UDC 544.421+577.151

# Denaturation of Cellulosolytic Enzymes in the Presence of Water

A. L. BYCHKOV, V. A. BUKHTOYAROV and O. I. LOMOVSKY

*Institute of Solid State Chemistry and Mechanochemistry, Siberian Branch of the Russian Academy of Sciences, Ul. Kutateladze 18, Novosibirsk 6300128 (Russia)*

*E-mail: bychkov.a.l@gmail.com*

(Received September 20, 2011)

## Abstract

Denaturation of cellulosolytic enzymes produced by microorganisms *Trichoderma viride* in the presence of water was studied. Effective kinetic constants characterizing denaturation process were determined. It was shown that the enzymes in solution possess a narrow zone of stability, are stable at a temperature below 55 °C and rapidly gets denaturated when heated above 65 °C. In the dry state, the enzymes possess higher stability and get noticeably denaturated only at temperatures above 80 °C. The effective rate constant of denaturation is exponentially dependent on the concentration of water in the enzymatic preparation.

**Key words:** enzymes, thermal denaturation, stability, mechanoenzymatic processes

## INTRODUCTION

Mechanoenzymatic processes allow successful processing of various kinds of unedible biorenewable raw material, first of all lignocellulose wastes from agriculture and forestry, into biofuel, conversion of industrially grown microorganisms into valuable expensive products [1–7]. Consequent combination of preliminary mechanical activation of the raw material with subsequent enzymatic hydrolysis allows one not only to increase the rate of enzymatic processes and the yield of target products but also solve a number of ecological and economic problems characteristics of enzymatic technologies.

Wide introduction of enzymatic processes is prevented by insufficient knowledge of the physicochemical processes that occur at each stage of treatment. From the economic point of view, the most important problem in the mechanoenzymatic treatment of renewable raw material is the gradual decrease in the catalytic activity of expensive enzymatic preparations due to thermal, mechanical denaturation or enzyme tanning by the phenol components of biomass, for example lignin. Most frequently, thermal denaturation is observed as a result of violation of the technological practices, local overheating, the use of preliminary mechanical activation of the dry mixtures of enzymes and substrates, ultrasonic treatment of reagents.

For many individual enzymes and complex preparations, the optimal reaction conditions were determined, inactivation and reactivation were studied, stabilization mechanisms were proposed [7–13]. However, the overwhelming majority of works consider only denaturation of dissolved enzymes almost ignoring the solid-phase processes. Results obtained under different conditions and with different enzyme preparations can hardly be compared with each other correctly. Mechanical denaturation of enzymes under technologically admissible conditions has not been considered sufficiently thoroughly yet.

The goal of the present work was the experimental investigation of the kinetics of denaturation of cellulolytic enzymes under heating in solution and in the dry form.

## EXPERIMENTAL

### *Reagents and materials*

TselloLyuks-A complex cellulolytic preparation (Sibbiofarm Co., Berdsk city, Novosibirsk Region) was used in the work. Determination of activity: chromatographic paper Whatman No. 1, acetate buffer pH 4.7. Cellulolytic preparation is a mixture of enzymes possessing the following activity profile: xylanase 8000 e.u./g, cellulase 2000 e.u./g, β-glucanase up to 1500 e.u./g, glucoamylase 20 e.u./g. Water content of the samples was controlled with the help of automatic humidity analyser Radwag WPS 50SX (Poland). Carbohydrate content was measured with an UNICO 2800 UV/VIS spectrophotometer (USA).

### *Enzyme denaturation in the dry form*

A weighed portion (100 g) of enzymatic preparation (humidity 2 %) was kept at a temperature of 60–115 °C. Samples were taken after definite time intervals in order to determine cellulolytic activity.

### *Enzyme denaturation in solution*

Aqueous solutions of the enzymatic preparation (2 mg/mL) were kept at a temperature of 50–70 °C. Samples were taken after definite time intervals in order to determine cellulolytic activity.

### *Denaturation at different humidities of the enzymatic preparation*

Weighed portion of air-dry enzymatic preparation was placed into a desiccator above the layer of distilled water. Every 3 h the preparation was thoroughly mixed. Due to the high hygroscopic properties of the samples and uniform mixing, the sorption of water vapour occurred rapidly (within 3–20 h depending on required humidity) and uniformly, which was controlled with the help of Radwag WPS 50SX instrument (Poland). The resulting samples were denaturated at 80 °C under the conditions used to study denaturation in the dry form.

### *Determination of cellulolytic activity*

To determine cellulolytic activity of the enzymes of initial preparation and the enzymes of the preparation subjected to thermal treatment, enzymatic hydrolysis of chromatographic paper was carried out, followed by the determination of the sum of reducing carbohydrates (calculated for glucose).

The portions of 4.0 mL of enzyme solutions with the concentration of 1.0 mg/mL were added to the weighed portions (100 mg) of filter paper Whatman No. 1. The reaction mixture was incubated at a temperature of 50 °C for 1 h. Then the mixture was kept for 15 min at 95 °C for complete denaturation of enzymes, and cooled to room temperature. Non-dissolved residue of filter paper was removed by centrifuging for 10 min at 7000 $min^{-1}$. The supernatant was used to determine the total amount of reducing carbohydrates.

### *Determination of the total amount of reducing carbohydrates*

The sum of reducing carbohydrates was determined by means of the reduction of potassium ferricyanide $K_3[Fe(CN)_6]$. For this purpose, portions of 3.0 mL of 0.06 % solution of $K_3[Fe(CN)_6]$ were added to 1.0 mL of carbohydrate solutions with concentrations 30 to 150 mg/L, then the mixtures were stirred and kept at 100 °C for 10 min. After cooling, the solutions were examined with an UNICO 2800 UV/VIS spectrophotometer at the wavelength of 420 nm against distilled water. Standard glucose solutions with concentrations 30–150 mg/L were used to draw calibration plots.

The amount of glucose (in milligrams) obtained during the hydrolysis of chromatographic paper with the enzymatic preparation having the mass 1 g for 1 h under the excess of the cellulose substrate was taken as the unity of enzymatic activity. The ratio of enzymatic activity after heating to the initial activity was used to determine the effective rate constants of denaturation.

Experiments on enzyme denaturation in solution and in the dry form were repeated three times. The average values of reaction extent were used to plot the kinetic curves. The correlation

coefficient between the points of the experimental curves and the trend line drawn according to the exponential law corresponding to the first-order reactions exceeded 93 % in all the cases.

## RESULTS AND DISCUSSION

To study enzyme denaturation kinetics during heating in solution and in the dry form, we carried out experiments in which the Tsello-Lyuks-AQ preparation was kept at a temperature of 50–115 °C. Results are presented in Fig. 1.

One can see that the enzymes in dissolved form posses extremely narrow stability zone (see Fig. 1, *a*). At 50 °C (the conditions that are most frequently used in technology) the activity of enzymes decreases insignificantly within technologically acceptable time. Temperature rise to only 60–65 °C causes rapid denaturation of enzymes (3 and 1.5 h, respectively), while at a temperature above 65 °C denaturation occurs almost immediately.

Enzymatic preparations in the dry form possess higher stability to heating (see Fig. 1, *b*). At temperatures below 80 °C a decrease in activity is insignificant, only at temperatures above 100 °C denaturation occurs almost immediately.

It is known [12, 14, 15] that the overall enzyme denaturation process is described within the first-order kinetic equations:

$$N \overset{k}{\longleftrightarrow} D \qquad (1)$$

This process may be decomposed into separate stages

$$N \overset{k_i}{\longleftrightarrow} N_i \overset{k_{ij}}{\longleftrightarrow} D_j \overset{k_j}{\longleftrightarrow} D \qquad (2)$$

where $N$ is the initial active form of enzymes; $N_i$ is the sum of the transition states of enzymes able to catalyse reactions; $D_j$ – the sum of transient states unable to catalyze reactions; $D$ is the final denaturated form of enzymes.

In this case, to determine the effective thermodynamic parameters of the process, we may use equations

$$-\ln \alpha = kt \qquad (3)$$

$$\frac{E_a}{R} \cdot \frac{1}{T} + \ln A = -\ln k \qquad (4)$$

where α is the transformation degree of the overall process (1); $k$ is effective reaction rate

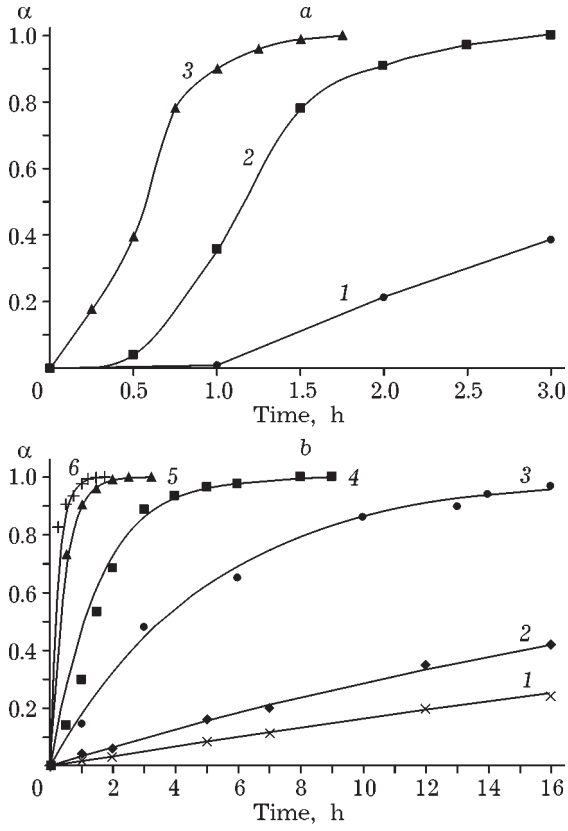


Fig. 1. Denaturation of cellulolytic enzymes in solution (*a*) and in the dry form (*b*) during heating. *T*, °C: *a* – 55 (*1*), 60 (*2*), 65 (*3*); *b* – 60 (*1*), 65 (*2*), 80 (*3*), 90 (*4*), 100 (*5*), 110 (*6*); α is the degree of transformation for denaturation process.

constant; $t$ is the time of reaction; $E_a$ is effective activation energy; $T$ is temperature at which the process takes place; $A$ is the preexponential factor; $R$ is the universal gas constant.

The calculated effective constants are presented in Table 1. A comparison between the constants at the same temperatures shows that the enzymes in the dry form are about two orders of magnitude more stable during heating than the enzymes in solution are; calculated effective activation energies are 118 kJ/mol for the solid-phase process and 251 kJ/mol for processes in solution.

The higher activation energy (accompanied by lower stability) for enzymes in solution can be explained in the following manner. First of all, water molecules may solvate proteins due to the formation of a developed network of hydrogen bonds, which increases the potential barrier through which the system is to pass in the course of the elementary stage of chemical transformation. However, in the dissolved

TABLE 1

Kinetic parameters of enzyme denaturation processes in solution and in the dry state

| Temperature, °C | Effective rate constant, $s^{-1}$ | |
|---|---|---|
| | In solution | In the dry form |
| ≤50 | Enzymes are stable for a long time | Enzymes are stable for a long time |
| 55 | $6.5 \cdot 10^{-5}$ | The same |
| 60 | $4.0 \cdot 10^{-4}$ | $5.0 \cdot 10^{-6}$ |
| 65 | $1.0 \cdot 10^{-3}$ | $9.4 \cdot 10^{-6}$ |
| 70 | Enzymes denaturate almost immediately | $1.7 \cdot 10^{-5}$ |
| 80 | The same | $5.5 \cdot 10^{-5}$ |
| 90 | « | $1.8 \cdot 10^{-4}$ |
| 100 | « | $6.3 \cdot 10^{-4}$ |
| 110 | « | $1.1 \cdot 10^{-3}$ |
| >110 | « | Enzymes denaturate almost immediately |

form also the mobility of separate fragments of the protein molecule with respect to each other increases, which causes a decrease in thermal stability with an increase in activation energy. Second, it is known [12, 16] that the overall process (1) of enzyme denaturation can be split into a sequence of stages (2) in which protein molecules pass from the initial to denaturated state through a number of intermediate states $N_i$ and $D_j$ that are able to catalyse reactions with different efficiencies. The progress of such a cascade of transformations can be evidenced by the induction period observed at the initial section of kinetic curves (see Fig. 1, *a*). The absence of induction period on the kinetic curves of denaturation of the enzymes in the dry form may suggest that during drying the enzyme solutions, the proteins acquire $N_i$ conformation, which is more like the denaturated state than the initial conformation *N*. Evidently, lower energy is necessary to complete the cascade of transformations during heating the proteins in this conformation.

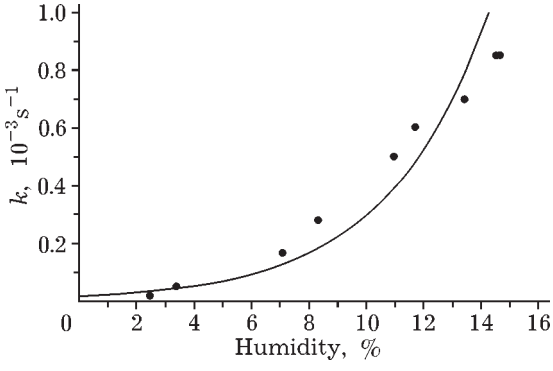


Fig. 2. Dependence of effective denaturation rate constant on the humidity of the enzymatic preparation.

To determine the effect of enzyme humidity on denaturation rate, samples with different water content were obtained. Heating of these samples at 80 °C (Fig. 2) showed that the effective rate constant increases exponentially with an increase in humidity. The most stable samples were those with water content not higher than 8 %. With an increase in humidity above 10 %, denaturation occurs rapidly, while for humidity about 30 % the rate constant in the solid phase is comparable with that in solution.

So, investigation of denaturation processes in solution and in the dry form showed that the humidity of enzymes has a strong effect on the rate of enzyme denaturation. Relying on the data presented here, for mechanoenzymatic processes, we can recommend the conditions providing operations with the dry mixtures substrate/enzyme or with enzyme solutions at temperatures not higher than 50–55 °C.

## CONCLUSION

It is demonstrated in the work that the solutions of the cellulolytic enzymatic preparation TselloLyuks-A possess low thermal stability and rapidly denaturate at temperatures above 60 °C. During heating the enzymes in the dry form, the effective rate constant of dena-

turation is much smaller than the rate constant in the presence of water; enzymes demonstrate higher stability and denaturate rapidly at temperatures above 100 °C. Effective denaturation rate constants are exponentially dependent on water content in preparations. For the processes taking place at increased temperatures and the processes connected with short-term local temperature rise, for example mechanical treatment, it is reasonable to use the dry form of enzymes, which is more stable.

## REFERENCES


1 Spitsyn A. P., Gusakov A. V., Chernoglazov V. M., Biokonversiya Lignotsellyuloznykh Materialov, Izd-vo MGU, Moscow, 1995.
2 Bychkov A. L., Korolev K. G., Lomovsky O. I., *Appl. Biochem. Biotechnol.,* 162 (2010) 2008.
3 Lomovsky O. I., Lomovsky I. O., in: Enhancing Extraction Processes in the Food Industry, in Lebovka N., Vorobiev E., Chemat F. (Eds.), Taylor & Francis, Raton–London–New York, 2011.
4 He X., Miao Y., Jiang X., Xu Z., Ouyang P., *Appl. Biochem. Biotechnol.*, 160, 8 (2010) 2449.
5 Perevolotskaya V. K., Afanasieva V. A., Golovina L. A., *Ros. Khim. Zh.*, 46, 2 (2002) 52.
6 Taherzadeh M. J., Karimi K., *Int. J. Mol. Sci.*, 9 (2008) 1621.
7 Bychkov A. L., Ryabchikova E. I., Korolev K. G., Lomovsky O. I., *Chem. Sust. Dev.*, 17, 5 (2009) 479. URL: http://www.sibran.ru/English/csde.htm
8 Vendruscolo M., *Trends in Biotechnol.*, 20 1 (2002) 1.
9 Yakusheva L. D., Dubinskaya A. M., *Biofizika*, 29, 2 (1984) 190.
10 Yakusheva L. D., Dubinskaya A. M., *Biofizika*, 29, 3 (1984) 365.
11 Dubinskaya A. M., *Biofizika*, 25, 4 (1980) 610.
12 Mozhaev V. V., Martinek K., *Mol. Biol.*, 16, 4. P. 676.
13 Ganesh K., Joshi J. B., Sawant S. B., *Biochem. Eng. J.*, 4 (2000) 137.
14 Kovaleva T. A., Kozhokina O. M., Bityutskaya L. A., Menshikova T. G., *Vestn. VGU. Ser. Khim. Biol. Farmatsiya*, 1 (2003) 57.
15 Cheshkova A. V., Fermenty i Tekhnologii dlya Tekstilya, Moyushchikh Sredst, Kozhi i Mekha, Izd-vo IGKhTU, Ivanovo, 2007.
16 Kozlov L. V., *Bioorgan. Khim.*, 6, 8 (1980) 1243.