Purification and investigation of physico-chemical properties of β-glucanase

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Abstract. From the complex enzyme preparation Bruzaim BGX, β-glucanase enzyme with a specific activity of 369.5 units/mg of protein and a degree of purification of 57.7 was isolated by ethanol precipitation and purified by gel filtration on Sephadex G-25 and G-150 and ion-exchange chromatography on DEAE - cellulose. The influence of temperature and pH on the activity and stability of the enzyme was studied in the temperature range 30–70°C and pH 4.0–7.0. It was found that the optimum effect of β-glucanase corresponds to pH 4.8 and a temperature of 56°C, the enzyme has a sufficiently high thermal and acid stability, hydrolyzes non-starch polysaccharides containing both β-1,3 and β-1,4 - glucosidic bonds, which makes its use in biotechnology promising for the hydrolysis of non-starch polysaccharides of grain.

1. Introduction
The enzyme β-glucanase (EC 3.2.1.73) belongs to the class of hydrolases and gyrolizes β-glucans contained in the cell walls of the endosperm, aleuron, scutellum of rye and barley grains. β-glucan is long chains of glucose molecules linked to each other at position 1.3 and, more often, at position 1.4. The β – 1.4 bonds account for 70–74% of the total number of bonds in the β – glucan molecule and 26–30% account for β – 1.3 bonds. The enzyme β-glucanase is currently widely used in the brewing and alcohol industries to reduce the viscosity of the resulting intermediates. The study of the physicochemical properties of enzymes, their specificity of action is important in terms of rational use of enzyme preparations and the intensification of the technological process for producing alcohol and beer.

The study of the physicochemical properties of enzymes covers a wide range of issues. However, for such an industry as biotechnology, it is of interest to study properties of applicable nature: optimal conditions for the action of enzymes (pH, temperature) and their thermal and pH stability, which allows the substrate to be processed at elevated temperatures and low values of the pH, thereby ensuring the sterility of the process.

The purpose of this work was to purify and study the physicochemical properties and substrate specificity of β-glucanase of the enzyme preparation Bruzaim BGX.

2. Material and research methods
The complex enzyme preparation Bruzaim BGX from Diadic International Inc. (the USA) was used, obtained by submerged cultivation of the Trichotheca lorgibranchiatum micromycete strain.
containing β-glucanase with an activity of 180 units/cm³ and xylanase - 3700 units/cm³. This is concentrated amber to brown liquid enzyme preparation with a specific density of 1.16 g/cm³. The effective range of the preparation is pH 3.5 - 6.0; temperature 45 - 65°C.

The concentrated enzyme preparation was precipitated with ethanol in a ratio of 1:4 at a temperature of 2 - 4°C. The precipitate was separated by centrifugation, dried and dissolved in 0.5 M Tris/ HCl buffer, pH 6.5.

A solution of an alcohol-precipitated enzyme preparation was passed through a column (1.5×20 cm) with Sephadex G-25 to purify low molecular weight impurities and was eluted with a speed of 0.5 cm³/min with 0.5 M Tris HCl buffer, pH 6.5. The volume of the applied sample did not exceed 4 cm³. After applying the sample, the column was filled with buffer solution and elution started. The volume of fractions was 3 cm³.

A desalted enzyme solution was applied to a column (1.5×12 cm) with DEAE cellulose balanced with 0.5 M Tris/HCl buffer pH 6.5. Elution was carried out by a linear gradient of KCl 0.2 M in the same buffer. Fractions were collected at 3 cm³ and enzyme activity was defined. The elution rate was 0.3 cm³/min.

The obtained fraction was applied to a column (1.5×25 cm) with Sephadex G-150 (Pharmacia; Sweden) for purification from high molecular weight impurities. Sephadex G-150 swelled for 3 days in 0.5 M Tris/HCl buffer. 2 ml of a 0.2% “blue dextran 2000” solution was passed through the prepared column to measure the free volume of the column. The elution rate was 0.3 ml/min [1].

At each stage, β-glucanase activity, the protein content on a spectrophotometer at 260 and 280 nm and according to Lowry were defined [4].

3. Research results and discussion
The study of the properties of enzymes is possible only if they are isolated with a high degree of purification. To do this, first of all, the enzymes were precipitated with ethanol from the complex enzyme preparation Bruzaine BGX.

The pH value, which should be close to the isoelectric point of the precipitated protein, has a significant effect on the precipitation effect. Considering that many proteins undergo denaturation upon contact with organic solvents, precipitation is carried out at low temperatures close to the freezing temperature of the solvent – water mixture [1, 2].

The precipitation of the enzyme from the complex preparation was carried out with 96.3% vol. ethanol in the ratio 1:4 at pH scale from 3.0 to 8.0 and a temperature of 2 - 4°C. The precipitate formed was separated by centrifugation, dried in air, weighed, after which β-glucanase activity and the amount of protein were determined according to Lowry.

The maximum production of β-glucanase is observed at a pH of 5.0. It is likely that the isoelectric point of β - glucanase is in the pH range close to 5.0. The production of β-glucanase is 48.1% with a purification ratio of 1.95.

To remove low molecular weight impurities, gel filtration was used on a Sephadex G-25 column, as a result of which the specific activity of β-glucanase increased 2.1 times.

The most important stage in the purification scheme for the separation of proteins with similar physicochemical properties is ion-exchange chromatography on DEAE cellulose. When a certain eluting solution is passed through a column, chromatographic separation of proteins occurs depending on the degree of their interaction with the ion-exchanger. The breaking of bonds is achieved by changing the pH and ionic strength of the solution.

Most of the active protein was eluted in the initial buffer with a KCl concentration of 0.03 to 0.08 M. When the enzymes were purified after ion-exchange chromatography on DEAE cellulose, a significant elution peak of enzymatic activity was observed — fraction 4 — corresponding to β-glucanase activity . The specific activity of β-glucanase increased by 5.7 times.

At the final stage of purification, a gel filtration method was used on a column with Sephadex G-150. 3 cm³ 4 fractions were applied to a Sephadex G-150 column after ion-exchange chromatography on DEAE-cellulose and eluted at a rate of 6-8 cm³/h with 0.1 M Tris-buffer pH 7.0. The eluate
released from the column was collected in 1 cm³ fractions and the activity of β-glucanase and protein was determined in them. Fractions with the highest enzyme activity were combined. The specific activity of β-glucanase increased 2.5 times.

Table 1. Purification of β-glucanase.

| Stages of purification    | Vol, cm³ | Total protein, mg | Activity | Degree of purification | Activity Output, % |
|---------------------------|----------|-------------------|----------|------------------------|-------------------|
| Concentrated preparation  | 20       | 449               | 2875     | 6,4                    | 1                 | 100               |
| Ethanol precipitation     | 4        | 110,6             | 1382,9   | 12,5                   | 1,95              | 48,1              |
| Sephadex G-25             | 3        | 5,7               | 149,5    | 26,1                   | 4,06              | 5,2               |
| DEAE cellulose            | 3        | 0,89              | 132,3    | 148,4                  | 23,1              | 4,6               |
| Sephadex G-150            | 3        | 0,29              | 106,4    | 369,5                  | 57,7              | 3,7               |

As a result of the developed scheme (Table 1), the degree of purification of β-glucanase in terms of specific activity was 57.7 when the enzyme production was 3.7% for this indicator. A highly purified β-glucanase enzyme was obtained with a specific activity of 369.5 units/mg protein.

The molecular weight of the studied enzyme was determined by gel filtration using Sephadex G-150, which amounted to 112.2 kDa.

The use of enzymes in biotechnology involves the determination of optimal conditions for their action. For this purpose, the effect of pH on the activity of β-glucanase of the enzyme preparation Bruzaime BGX was studied in the range of pH scale from 2 to 7. The target pH of the substrate was maintained with 0.1 M acetate buffer.

![Figure 1. The effect of pH on the activity of β-glucanase.](image1)

![Figure 2. The effect of temperature on the activity of β-glucanase.](image2)

The dependence curves have a bell-shaped appearance (Figure 1) with a maximum of β-glucanase activity at pH 4.8. The presence of such an optimum may have several reasons: the true reversible effect of pH on the reaction rate upon saturation of the enzyme with a substrate; the effect of pH on the affinity of the enzyme for the substrate; the effect of pH on enzyme stability. For β-glucanase, there is a significant increase in activity in the pH range from 3 to 4.8. The enzyme β-glucanase is sharply inactivated with an increase in pH above 5.5.

The optimum temperature of action of β-glucanase was found out in the range of 30–70°C. The dependence curve had a profile typical for enzymes (Figure 2): with an increase in temperature, the reaction rate increased, reaching a maximum, and then decreased, due to the protein nature of the
enzymes. The shape of the curve is close to bell-shaped, with a marked temperature optimum of the reaction.

The effect of temperature on enzyme activity has a twofold effect. On the one hand, it leads to protein denaturation and a decrease in catalytic function; on the other hand, it intensifies the rate of formation of the enzyme – substrate complex and all subsequent stages of substrate transformation [2, 3].

Figure 2 shows that the activity of enzymes increases with increasing temperature, but up to a certain maximum of the optimum enzyme action, which for β-glucanase is 56°C, then drops sharply, which indicates the inactivation of the enzyme.

Thus, the optimum action of β-glucanase is at temperature - 56 °C, pH - 4.8.

Currently, questions remain open explaining the high activity and specificity of biological catalysts - enzymes. In their study, one of the most relevant is the study of the influence of various physicochemical factors on the conformation of proteins and the mechanisms of activation and inhibition associated with it [5]. In addition, as noted previously, the thermal and pH stability of enzymes are important characteristics in the technology of enzyme preparations and their use at various stages of the biotechnological process [3]. Little research on characteristics of β-glucanase has been carried out, therefore, their study deserves special attention.

Figure 3. The effect of pH and temperature on the stability of β-glucanase.

The stability of β-glucanase was studied upon keeping the enzyme preparation in 0.1 M phosphate-citrate buffer with different pH values from 4.0 to 7.0. The enzyme preparation was kept at temperatures from 30 to 70°C for 1 h and the residual activity was determined. The effect of temperature on the stability of β-glucanase is shown in Figure 3.

It was found that β-glucanase is most stable at pH 5.0. The residual activity at 50° was 97.5%, at 60°C - 89.5%, at 70°C - 57.3%.

Table 2. The action of β-glucanase on some poly- and disaccharides.

| Substrate       | Type of communication | The relative activity of β-glucanase, % |
|-----------------|-----------------------|----------------------------------------|
| Barley β-Glucan | β – 1,3; β – 1,4       | 100,0                                  |
| Lichenin        | β – 1,3; β – 1,4       | 92,5                                   |
| Yeast β-Glucan  | β – 1,3; β – 1,6       | 0,0                                    |
| Laminarine      | β – 1,3               | 0,0                                    |
| Na - CMC        | β – 1,4               | 0,0                                    |
| Cellobiosis     | β – 1,4               | 0,0                                    |
| Starch          | α – 1,4; α – 1,6       | 0,0                                    |
| Maltose         | α – 1,4               | 0,0                                    |
Thermal inactivation of enzymes is associated with a direct effect of temperature on the protein globule, which leads to its destruction. Since proteins contain numerous groups capable of ionization, pH changes will affect the catalytic sites and the shape of the enzyme molecules. β-glucanases are strictly specific to the configuration of glucose residues in the molecules of the substrates.

Table 2 shows the results of a study of the substrate specificity of β-glucanase. It is obvious that the studied enzyme does not cleave α-glycosidic bonds and does not act on saccharides containing only β-1,3 or β-1,4 glycosidic bonds. At the same time, the enzyme catalyzes the hydrolysis of barley glucan and lichenin, in which glucose residues are connected by alternating β-1,3 and β-1,4 glycosidic bonds, moreover, the activity when using lichenin is slightly lower than when using barley glucan.

Thus, when studying the substrate specificity of β-glucanase, it was found that the enzyme hydrolyzes non-starch polysaccharides containing both β-1,3 and β-1,4-glucosidic bonds, which makes it effective when used for hydrolysis of the hemicellulose fraction of grain.

4. Conclusion
Highly purified β-glucanase was obtained with a specific activity of 369.5 units/mg and a degree of purification of 57.7. When studying the effect of pH and temperature on the activity of β-glucanase, it was found that the maximum value of β-glucanase activity corresponds to a pH of 4.8 and a temperature of 56°C. The study of acid and thermal inactivation showed a rather high acid and thermal stability of the enzyme, which makes its use in biotechnology promising. The studied substrate specificity of the enzyme action allows its use for hydrolysis of non-starch polysaccharides of grain.

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