Refining process and properties of polysaccharide xylanase

A N Yakovlev, G V Agafonov, S F Yakovleva, T I Romanyuk, N V Zueva and T S Kovaleva

Voronezh State University of Engineering Technologies, 19, Revolution ave., Voronezh, 394036, Russia

E-mail: tafursova@yandex.ru

Abstract. The xylanase enzyme with a specific activity of 5676.4 units/mg of protein and a purification degree of 53.1 was isolated and purified from the complex enzyme preparation Brusiem BGX. The enzyme was isolated by ethanol precipitation, and purification was performed by gel filtration on sefadex G-25 and G-150 and ion exchange chromatography on DEAE-cellulose. The effect of temperature and pH on the activity and stability of the enzyme in the temperature range of 30-70 °C and pH 4.0-7.0 was studied. The optimum action of xylanase is a pH of 5.5 and a temperature of 50 °C. The enzyme has a sufficiently high thermal and acid stability, hydrolyzes non-starchy polysaccharides containing (1,4) - β-D-xyloside bonds. In this regard, its use in biotechnology for hydrolysis of non-starchy polysaccharides of grain in the brewing and in the production of ethanol is prospectively.

1. Introduction
Xylanase or endo-1,4-β-xylanase (EC 3.2.1.8) (1,4-β-D-xylan xylanohydrolase) belongs to the class of hydrolases and hydrolyzes the (1,4)- β-D-xyloside bonds in xylans. Xylans are found in the cell walls of the endosperm, aleyron of rye, barley, and oats. Xylans are called hemicelluloses, the main structural element of which is a linear or a few branched polysaccharide formed by residues of β-D-xylopyranose, connected by β-(1-4) - bonds. The side branches of some xylans also contain arabinose, glucose, galactose, mannose, and glucuronic acid [1, 2].

The xylanase is now widely used in the alcohol and brewing industry to reduce the viscosity of the resulting intermediates. To intensify the technological process of producing alcohol and beer, it is important to study the physical and chemical properties of enzymes and their specificity of action [6, 7].

The study of the physical and chemical properties of enzymes is multifaceted. However, for the production of alcohol and beer, it is necessary to study the applied properties: optimal conditions for the action of enzymes (pH, temperature) and their thermo- and pH-stability. This will allow the substrate to be processed at elevated temperatures and low pH values, thereby ensuring the sterility of the process.

We purified and studied the physical and chemical properties and substrate specificity of the xylanase enzyme preparation Brusiem BGX.
2. Material and methods

We used the complex enzyme preparation Brusiem BGX of the company "Diadic International Inc." (USA), obtained by deep cultivation of a strain of Trichoterma lorgibranchiatum micromycete containing β-glucanase activity of 180 units/cm³ and xylanase – 3700 units/cm³. It is a concentrated liquid enzyme preparation from amber to brown with a specific density of 1.16 g/cm³. The range of action of the preparation: 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 a 0.5 M Tris-HCl buffer, pH 6.5.

The solution of the alcohol-precipitated enzyme preparation was passed through a column (1.5×20 cm) with sefadex G-25 for cleaning from low-molecular impurities and eluted at a rate of 0.5 cm³ / min with 0.5 M Tris - HCl buffer, pH 6.5. After applying the sample, the column was filled with a buffer solution and elution was started. The volume of fractions was 3 cm³.

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

The received fraction was applied to a column (1.5 × 25 cm) with Sefadex G-150 (Phamacia; Sweden) for cleaning from high-molecular impurities. Sefadex G-150 swelled for 3 days in a 0.5 M Tris - HCl buffer. 2 ml of 0.2% solution of "blue dextran 2000" was passed through the finished column to measure the free volume of the column [1].

At each stage, xylanase activity and protein content were determined using a spectrophotometer at 260 and 280 nm and by the Lowry method [4].

3. Results and discussion

Isolation and purification of enzyme preparations from impurities is an important step in the study of their properties. The enzymes were precipitated with ethanol from the complex enzyme preparation Brusiem BGX.

The pH value, which must be close to the isoelectric point of the deposited protein, has a significant effect on the deposition effect. Since many proteins are denatured in contact with organic solvents, precipitation is carried out at low temperatures close to the freezing point of the solvent-water mixture [1, 2].

Precipitation of the enzyme from the complex preparation was performed by 96.3 vol. % ethanol in a ratio of 1: 3 at a pH of 3.0 to 8.0 and a temperature of 2-4 °C. The precipitate was separated by centrifugation, air-dried, weighed, then xylanase activity and protein amount were determined by Lowry.

The maximum xylanase yield was at pH 6.0. It is likely that the isoelectric point of xylanase is in the pH range close to 6.0. The effect of ethanol concentration on xylanase deposition was studied at pH 6.0. The results are presented in Table 1.

The concentration of ethanol significantly affects the yield of enzymes and their degree of purification. The best results are obtained at an ethanol concentration of 77 vol. % (water/ethanol 1: 4.0). The yield of xylanase is 35.7 %, with a purification level of 1.45.

To remove low-molecular impurities, a gel filtration method was used on a cell with sefadex G-25, resulting in a 2.6-fold increase in the specific activity of xylanase.

Ion exchange chromatography on DEAE-cellulose is the most important stage in the purification scheme for separating proteins with similar physical and chemical properties. When a certain eluting solution is passed through the column, chromatographic separation of proteins occurs depending on the degree of their interaction with the ion exchanger. The bond break is obtained by changing the pH and ionic strength of the solution.
Table 1. Deposition of xylanase with ethanol.

| Amount of ethanol in volume ratios, % | Total protein, mg | Xylanase activity total, unit | Yield by activity, % | Purification degree |
|-------------------------------------|------------------|------------------------------|---------------------|--------------------|
|                                     |                  | Xylanase activity specific, unit/mg protein |                     |                    |
| Concentrated preparation            | -                | 449                          | 48000               | 100,0              | -                  |
| 1:1,0                               | 48.2             | -                            | -                   | -                  | -                  |
| 1:1,5                               | 57.8             | 41.5                         | 5316.2              | 128.1              | 11.1               | 1.20               |
| 1:2,0                               | 64.2             | 53.8                         | 6960.0              | 129.3              | 14.5               | 1.21               |
| 1:2,5                               | 68.8             | 80.4                         | 10886.2             | 135.4              | 22.7               | 1.27               |
| 1:3,0                               | 72.2             | 96.3                         | 13501.3             | 140.2              | 28.1               | 1.31               |
| 1:3,5                               | 74.9             | 104.9                        | 15408.1             | 146.9              | 32.1               | 1.37               |
| 1:4,0                               | 77.0             | 110.6                        | 17143.6             | 155.0              | 35.7               | 1.45               |
| 1:4,5                               | 79.0             | 124.7                        | 17744.8             | 142.3              | 37.0               | 1.33               |

Most of the active protein was eluted in the initial buffer with a KCl concentration of 0.03 to 0.08 M. When purification enzymes after ion-exchange chromatography on DEAE-cellulose, there was a pronounced peak in the elution of enzymatic activity - fraction №8, which corresponds to the activity of xylanase. The specific activity of xylanase increased 5.9 times.

At the final stage of purification, the gel filtration method was used on a column with sefadex G-150. 3 cm³ fractions № 8 obtained by ion exchange chromatography on DEAE cellulose were applied to a column with sefadex G-150 and eluted at a rate of 6 – 8 cm³/h 0.1 M with a pH 7.0 Tris - buffer. The eluate coming out of the column was collected in fractions of 1 cm³, in which the xylanase activity and the amount of protein were determined. Fractions with the highest enzyme activity were combined. Fractions with the highest enzyme activity were united. The specific activity of xylanase increased 2.4 times.

Table 2. Xylanase purification.

| Purification stages | Volume, cm³ | Total protein, mg | Activity total, unit | Activity specific, unit/mg | Purification degree | Yield by activity, % |
|---------------------|-------------|------------------|----------------------|---------------------------|-------------------|----------------------|
| Concentrated preparation | 20         | 449             | 48000               | 106.9                     | 1                 | 100                  |
| Ethanol deposition  | 4           | 110.6           | 17143.6             | 155.0                     | 1.45              | 35.7                 |
| Sephadex G-25      | 3           | 5.7             | 2304.0              | 404.2                     | 3.8               | 4.8                  |
| DEAE-cellulose     | 3           | 1.3             | 1872.0              | 2394                      | 22.4              | 3.9                  |
| Sephadex G-150     | 3           | 0.26            | 1488.0              | 5676.4                    | 53.1              | 3.1                  |

As a result of the developed scheme (table 2), the degree of purification of xylanase by specific activity was 53.1, with the yield of the enzyme was 3.1 %. A highly purified β-xylanase enzyme with a specific activity of 5676.4 unit/mg of protein was obtained.

The molecular weight of the studied enzyme was determined by gel filtration using sefadex G-150, it was 91.22 kDa.

The use of enzymes in biotechnology requires determining the optimal conditions for their action. The effect of pH on the xylanase activity of the enzyme preparation Brusiem BGX in the pH range from 2 to 7 was studied. The specified pH value of the substrate was maintained with 0.1 M acetate buffer.
Dependency curve is bell-shaped (figure 1) with a maximum of xylanase activity at pH 5.5. Xylanase activity increases significantly in the pH range from 4 to 5.5. Xylanase enzymes are sharply inactivated when the pH increases above 6.5. Determination of the optimal temperature of xylanase action was carried out in the range of 30-70 °C. The dependence curve had a characteristic profile for enzymes (figure 2): as the temperature increased, the reaction rate increased, reaching a maximum, and then decreased, due to the protein nature of the enzymes. The shape of the curve is similar to bell-shaped, with a shown temperature optimum of the reaction.

Figure 2 shows that the activity of the enzyme increases with increasing temperature, but up to a certain maximum corresponding to the optimal action of the enzyme, for xylanase it is 50 °C, then drops sharply, which is due to inactivation.

Thus, the optimum action of xylanase: temperature-50 °C, pH-5.5.

Now there are open questions that explain 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 physical and chemical factors on protein conformation and associated mechanisms of activation and inhibition [5]. In addition, as noted earlier, 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]. These characteristics of xylanase have not been studied much, so their study requires special attention.

The stability of xylanase was studied when the enzyme preparation was maintained in a 0.1 M phosphate-citrate buffer with a pH from 4.0 to 7.0. The enzyme preparation was maintained at temperatures from 30 to 70 °C for 1 hour and the residual activity was determined. The effect of temperature on stability xylanase is shown in Figure 3.

Xylanase is most stable at pH 6.0. Residual activity at 50 °C was 95.6 %, at 60 °C – 79.6 %, at 70 °C – 44.5%.

Thermal inactivation of enzymes is associated with the direct influence of temperature on the protein globule, which leads to its destruction. Since proteins contain numerous ionization-capable groups, changes in pH will affect the catalytic centers and the shape of the enzyme molecules.

Conventionally, xylanases of microorganisms can be subdivide into enzymes that hydrolyze substrates to xylose and xyloooligosaccharides, and into enzymes that hydrolyze substrates only to xyloooligosaccharides, among which the main product is xylobiose [8].

**Figure 1.** Effect of pH on xylanase activity.

**Figure 2.** Effect of temperature on xylanase activity.
Figure 3. Effect of pH and temperature on xylanase stability.

Gel chromatography using sehadex G-50 was used to determine the final products of xylan hydrolysis by xylose of the complex preparation Brusiem BGX.

Xylan, xylotriose, and xylose were used as markers to calibrate the column. We determined the output of these carbohydrates from the column with sehadex G-50. Based on the results of the experiment, a calibration graph of the dependence of the decimal logarithm of the molecular weight of a substance on the volume of eluate in which this substance left the column is drawn. The calibration graph allowed us to determine the molecular weight of the substance that comes out of the column in this sample of eluate.

Hydrolysis of a 2% xylan solution with the enzyme xylanase was performed: 3 cm$^3$ of the hydrolysate was applied to a column with sehadex G-50 and eluted at a rate of 10–12 cm$^3$/h 0.1 M with an acetate buffer solution of pH 6.0. The eluate coming out of the column was collected in fractions of 3 cm$^3$ and dry substances were determined in them. The content of each fraction in the hydrolysate was determined by the peaks.

It was found that with increasing duration of hydrolysis in the composition of the hydrolysate, the content of xylan decreases, the content of xylooligosaccharides with a degree of polymerization greater than three increases, and a small amount of xylose also accumulates. This fact confirms that the enzyme belongs to endo-1,4-β-xylanases.

4. Conclusion

A highly purified xylanase was obtained with a specific activity of 5676.4 unit/mg and a purification degree of 53.1. When studying the effect of pH and temperature on xylanase activity, it was found that the maximum value of xylanase activity corresponds to a pH of 5.5 and a temperature of 50 °C. The study of acid and thermal inactivation showed a sufficiently high acid and thermal stability of the enzyme, which makes its application in biotechnology promising. The substrate specificity of the enzyme's action has been studied, which makes it possible to use it for hydrolysis of non-starchy polysaccharides of grain in the biotechnology of alcohol and beer.
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