ultra-gel in 0.05 M $(NH_4)_2CO_3$, pH 8.3. The active fraction was freeze-dried.

Electrophoresis. PAAG electrophoresis in a 10–20% acrylamide concentration gradient was performed in the presence of SDS-Na according to the Laemmli method [19]. The protein content in the fractions was determined by the Lowry, et al. [20]. Amylolytic activity was determined by the iodometric method [21]. Proteinase activity was measured according to the modified Anson method [22] in 0.1 M universal buffer, pH 2.3. 1 ml of 2% BSA solution was used as a substrate.

Determination of α-amylase activity. The activity of α-amylase was determined by the iodometric method [23]. For this, 1 ml 1% starch pH 5.0 (pH adjusted with 0.1 M CH_3COOH buffer) was added to 0.5 ml enzyme solution. Starch hydrolysis was carried out for 10 minutes at 37°C. Then 0.1 ml aliquots of the solution were transferred into a 10 ml tube working solution of iodine (5.0 mg of J_2 and 50 mg of KJ in 100 ml of 0.1 N HCI). In the control experiment, distilled water was taken instead of the enzyme. The colour intensity was determined by calorimetrically against the control sample.

As a unit of activity was taken as the amount of enzyme that catalyzed the hydrolysis of 10 ml for 10 minutes at 37°C 1% starch solution so that the degree of conversion of the substrate is 30%.

Calculation of enzymatic activity according to the depth of hydrolysis in % was carried out according to the formula:

Obtained data was calculated by the following formula:

$$C = \frac{D1-D2}{D1} * 100$$

Where:

D_1 – optical density of the control solution;

D_2- optical density of the sample.

The calculation of enzymatic activity by the amount of hydrolysed starch was carried out according to the formula:

$$C = \frac{D1-D2}{D1} * n$$

Where:
D_1 – optical density of the control solution;
D_2- optical density of the sample.
n-amount of starch, taken for analysis as a substrate (g)

Amylase activity was calculated according to the formula:

$$A = \frac{7.264*C-0.03766}{n*5} * 1000$$

Where:
C – amount of hydrolysed starch, mg;
n- amount of enzyme, mg;
1000- coefficient to calculation from milligram to gram;
7,264 and 0,03766- calculation equation coefficients.

Synthesis of α-amylase conjugates. The antigens listed above were used to obtain conjugates and purify them. As examples, we describe the preparation of conjugates with antibodies to diphtheria toxin.

To a 1.15 mg of α-amylase in borate buffer pH 8.3 was added 0.5 mg of antibodies and 0.1 M borate buffer, pH 8.3 to a volume of 5 ml. Then, 0.1 ml of a 0.01% solution of glutaric dialdehyde in the same buffer was added dropwise to this mixture with vigorous stirring. After incubation (8 hours), dialysis was performed against 0.15 M NaCl solution, centrifuged, and protein was determined by the Lowry, et al. [20]. The conjugate was purified by gel chromatography on TSK gel HW-55.

3. Results and Discussion

To obtain the enzyme α – amylase with high activity, we carried out two approaches: purification of amylase on DE-52 cellulose, without pre-sorption of proteinases, and the same, after treatment of the initial solution with an affine sorbent.

Experiments have shown that during purification, amylase was indeed subjected to enzymatic cleavage by proteolytic enzymes contained in the composition of CL. Moreover, proteins adsorbed on DEAE cellulose were more efficiently subjected to enzymatic hydrolysis by acid proteinase. This has been proven using artificial mixtures of BSA and proteinase.

In the case when proteolytic enzymes were first removed from the initial solution during amylase purification, fairly good data were obtained. Removal was carried out using a biospecific sorbent, which is polyethylene terephthalate impregnated with BSA. At the same time, the level of proteinase activity in the enzyme solution was significantly reduced. The results of the experimental data obtained are presented in the Table 1. As can be seen from the table, the removal of proteinases had a positive effect on the processes of amylase purification. Such treatment of the solution reduced the proteinase activity by three times, and the amylase activity increased by 3,5 times.

Table 1.
Purification steps of α-amylase.

Cleaning stage	Protein concentration. mg/ml	Total protein. mg	Amylase activity. units/g	Protease activity. units/g	The frequency of purification by amylase activity
Concentrated CL	25.5	670.0	2200	180	1.0
Sorbent treatment	17.0	370.0	7100	51.3	3.2
Salting out	6.0 (12.5)*	130.0 (260)	16600 (6100)	6.8 (49.2)	6.9
Ion exchange chromatography	0.75 (0.5)	8.4 (14.2)	260000 (61200)	0.051 (14.2)	118.2
Gel chromatography	0.59	3.65	610000	0.03	277

Note: *Data in parentheses are obtained without using the procedure for removing proteinase by biospecific chromatography.

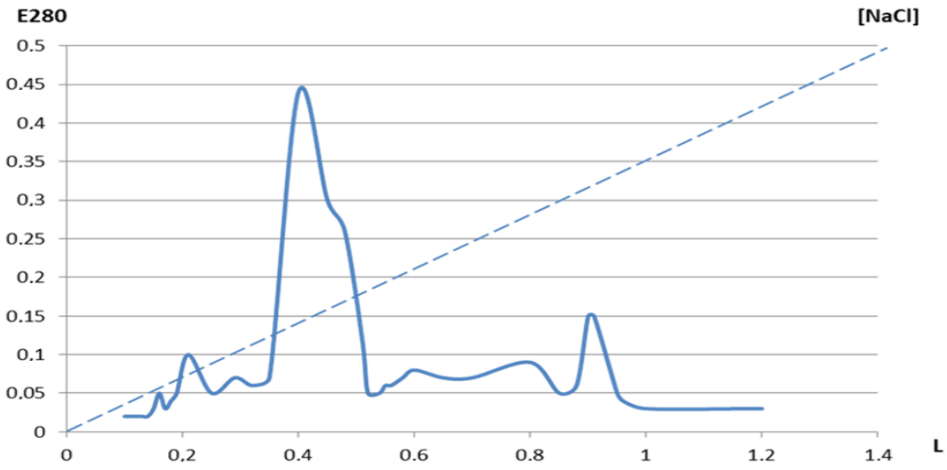


Figure 1.
Ion exchange chromatography of concentrated CS of the fungus Aspergillus oryzae on the DEAE DE-52. Column 2,5x20 cm, in 0,05 M tris-HCl buffer, pH 8,3, linear concentration gradient [NaCl] from 0 – 0,5 M. Elution rate 50 ml /h. 3 - active fraction.

At the next stage, the enzyme solution was salted with ammonium sulfate to 80% saturation. In a result, proteinase activity decreased by 24 times, and amylase activity increased by 7.5 times. During ion-exchange chromatography (Figure 1) of the obtained fraction on DEAE cellulose, there was a further increase in amylase activity by 118 times. At the same time, proteinase activity decreased by 3529 times.

Further purification of the active fraction by gel chromatography on the AcA-54 ultra-gel, a highly purified amylase with an activity of 610000 U/g was obtained (Figure 2). Proteinase activity was insignificant (0.03 U/g). The degree of purification in this case was 277 times. The resulting α -amylase preparation had a sufficiently high degree of purity.

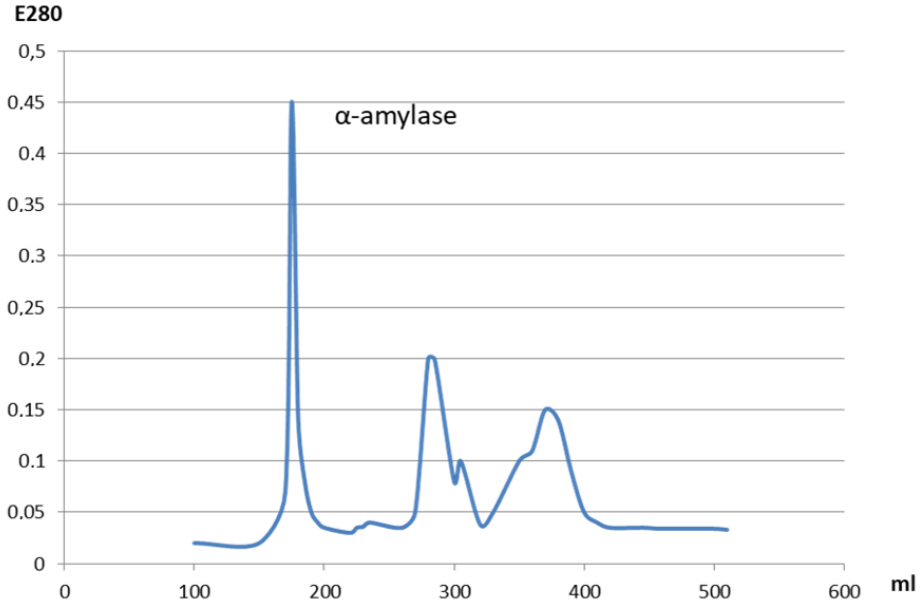


Figure 2.
Gel chromatography of the active fraction on a column (2,5x100cm.) with ultra-gel AsA - 54 in 0,05 M $(NH_4)_2CO_3$, pH 8,1. Elution rate 35 ml/h.

Thus, electrophoresis performed in the presence of SDS-Na PAGE showed a single band in the anode area (Figure 3).

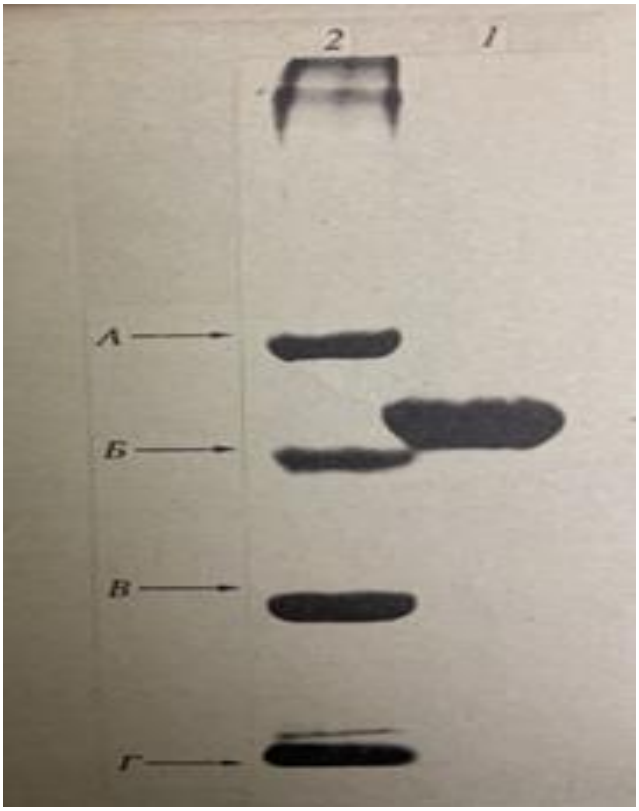


Figure 3.
Electrophoresis of α - amylase (1) in PAAG in the presence of SDS-Na in an acrylamide concentration gradient of 10-20%. 2 - marker proteins (kD) A- BCA, Б-ovalbumin, B-chymotrypsin, G-cytochrome C.

Thus, when purifying amylase from a mixture of amylolytic enzymes and proteinases, the preliminary removal of proteinase by its biospecific sorption contributes to an increase in the efficiency of enzyme purification. This approach can be successfully used for the separate isolation of amylases and proteinases from various solutions containing these enzymes.

Further, the resulting highly purified α-amylase was studied to clarify some of the physicochemical characteristics of the enzyme. Thus, the results of electrophoresis performed in the presence of SDS-Na showed that the molecular weight of α - amylase is 52000 D.

It is known that all α - amylases belong to metalloenzymes and they exhibit greater stability and activity in the presence of Ca^{2+} ions. The content of Ca^{2+} ions in them ranges from 1 to 30 g-atom per 1 g-mole of the enzyme [24]. The determination of the activity of the α – amylase purified by us showed that at 350 mM of calcium concentration, the enzyme shows maximum activity. A further increase in the concentration of Ca^{2+} ions in the incubation medium adversely affects the activity of the enzyme, and a decrease in the activity of α – amylase is observed.

In addition, we have studied some biological properties of purified α - amylase. It can be seen from Figure 4 that the enzyme exhibits maximum activity at a pH value of 5.2.

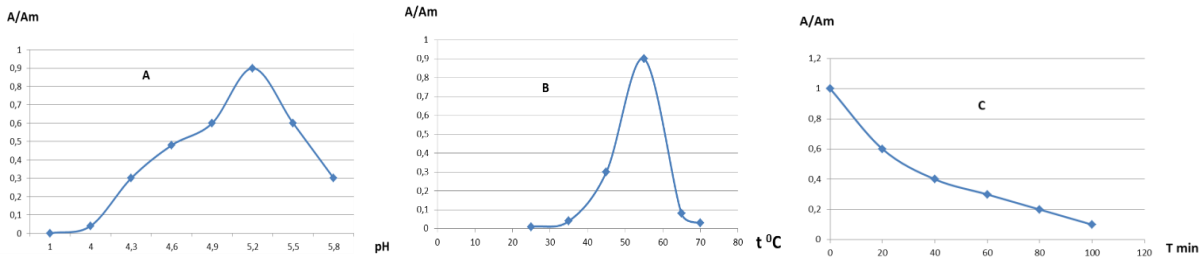


Figure 4.
Some catalytic properties of α - amylase. A - pH-optimum, B - dependence of activity on temperature, C - thermal inactivation at 50 ^{0}C.

At the temperature of 55°C, thermal stability of amylase is observed (Figure 4 C). As can be seen from Figure 4C, the half-inactivation time of the native enzyme at 55°C is 30 minutes. This means that the enzyme, despite the high degree of purity, is quite stable. In solution, the activity of the enzyme at +4°C was maintained for several weeks.

It is known that all α-amylases are metalloenzymes and exhibit greater stability and activity in the presence of Ca^{2+} ions. The content of Ca^{2+} ions in them varies from 1 to 30 g-atom per 1 g-mol of enzyme [21]. Determination of the activity of α-amylase purified by us showed that at a calcium concentration of 350 mM, the enzyme exhibits maximum activity. A further increase in the concentration of Ca^{2+} ions in the incubation medium negatively affects the activity of the enzyme, while a decrease in the activity of α-amylase is observed (Figure 5).

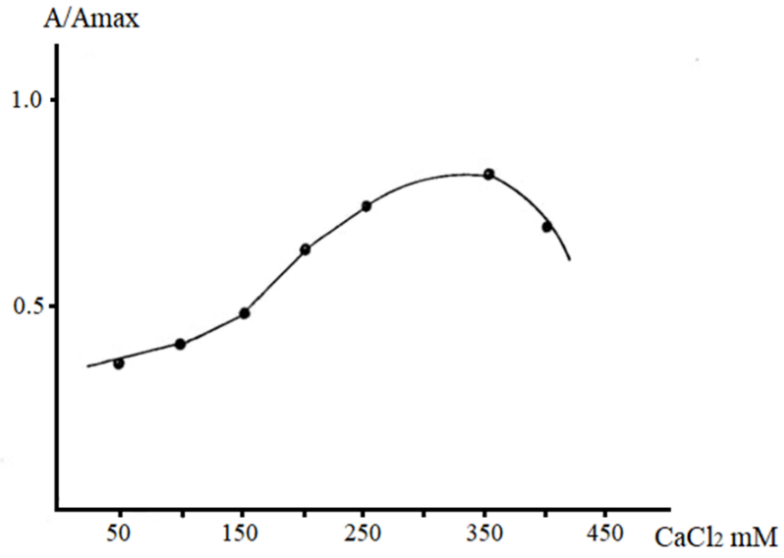


Figure 5.
Influence of Ca2+ ions on the enzymatic activity of α-amylase.

Thus, in the process of isolating α – amylase from the CL of the fungus *Aspergillus Oryzae*, the preliminary removal of proteinase by its biospecific sorption contributes to an increase in the efficiency of enzyme purification. The isolated highly purified enzyme can be successfully used in biotechnological research, for example, in immunodiagnostics, in particular in the development of ELISA as an enzyme label.

3.1. Preparation And Purification of Conjugates and Their Properties

When developing an ELISA, one of the defining moments is the introduction of an enzyme label into the composition of antibodies [22]. Currently, two methods are mainly used for the synthesis of conjugates: one-stage and two-stage methods for obtaining conjugates. In the one-step synthesis of conjugates, a cross-linking reagent, for example, glutaric dialdehyde, is added directly to the mixture of the enzyme with antibodies (or antigen) [23] while in the two-step method, the enzyme is first pretreated with a cross-linking reagent, i.e., modified with glutaric dialdehyde, and then the desired component of the immunochemical reaction [25].

When obtaining an antibody-enzyme conjugate using cross-linking agents by a one-step method, the selection of the concentrations of these reagents plays a decisive role. High concentrations of crosslinkers in the reaction mixture increase the likelihood of conjugation of the same type of antibody-antibody and enzyme-enzyme molecules, and, conversely, low concentrations of crosslinkers form too few mixed-type conjugates, as a result of which the enzymatic activity of the conjugates will be reduced. Therefore, we carried out preliminary experiments with various concentrations of glutaric dialdehyde in order to find the optimal concentration of the latter. At the same time, the conjugate obtained at a concentration of glutaric dialdehyde of 0.01% had the maximum enzymatic activity, which was 55% of the activity of the original α-amylase (Figure 6). Lower concentrations and higher concentrations give reduced activity.

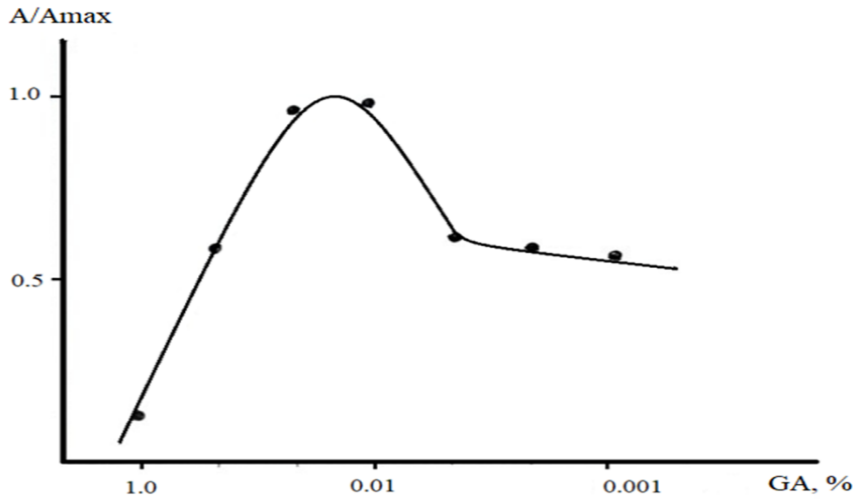
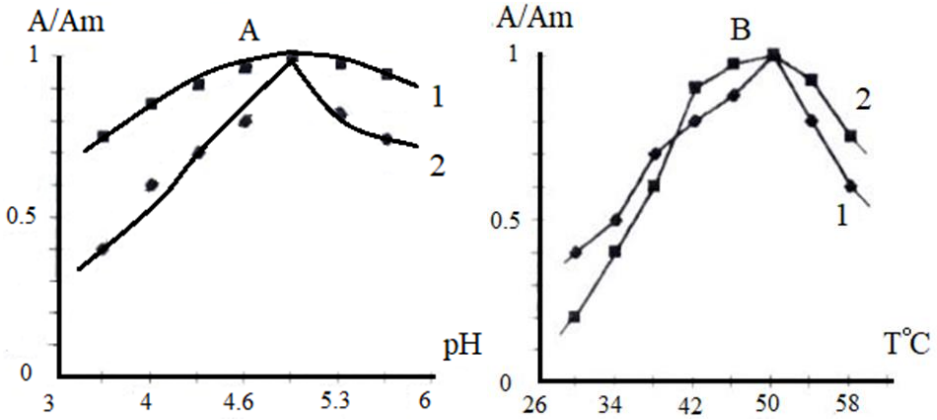


Figure 6.
Dependence of the enzymatic and antigenic activity of the conjugate on the concentration of glutaric dialdehyde in the incubation medium.

3.1.1. GA -- Glutaric Dialdehyde

The results of preliminary studies have shown that simply mixing antibodies with the enzyme α-amylase does not adversely affect the ability of antibodies to react with antigens. It does not change the activity of the amylase.

The study showed that when antibodies are covalently bound to α-amylase, the activity of the enzyme decreases, which is apparently due to the difficulty of access of the macromolecular substrate (starch) to the enzyme molecule that has passed into the bound state. This assumption is supported by the data characterizing the dependence of the rate of the reaction catalyzed by amylase on the concentration of substrates in Leunweaver–Burk coordinates (Figure 7D).



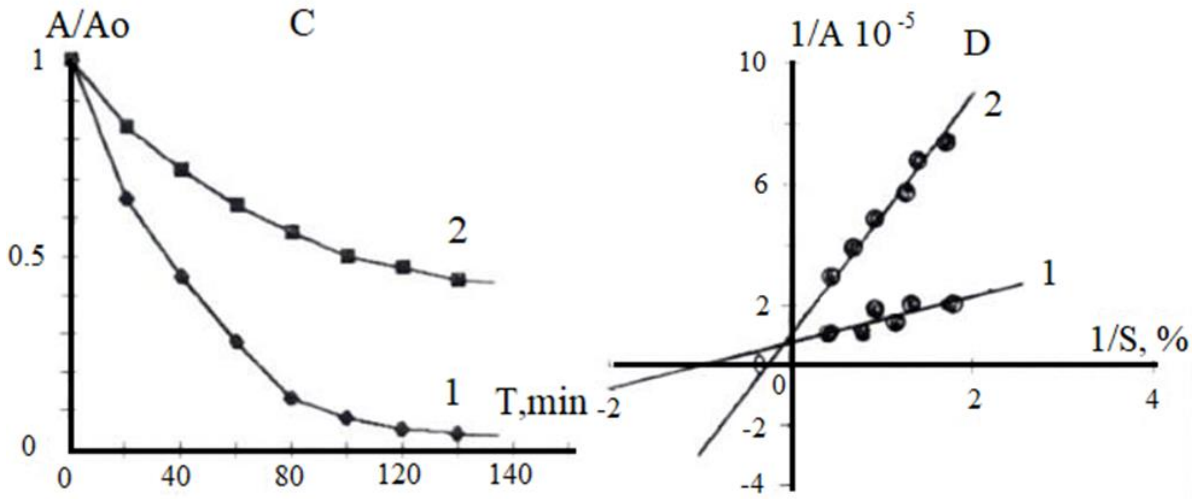


Figure 7.
Changes in some of the catalytic properties of a-amylase during the formation of an enzyme-antibody conjugate.

A - pH-optimum: B - dependence of activity on temperature; C - thermal inactivation at 50°C; D - dependence of activity on the concentration of the substrate in the Lineweaver - Berkk coordinates.
1- original a-amylase; 2- a-amylase conjugate.
Ao - specific activity of the enzyme before incubation;
Am - the maximum value of the specific activity.

Figure 7D shows that the Km value increases from 0.91 to 2.85 during enzyme conjugation. This indicates that binding to the substrate is difficult for the enzyme present in the conjugate. The decrease in the activity of amylase in conjugates compared with the initial activity of the enzyme is the more significant, the higher the concentration of the cross-linking agent and both components forming the conjugate. At the same time, the obtained conjugates have a sufficiently high amylase activity.

After binding of amylase with antibodies, one can expect changes in the individual properties of the enzyme. In this regard, in a comparative aspect, some properties of the conjugated and native enzyme were studied. On Figure 7A shows the pH-dependence of the activity of α-amylase in native and conjugated states with antibodies. The pH optimum for the manifestation of amylase activity does not change after conjugation; however, there is some broadening of the profile of optimal pH values in comparison with the native enzyme. The temperature optimum of the enzyme action before and after conjugation practically does not change (Figure 7 B).

In addition, after conjugation, a slight increase in the inhibitory ability of amylase is observed. As can be seen from Figure 7 B, the half-inactivation time of the native enzyme upon incubation at 50°C is 30 min, and for the amylase in the antibody conjugate, it is more than 100 min.

Studies of the dependence of the enzymatic activity of the conjugate on its concentration in the reaction medium showed that in the medium of a dilute solution, the conjugates have a higher catalytic activity (Table 2).

Table 2.
α-amylase activity.

No.	α-amylase. mg	Antibody. mg	GA.%	Volume.ml	Activity of the conjugate. U/g
1	1	0.3	0.05	2	24160.05
2	1	0.3	0.05	3	35364.25
3	1	0.3	0.05	5	47837.32
4	1	0.3	0.05	7	49768.12

Source: GA - glutaric dialdehyde, Initial activity of α-amylase 103000 ED/g.

The next stage of research was to determine the optimal concentration of the conjugate for ELISA (Figure 8 A, B, curves 1-4). The results of testing the antigen at concentrations of 5 ng/ml, 50 ng/ml, 500 ng/ml and 5000 ng/ml on 16.2 mg membranes (25 µg of antibodies were used for immobilization) shown in the figure show that a higher percentage of enzymatic reaction products formed after 10 minutes of hydrolysis, when using conjugates at a concentration of 20 µg/ml.

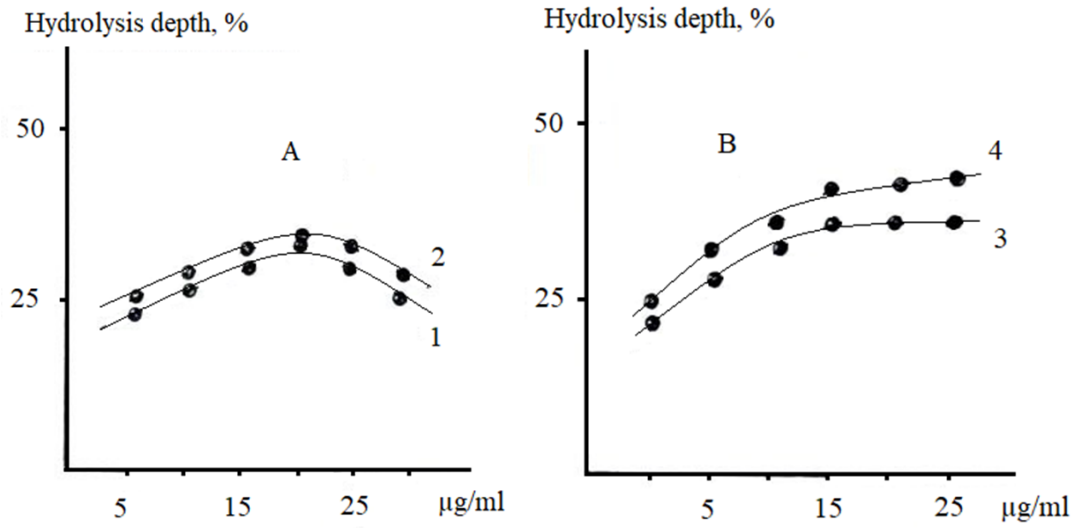


Figure 8.
Determination of the concentration of the conjugate, optimal for setting the "sandwich" variant of the ELISA.

1. The content of the antigen in the sample is 5.0 ng/ml.
2. The content of the antigen in the sample is 50 ng/ml.
3. The content of the antigen in the sample is 500 ng/ml.
4. The content of the antigen in the sample is 5000 ng/ml.

It should be noted that when testing the antigen at a concentration of 5 ng/ml, 50 ng/ml, 500 ng/ml, the numerical value of the amount of hydrolysis products is at the same level, although the greatest depth of hydrolysis is observed when testing at a concentration of 5000 ng/ml. Perhaps this result is achieved due to the specifically bound conjugate with the antigen, that is, with an increase in the concentration of the antigen in the medium, the amount of the specifically bound conjugate increases proportionally.

The α-amylase-antibody conjugate was prepared by cross-linking with glutaric dialdehyde. The optimal ratio of antibodies to α-amylase and the effect of covalent crosslinking on the preservation of antigenic and enzymatic activity in a medium with different number of antibodies and the constant concentration of other components (Table 3).

As the table data show, when α-amylase is conjugated with antibodies, a decrease in enzyme activity is observed, and the more significant, the higher the concentration of antibodies in the medium. The resulting conjugate retains a sufficiently high specific activity and antigenic specificity. As evidenced by the results of testing antigens of bacterial toxins in the range of 0.005-5000 ng, in the sample, with a decrease in the concentration of antigens in the medium, the enzymatic activity of the specifically bound conjugate also decreases proportionally. However, in the third and fourth variants of the ratio of antibodies and enzyme, conjugates are formed that are deficient in the content of the enzyme, which reduces the efficiency of their use, especially in the region of low antigen concentrations, and the sensitivity of the analysis decreases.

From the test results, we can conclude that the most effective binding of α-amylase molecules and antibodies occurs at a ratio of 1:0.5 (Table 3, option 1) and an aldehyde concentration of 0.01% in an incubation medium with a volume of 5 ml. This ratio is optimal for the use of the resulting conjugate when conducting ELISA for bacterial toxins [26, 27]. When conjugated with α-amylase, along with antibodies labeled with the enzyme, components of various compositions are formed in solution, i.e., the resulting conjugates can be contaminated with impurities of antibodies that have not reacted with the enzyme. These impurities interfere with the practical application of ELISA and reduce the sensitivity of the method. Therefore, it was necessary to separate them from the reaction mixture.

The conjugate was purified by gel chromatography on TSK gel HN-55. As can be seen from Figure 9, 4 fractions were obtained as a result of chromatography. Of the obtained fractions, only fraction 2 had both serological and enzymatic activity.

Table 3.
Dependence of the Enzymatic and Antigenic Activity of the Antibody Conjugate on the Content of the Reacting Components.

Number of conjugates	Reacting components			Specific activity of the conjugate. U/g	Volume. ml	Activity of the conjugate in deep hydrolysis % at ELISA						
	GA. %	Antibody.mg	α-amylase.mg			Amount of antibody in sample. ng/ml						
						5000	500	50	5	0.5	0.05	0.005
1	0.01	0.5	1.15	48176.4	5	29.7	25.4	19.6	14.3	9.3	2.5	0
2	0.01	1.0	1.15	39092.8	5	26.1	23.2	17.7	14.0	7.9	1.9	0
3	0.01	2.0	1.15	36893.5	5	19.0	15.2	12.1	8.3	6.4	1.02	0
4	0.01	3.0	1.15	23108.1	5	15.4	12.7	10.2	6.5	3.7	0.18	0

Source: GA – glutaric dialdehyde. The data presented in the table corresponds to the difference between the ELISA data and the background.

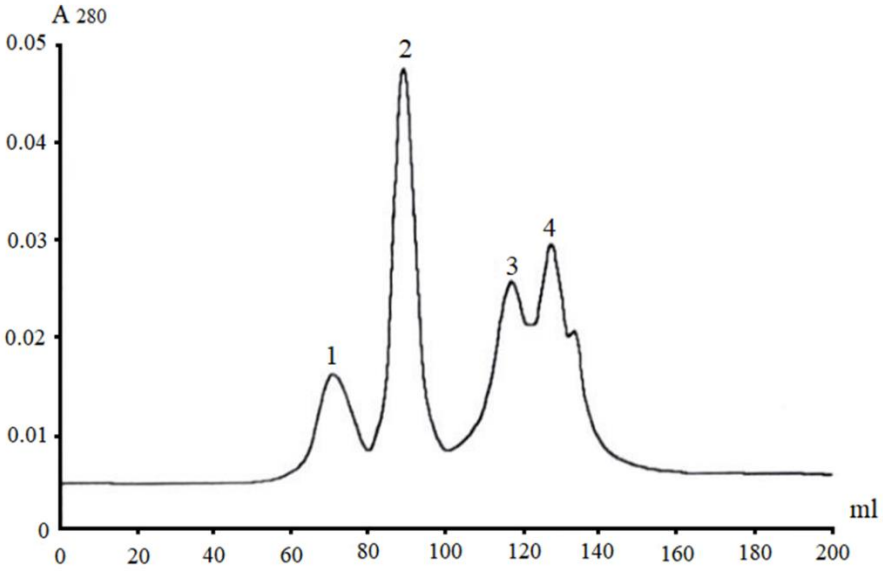


Figure 9.
Gel chromatography of the conjugate of antibodies with diphtheria toxin on a column (1.5x100 cm) with TSC-HW55 in 0.05 M Tris-chloride buffer, pH 8.2. Elution rate 42 ml/h.

It is likely that the antibody-antibody complex (AT-AT) is contained in fractions 1, since the 1st fraction has the highest molecular weight, and the enzyme-enzyme complex and unreacted enzyme and antibody are contained in fractions 3 and 4. Then fraction 2, having the highest enzymatic and serological activity, subjected to dialysis and concentrated to a volume of 1 ml. After dialysis, the activity of the purified conjugate was checked by the Uchterlony method. The obtained data confirmed that the conjugate has sufficient amylase activity and simultaneously enters into a serological reaction with antibodies to diphtheria toxin.

The total yield of the conjugate after dialysis was 33.48% of the activity of the initial mixture taken for purification. Thus, the conjugate of antibodies to diphtheria toxin, which has a high enzymatic and serological activity, has been purified. The purified conjugate was subsequently used for diphtheria toxin antigen detection by ELISA.

It is known that at present in enzyme immunoassay (ELISA) polystyrene plates with adsorption-immobilized antibodies are very widely used as a substrate. However, adsorption does not provide sufficient strength of ligand binding, which leads to errors in the analysis and is the main factor in the scatter of the obtained data [25]. Therefore, more preference in ELISA has recently been given to covalent methods of binding to carriers. Materials such as cellulose, sepharose, sephadex, silochrome, porous glass, etc. are widely used as carriers [28, 29]. But it should be noted that one of the significant drawbacks of granular carriers is the difficulty of their accurate dosing and the difficulty of washing without losing microquantities of the immunosorbent used in the analysis.

In this regard, it seemed to us appropriate to work on membrane carriers, since their use simplifies the process of enzyme immunoassay and eliminates losses during dosing, washing, etc. Therefore, we chose polyamide membranes as carriers, which have a number of advantages: non-swelling in water and buffer solutions, availability, easy of conversion into a reaction form, etc.

Based on the data obtained, an enzyme-linked immunosorbent assay (ELISA) "Sandwich" variant for viral, bacterial and fungal antigens was developed (Scheme).

The "sandwich" method is based on the use of immobilized and labeled specific antibodies immobilized on a carrier, as well as labeled antibodies (antibody-enzyme conjugates).

The immune reaction is carried out in two stages: the first stage consists in adding a detectable sample to the immobilized antibodies. After washing off the impurities, a repeated immune reaction is carried out. An antibody-enzyme conjugate is added to the antibody-antibody complexes. Conjugates of enzymes with antibodies are able to bind only in those places where the antigen molecules are located. This means that the more antigen was in the test sample, the more antibodies labeled with the enzyme will bind. Then the medium is washed a second time, the substrate is added and the amount of the label associated with the immune complex is recorded. The antigen sandwiched between the molecules of the immobilized antibody and the antibody bound to the enzyme is a "sandwich" complex.

Immune enzyme analysis scheme

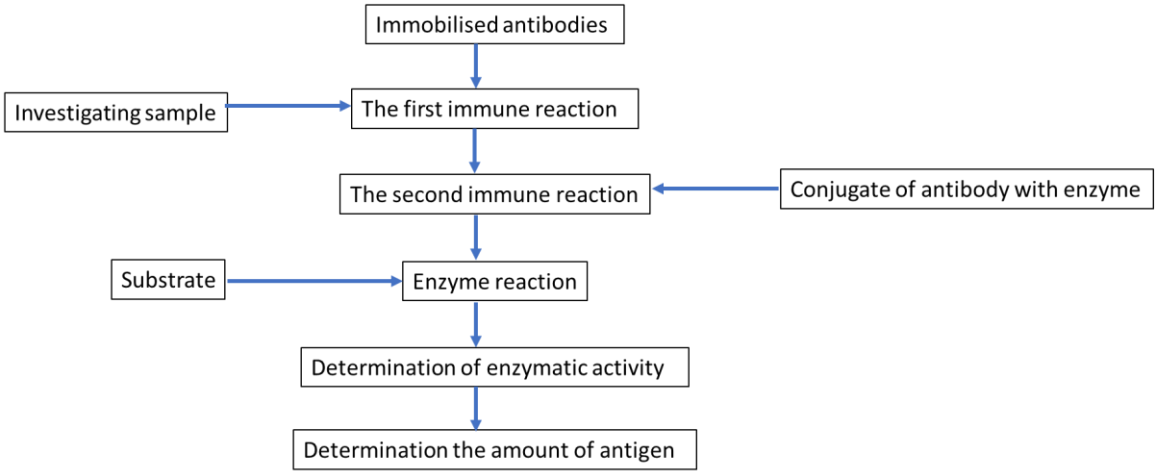


Figure 10.
Immune enzyme analysis scheme.

Next, we present the ELISA developed by us according to the above scheme for a number of objects under study.

3.2. Analysis of Diphtheria Antigens

The diphtheria diagnosticum developed by us includes: a) purified antigenic preparation (for calibration blood); b) polyamide membranes with immobilized antibodies; c) conjugate of antibodies with α-amylase; d) 0.5% starch solution.

The analysis procedure is simple, does not require special equipment, and consists in successive immersion of membranes with immobilized antibodies.

Before using for ELISA, the membrane with immobilized antibodies is washed three times with 0.05 M Tris-HCl buffer pH 7.5 containing 0.01% Tween-20 and balanced with 0.05 M Tris-HCl buffer pH 7.5. Then pour 0.5 ml of a pure preparation of the sample to be determined.

Although the reaction of the formation of the antibody-antigen complex in solution proceeds rather quickly, it should still be noted that the determining factor affecting the rate of formation of the antibody-antigen complex is low concentrations of the components. In this regard, it was decided to increase the incubation time from 10 to 20 minutes.

In this case, the antigen of the sample to be determined specifically binds to the antibodies immobilized on the membrane. After incubation, the membrane is washed from unbound antigen and poured into 0.9 ml of Tris-HCl buffer pH 7.5 containing 20 μg (by protein) of the antibody conjugate with α-amylase, and left for 20 minutes with continuous stirring. During incubation, the antibody conjugate on the membrane binds specifically to the antigen of the sample to be determined on the membrane. The membrane is washed from unbound Tris-HCl conjugate with a buffer containing 0.01% Tween-20 to completely remove non-specifically bound proteins and 0.05 M Tris-HCl buffer pH 8.1.

After enzyme immunoassay (ELISA), 0.2 ml of the substrate solution was added to the membrane and hydrolysis was carried out for 10 minutes at 37 0C with constant shaking. Then, a 0.1 ml aliquot of the solution was transferred into a preliminarily prepared test tube with 10 ml of iodine working solution. In control experiments, a solution of the substrate was taken. By changing the intensity of the color of the iodine solution, you can visually determine the presence of antigen in the sample or use a photo-colorimeter to determine the quantitative content of the antigen using a calibration curve.

A calibration curve was built using a purified preparation of diphtheria toxin in the concentration range from 0.032 to 3200 ng/ml.

3.3. Analysis of the HSV-TMV virus

Taking into account certain conditions, ELISA TS-TMV was carried out in a purified virus preparation in the concentration range from 50 ng/ml to 0.025 ng/ml and in the juice of virus-infected plants in a dilution range from 1:2 to 1:5000. Experiments have shown that, starting from a virus concentration of 6.25 ng/ml, and in the juice of charged plants from a dilution of 1:64, there is a proportional dependence of the rate of the enzymatic reaction of the conjugate specifically associated with the virus on the membrane on the concentration of kills and the activity of the virus, which also indicates preservation of the enzymatic and antigenic activity of the conjugates. At high concentrations of the virus in the incubation medium, inhibition of the immunological reaction by an excess of the virus is observed. It should be noted that the lower value of the virus concentration by protein, which can be determined using ELISA, is 0.048 ng/ml on membranes weighing 10-15 mg. The lower value of the juice dilution is 1:1024 (see Table 4).

3.4. P. varioti antigen analysis

To determine the pathogenic fungus P. varioti in the blood of patients, microbiological methods are currently used mainly (blood cultures, sputum of patients). Immunological methods for this purpose are practically not used. ELISA for the detection of P. varioti antigens was developed by us for the first time. The optimal conditions for conducting ELISA for the fungus P. varioti were as follows: 0.05 M Tris-HCl buffer pH 7.5 containing 0.1% BSA was used for analysis; the concentration of the conjugate at low concentrations of the antigen was 16 μg/ml, at high concentrations up to 22 μg/ml;

membrane weight 15 mg; the volume of the substrate is 0.2 ml. The minimum value of the protein antigen concentration of the fungus P. varioti is presented in the table.

Table 4.
Dependence of the enzymatic activity of conjugates of antibodies to bacterial, viral and fungal antigens and the sensitivity of ELISA on the reacting components of the medium.

Conjugates and objects	Reacting components			Conjugate activity, U/g	Lower limit of sensitivity, ng/ml
	GA,%	Antibody, mg	α-amylase, mg		
Diphtheria	0.05	0.5	1	44494.47	0.032
TSh-VTM	0.01	3	1	19816.2	0.048
P. varioti fungus	0.05	3	1	21124.02	0.040

Based on the research carried out, it was possible to develop an original, simple, affordable, highly sensitive method for enzyme immunoassay (ELISA) for the determination of viral, bacterial and fungal antigens. Its applicability for the analysis of a wide range of objects of varying complexity is shown. Among them, diphtheria toxin can be determined from microbial toxins up to a concentration of 0.032 ng/ml. more complex entities such as the HSVTM tobacco mosaic virus and P. varioti antigen can be detected down to an antigen concentration of 0.048 ng/mL. and 0.04 ng/ml. respectively.

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