

Purification and Characterization of Highly Active Glucoamylase and Pre-Clinical Study of General Toxicology of Fungus *Aspergillus oryzae* UzMU K-14

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Abstract Highly active and thermostable amylolytic enzymes are currently being investigated to improve industrial processes of starch degradation. In this study, we developed obtaining of highly activity fungi strain *Aspergillus oryzae* UzMU K-14 producers of thermostability glucoamylase and studied some cultural-morphological properties got strain, the method fungi is received allowing get in laboratory condition, purification and characterization of a thermostable glucoamylase from *Aspergillus oryzae* UzMU K-14. A highly active extracellular glucoamylase (exo-1,4- α -D-glucanohydrolase, E.C.3.2.1.3) from the culture supernatant of a thermophilic fungus *Aspergillus oryzae* UzMU K-14 was purified by gel-filtration, gel electrophoresis (SDS PAGE) homogeneity by using ammonium sulfate fraction. Enzyme was purified until homogeneous electrophoretically by gel-filtration over HW-55 TSK-gel with specific activities of 198.5 U/mg for glucoamylase. SDS-PAGE of the purified enzyme showed a single protein band of molecular weight 56 kDa. The glucoamylase exhibited optimum catalytic activity at pH 6.0 and 65-70°C. It was thermostable at 50°C and 60°C, and retained 80% activity after 30 min at 60-65°C. The half-life of the enzyme at 85°C was 20 min. Different metal ions showed different effects on the glucoamylase activity. Ca²⁺, Mg²⁺, Na, and K enhanced the enzyme activity, whereas Fe²⁺, Ag, and Hg²⁺ cause obvious inhibition. Acute toxicity of *Aspergillus oryzae* UzMU K-14 culture liquid on rats with oral and dermal introduction, as well as cumulative and local irritant effect (subacute toxicity) in mice is studied. It was determined that the strain of fungus is non-toxic. These properties make it applicable to other biotechnological purposes and to improve industrial processes of starch degradation.

Keywords *Aspergillus oryzae*, Microorganism, Fungi, Degradation, Protein, Characterization, Purification, Cultural-Morphological properties, Toxicology, Glucoamylase, Ion-Exchange, Gel-Filtration

1. Introduction

Glucoamylase is an exo-hydrolase, exo-acting enzyme that removes the β -D-glucose units from the non-reducing ends of starch and related oligo- and poly-saccharides, amylose, amylopectin and glycogen by hydrolyzing α -1, 4 linkages in a consecutive manner, and producing D glucose as the sole product. It also hydrolyzes α -1, 6 and the rare α -1, 3 linkages although at a much slower rate [1]. Glucoamylases are an important group of enzymes in starch processing. They are second to protease in worldwide distribution and sales among industrial enzymes. Glucoamylases find many applications in industry. This enzyme is used in dextrose production, in the baking industry, in the brewing of low-calorie beer and in whole grain

hydrolysis for the alcohol industry [2]. The primary commercial application of glucoamylases is the production of glucose syrups from starch [3]. The primary commercial application of glucoamylases is the production of glucose syrups from starch. A number of glucoamylases from cultures of various microorganisms, including *Aspergillus*, *Rhizopus*, and *Saccharomyces* species have been screened for potential industrial application. In recent years glucoamylases from thermophilic fungi, which are expected to be thermostable, have aroused increasing attention among researchers. Various methods for isolating highly purified and homogeneous forms a fungal glucoamylase have been reported [4-6].

In the world practice as producers of commercial preparations of highly active and highly specific glucoamylase most widely used strain of *Aspergillus* fungi [7]. Here, we describe the purification and biochemical characterization of thermostability glucoamylase from *Aspergillus oryzae* UzMU K-14. The strain *Aspergillus oryzae* UzMU K-14 differed from the previously studied

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strains glucoamylase activity and in certain cultural sennatures [8-9]. Earlier we obtained by screening high-yield strain of fungus *Aspergillus oryzae* UzMU K-14 producing glucoamylase [10].

The aim of this work is to study the general toxicology of fungus *Aspergillus oryzae* UzMU K-14. The objective of study included: 1. Determination of acute toxicity by oral and dermal introduction. 2. The study of cumulative effects (subacute toxicity) in mice. 3. Definition of locally irritating.

2. Materials and Methods

2.1. Grown Culture

We used mesophilic spore-forming *Aspergillus sp.* fungus UzMU K-14, isolated from the rhizosphere of rice grown in the Kashkadarya region and preserved laboratory at number № UzMU K-14 in the culture collection of microorganisms of the National University of Uzbekistan named after M.Ulugbek, starter culture used for the production of alcohol by local grows optimally at 45-50°C and secretes a significant amount of glucoamylase at pH 6.0 into the culture media. For the production of the enzyme, actively growing fungal mycelium was an inoculation (0.25%) of the nutrient medium was transferred to optimized fermentation medium and grown for 120 h at 45-50°C on a rocker rotating at 150 rpm, to choose the optimal liquid medium for fermentation 250 ml Erlenmeyer flasks with 50 ml liquid medium containing the following (g/L): maize flour, 30.0; wheat bran, 5.0; yeast extract, 3.0; (NH₄)₂HPO₄, 5.0; MgSO₄, 3.0; CaCl₂, 0.5, KCL, 0.5. The culture fluid was filtered and centrifuged at 8000 rpm for 20 min, 4°C, and the supernatant was used as a crude enzyme extract preparation.

2.2. Enzyme Assay

Determination of glucoamylase activity was carried out according to GOST - 20264.4-89. One unit of glucoamylase activity adopted by the ability of the enzyme to catalyze the hydrolysis of soluble starch at 50 °C and pH 6.0 for 1 min releasing 1 micromol of glucose.

2.3. Determination of Protein

Protein concentration was determined by the Lowry method. As protein standard bovine serum albumin was used. In the fractions obtained after chromatography, the protein was determined spectrophotometrically by absorbance at 280 nm using a spectrophotometer SF-26 "Lomo" [11].

2.4. Pre-Clinical Study of General Toxicology

Fungus *Aspergillus oryzae* UzMU K-14 was transferred from the National University of Uzbekistan named after M. Ulugbek to the laboratory of Pharmacology IBOC (Institute of Bio-Organic Chemistry) to conduct pre-clinical studies of general toxicology.

The general action and "acute" toxicity of *Aspergillus oryzae* UzMU K-14 was determined on 36 white outbred

mice and on 12 white rats of both sexes weighing 25 ± 1.5 and 150 ± 10 g after single, oral introduction. To define "acute" toxicity method of Litchfield and Wilcoxon [12] was used, and dermal application method of Noakes and Sanderson at seven animals per group [12] was used. The determination of *Aspergillus oryzae* UzMU K-14 cumulation was conducted by method of Lim et al. in 10 mice and eye mucosa of 10 rabbits [13]. On the local irritant effect of *Aspergillus oryzae* UzMU K-14 was judged by the impact of the fungus on the skin of 20 rats [14].

2.5. Isolation, Purification and Physic-Chemical Characterization of the Enzyme

Fungal cells were separated from cultural liquid (CL) by centrifugation at 8000 rpm for 20 min. CL was precipitated using of ammonium sulphate 60%. Fractional salting out of enzyme protein with ammonium sulfate was carried out at 4°C for 24 hours. The precipitate was collected by centrifugation the solution at 8000 rpm/min for 20 min. Desalting was performed with the enzyme solution by dialysis first against a 0.05 M phosphate buffer (pH 6.0) and then against distilled water for 24 hours. The precipitate obtained after 60% saturation of ammonium sulfate was dissolved in 3 ml of 0.05 M phosphate buffer and used for further purification of glucoamylase.

Gel-filtration of the active protein fraction after precipitation of CL by (NH₄)₂SO₄ was performed on a column (20×600 mm) packed with HW-55 TSK-gel (Toyopearl, Japan) equilibrated with potassium phosphate buffer (0.05 M) containing KCl (0.1 M) at pH 6.0 with elution rate 48 mL/h and fraction volumes 1.5 mL.

The homogeneity of amylolytic enzymes was monitored at all purification stages by electrophoresis in PAAG (12%) with SDS using the Laemmli method [15].

2.6. Optimum Temperature and Thermostability

The optimum temperature of the enzyme was established by incubating the enzyme reaction mixture with substrate for 10 min at 20-90°C in phosphate buffer (0.05 M) at pH 6.0. Thermostability was investigated by incubating the enzyme at 40, 50, 60, 70, 80, and 90°C in the same buffer without substrate.

2.7. pH Optimum and pH Stability

The optimum pH and pH stability of the studied enzyme toward soluble starch was determined in phosphatebuffer (0.05 M) at pH 3-10 and 50°C. Enzyme without substrate at different pH values of phosphate buffer (0.05 M) was incubated for 30 min after which the activity toward soluble starch was determined.

2.8. Effects of Various Metal Ions on the Enzyme Activity

The effects of various metal ions on enzyme activity were determined in phosphate buffer (0.05 M) at pH 6.0 and metalion concentrations of 1 mM.

3. Results and Discussion

The methods screening has been obtained for production of high activity strain *Aspergillus sp. UzMU K-14*, which produces thermostability glycoamylase, which allows obtaining glycoamylase with activity up to 170.6 U/mL in laboratory scale. *Aspergillus sp. fungus UzMU K-14*, which is isolated from the sources of the local region of the Republic of Uzbekistan, isolated from the rhizosphere soil of wheat Kashkadarya region starter culture used for the production of alcohol by local grows optimally at 45-50°C and secretes a significant amount of glycoamylase at pH 6.0 into the culture media.

By studying the morphology and physiology of *Aspergillus sp. UzMU K-14* fungi it this determined that it belongs to *Aspergillus oryzae*. It this also determined the conidial heads of *Aspergillus sp. UzMU K-14* develops more tightly and length of gifs consist about 120-400 x 20-40 mkm, and colony haslight color (Fig. 1).

Studies show that *Aspergillus sp. UzMU K-14* morphology and physiology of fungi that produce a yellowish-green color, appearance, and observed under a microscope stage of development of this mark indicates *Aspergillus oryzae*. These references are given in comparison with the strain *Aspergillus oryzae* proof of this generation and the type of background [16]. Accordingly, for future research this strain *Aspergillus oryzae UzMU K-14*.

Dalee, we studied the isolation, purification and investigation of the physical chemical and catalytic properties of purified glucoamylase from *Aspergillus oryzae UzMU K-14*. We precipitated the protein fraction from culture liquid (CL) using $(\text{NH}_4)_2\text{SO}_4$, ethanol, and isopropanol. A high yield by activity (62.6%) was obtained for precipitation of proteins by isopropanol in a 1:1 ratio. α -Amylase was lesssensitive to the action by fractional

precipitatio $(\text{NH}_4)_2\text{SO}_4$. The resulting enzyme preparation had specific activity 246.0 U/mg (Table 1).

These results show that production of active glucoamylase *Aspergillus oryzae UzMU K-14* should be precipitated proteins CL using $(\text{NH}_4)_2\text{SO}_4$ and this fraction was used for further purification. To clean glucoamylase performed gel-filtration.

Gel-filtration of partially purified glucoamylase preparation was performed over a column (20 × 600 mm) packed withHW-55 TSK-gel (Toyopearl, Japan). The results showed that the eluted protein fractions 20-21 contained active proteins glucoamylase and fractions corresponding topeak maxima were used to analyze the purity of proteins.

Thus, the aforementioned methods were used to prepare electrophoretically homogeneous fraction of glucoamylase with rather high activitie of 198.5 U/mg for glucoamylase and yield of 2.5% (for glucoamylase) (Table 1).

The homogeneity and molecular weight of purified glucoamylase was determined by electrophoresis in PAAG (12%) with SDS (Laemmli, U. K. (1970). It was found that glucoamylase had a molecular weight of about 56 kDa (Fig. 2).

The physical chemical and catalytic properties, optimum temperature and pH, and stability and effect of various metalions on the enzyme activity were studied to characterize and compare the purified glucoamylase preparation produced by us.

The effect of pH on enzyme activity and stability was examined in the pH range 3.0-10.0. Figure 3 shows the optimum pH for purified glucoamylase from *Aspergillus oryzae UzMU K-14* and its stability at various pH values. It can be seen that glucoamylase is more stable at pH values 5.0-9.0. The enzyme had the highest activity at pH 6.0 whereas it retained 20-25% of the activity at pH 9.0-10.0.



Figure 1. *Aspergillus sp. UzMU K-14* strains of development

Table 1. Purification of glucoamylase from *Aspergillus oryzae UzMU K-14*

Preparation and purification step	Protein, mg	Purification degree	Glucoamylase activity		
			total, U	specific U/mL	yield, %
CL supernatant	770	1	43650	56.7	100
Precipitation $(\text{NH}_4)_2\text{SO}_4$, (60%)	116.0	6.6	28560	246.0	65.4
Gel-filtration over HW-55 TSK-gel: glucoamylase fraction	5.5	140	1092	198.5	2.5

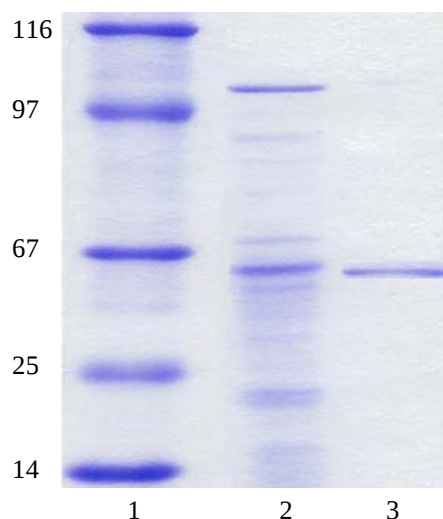


Figure 2. Electrophoresis (PAAG-SDS, 12%) of glucoamylase preparations from *Aspergillus oryzae* UzMU K-14: 1. Markers; 2. CL of *Aspergillus oryzae* UzMU K-14; 3. purified glucoamylase after gel-filtration over TSK-gel (HW-55)

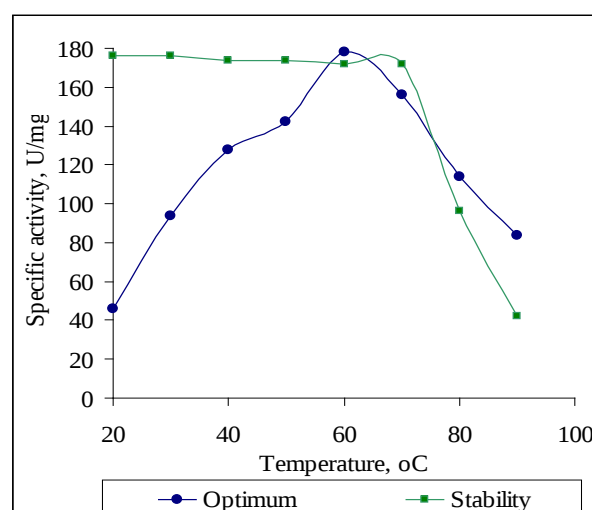
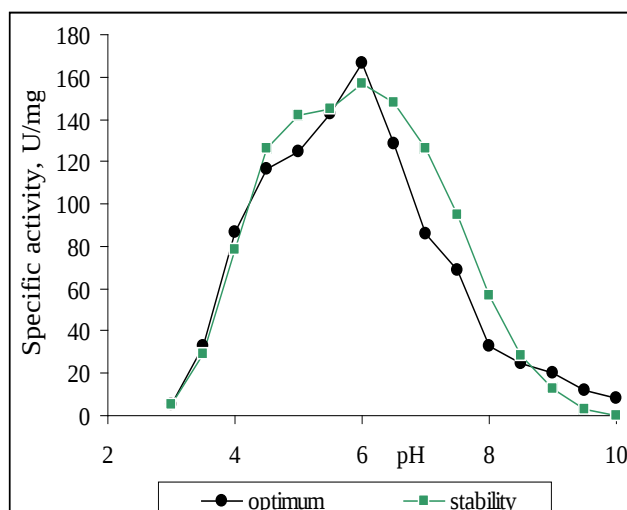


Figure 3&4. Effect of pH and of temperature on activity and stability of purified glucoamylase from *Aspergillus oryzae* UzMU K-14

The activity of glucoamylase from *Aspergillus oryzae* UzMU K-14 was studied as a function of temperature in the range 20-90°C (Fig. 4). Figure 4 shows the optimum temperature for activity and stability of purified glucoamylase from *Aspergillus oryzae* UzMU K-14. The enzyme had the highest activity at 60-70°C for 10 min. At 80°C, the enzymatic activity was 90% of the maximum whereas at 70°C, it was about 50% of the initial activity. After incubation of glucoamylase for 30 min at 50 and 60°C, the enzyme retained 75 and 60% of its activity, respectively. The enzyme retained only 10-12% of its maximum activity after incubation at higher (80-90°C) temperatures.

As noted previously, amylolytic enzymes are metallo enzymes with up to six Ca^{2+} atoms at the active site [12, 13]. Therefore, we studied the effects of various concentrations and salts of Ca^{2+} and mono- and divalent metals on the activity of *Aspergillus oryzae* UzMU K-14 glucoamylase. As it turned out, ions of certain metals at final concentrations of 1 mM had positive effects on the amylase activity. For

example, Ca^{2+} increased the enzyme activity by 50%; Mg^{2+} and Na^+ , 30; Mn^{2+} , 30; K^+ , 15; and Co^{2+} , 10; whereas Li^+ (8), Fe^{2+} (25), Cu^{2+} (12), Ni^{2+} (8), and Ba^{2+} (12) inhibited the activity from 8 to 25%.

Dalee, to define the "acute" toxicity at oral introduction method of Litchfield and Wilcoxon was used. Each dose of substance was investigated on 10 animals. The monitoring was conducted within 14 days. *Aspergillus oryzae* UzMU K-14 was introduced orally in doses 2000, 4000, 5000, 6000, 8000 and 10000 mg/kg. The study of the general action of *Aspergillus oryzae* UzMU K-14 found that in doses 5000-8000 mg/kg the behavior of mice and rats did not differ from the intact animals. In doses 6000 and 10000 mg/kg suppression of animals for 120-240 minutes is noted. Animals' death didn't observe within 14 days. At single, skin application cultural liquid (CL) of fungi treated on the cut-off site of skin of rats' back and sides of 30 cm² (4x7.5 cm), to mice of 3 cm² (0.4x0.75 cm) after single putting solutions in a dose of 10 ml/kg of animals placed in separate

cages, a skin site with the studied substance left open, supervision was conducted hourly in day of introduction, by 3 times a day for 2-3 days and once a day in the next 7 days of experience. Considered the general behavior, conditions of integuments on a place of putting liquid, existence of reddening's, a condition of wool, physical activity and death of rats. The monitoring was conducted within 14 days. In case substances for external application when studying acute toxicity in a dose of 10 ml/kg don't show toxic action, it is considered to be similar means non-toxical.

Therefore, the study of general validity and "acute toxicity" of *Aspergillus oryzae* UzMU K-14 showed that this bacterial strain belongs to class V virtually no toxic compounds. LD₅₀-over 10000 mg/kg when introduced to mice does not cause the death of rats in a dose of 10 ml/kg at skin use.

Study of *Aspergillus oryzae* UzMU K-14 cumulation was conducted by method of Lim, and others, allows to evaluate not only the cumulation, but also addictive. The objective of the study was to - identify possible cumulative properties from *Aspergillus oryzae* UzMU K-14. Experiments were carried out on 10 mice of both sexes weighing 20 ± 2 g CL was introduced orally as following scheme (Table 2).

Table 2. These doses introduced and deaths of animals in the determination of sub-acute toxicity (cumulative) of *Aspergillus oryzae* UzMU K-14

Days of introduction	The number of animals (n=10)	Proportion of LD50	LD ₅₀ ≥10000 mg/kg
1-4	0/10	0.1	1000
5-8	0/10	0.15	1500
9-12	0/10	0.22	2200
13-16	0/10	0.34	3400
17-20	0/10	0.50	5000
21-24	0/10	0.75	7500
25-28	0/10	1.14	11400

The total dose for 24 days - 12.7 LD_{50} , the maximum duration of the experiment 24 ± 4 days. $K_k = \text{LD}_{50n} / \text{LD}_{50}^1$, where K_k - coefficient of cumulation, LD_{50n} -average lethal dose of the n-fold introduction, LD_{501} -average lethal dose of a single introduction. $K_k \geq 1$ addictive; $K_k \leq 1$ cumulation.

After refueling, euthanasia was performed under general anesthesia etaminal sodium. Macroscopic research of slaughtered animals found the correct location of the internal organs, "free" fluid in the pleural and abdominal cavities not

found. The mucous membrane of mouth was clean and moist, characteristic color, edema, hemorrhages not found. Lung tissue, stomach and intestines are also characteristic color, with no signs of edema, hemorrhage, and ulceration. Pancreas, kidneys and adrenal glands unchanged (Table 3).

As seen from the data in Table 2 cumulative properties of *Aspergillus oryzae* UzMU K-14 have not been established since the introduction of the dose did not cause the death of animals. The drug has no cumulative properties. Conjunctively sample is a very sensitive test. In some cases, even allows identifying the animal's reaction to allergens in weak allergy and negative skin tests. Experiments were performed on 10 rabbits, weighing 2.5-3.0 kg, which instilled in the left eye 0.1 ml of 0.5 and 5.0% solutions of *Aspergillus oryzae* UzMU K-14, to the second eye (control) was introduced 0.1 ml distilled water. Reaction was considered in 15 minutes (fast reaction) and in 24-48 hours (hypersensitivity of the slowed-down type) and estimated on the following scale (in points):

- A slight reddening of the tear duct;
- Redness of the sclera and tear duct in the direction of the cornea;
- Redness whole of the conjunctiva and sclera.

In addition, we calculated of redness, swelling, lacrimation. The results showed that 0.5 and 5.0% of the culture liquid of *Aspergillus oryzae* UzMU K-14 or not 15 minutes, or not at 24 and 48 hours did not cause even a slight reddening. On this basis, it can be concluded that *Aspergillus oryzae* UzMU K-14 does not have an irritating effect on the conjunctiva of the rabbit.

Locally - irritating liniment base and CL of *Aspergillus oryzae* UzMU K-14 was studied on the skin of 20 rats weighing 150 ± 10 g of animals wool cut out on either side of the spine (4 field) $2 \times 2 \text{ cm}$ size. Rats were shaved portion 2 on the left side of the back was applied at 0.5 ml, 0.5 and 5% solution of *Aspergillus oryzae* UzMU K-14. CL was applied for 10 days. The controls were clipped areas that are applied to distilled water in the same volume (2-a right field). Monitoring conducted within 14 days. We take into account the reaction of the skin on a daily basis on a volume of skin tests in points.

The studies showed that *Aspergillus oryzae* UzMU K-14 does not cause irritation, redness, swelling, or other visible changes in the skin and the effect of *Aspergillus oryzae* UzMU K-14 is evaluated on the 0 points.

Table 3. Averages mass of internal organs of mice after oral introduction of *Aspergillus oryzae* UzMU K-14 ($M \pm m$; n = 10; P < 0.05)

Groups	The mass of internal organs, gr								
	liver	kidneys	spleen	heart	lungs	stomach	adrenals	thymus	ovary
Control	1.42±0.2	0.47±0.015	0.18±0.02	0.22±0.015	0.32±0.01	0.39±0.01	0.004±0.001	0.019±0.001	0.14±0.002
<i>Aspergillus oryzae</i> UzMU K-14	1.78±0.1	0.49±0.02	0.31±0.01	0.22±0.05	0.34±0.01	0.46±0.02	0.005±0.002	0.026±0.002	0.12±0.001

4. Conclusions

Thus, a purified enzyme preparation of glucoamylase from *Aspergillus oryzae* UzMU K-14 was prepared by sequential use of precipitation by $((\text{NH}_4)_2\text{SO}_4)$, and chromatography over HW-55 TSK-gel and the results allow us to conclude that the *Aspergillus oryzae* UzMU K-14 is not an irritant to the skin and conjunctiva of the animal's eyes.

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