Journal of International Scientific Publications:
Materials, Methods & Technologies, Volume 5, Part 1
ISSN 1313-2539, Published at: http://www.science-journals.eu

THERMAL AND MECHANICAL DENATURATION OF CELLULASE IN SOLUTION AND IN THE SOLID PHASE

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Abstract

Decrease of cellulosolytic activity Trichoderma viride cellulase under heating and mechanical treatment in solution and in solid phase is investigated. A comparison between the stabilities of the enzymatic preparation to thermal denaturation in the dry and dissolved formed is carried out. Kinetic parameters characterizing denaturation process are determined. The solutions of enzymes are stable at temperatures below 55 °C and rapidly get denaturated at a temperature above 65 °C. Under heating in the solid phase, the enzymes exhibit higher stability and get denaturated rapidly only at temperatures above 100 °C. Rapid denaturation of the enzymatic preparation in the solid phase is observed during mechanical treatment with the intensity more than 1.25 W/g. The rate of mechanical denaturation is weakly dependent on the process temperature.

Key words: *enzymes, thermal inactivation, mechanical inactivation, stability, mechanoenzymatic processes.*

1. INTRODUCTION

Enzymatic processes allow successful utilization of the wastes from woodworking industry, agriculture, industrially grown microorganisms (Sinitsyn et al. 1995; Golyazimova et al. 2009; Bychkov et al. 2010; He et al. 2010). One of the important problems in enzymatic processing of renewable raw material is denaturation of enzymes. Thermal and mechanical denaturation can appear as a result of technological mishandling, local overheating, or during preliminary mechanical activation of dry mixtures of enzymes and substrates, and ultrasonic treatment of reaction solutions. In processing the wastes and cheap agricultural raw material, the price of enzyme preparations comprises the major part of expenses necessary for the process implementation. Studies of the conditions that allow one to prolong the time of enzyme action are promising from the economical point of view.

A lot of works dealing with the studies of denaturation of individual enzymes had been published. For the majority of individual enzymes and complex preparations, optimal reaction conditions were determined, inactivation and reactivation were studied, stabilization mechanisms were proposed (Dubinaskaya 1980; Mozhaev and Martinek 1982; Yakusheva and Dubinskaya 1984a, 1984b; Ganesh et al. 2000; Vendruscolo 2002). However, the overwhelming majority of publications consider only denaturation of dissolved enzymes paying almost no attention to the processes in the solid phase. In addition, results obtained under different conditions and with different enzymatic preparations can hardly be correctly compared with each other. Mechanical denaturation of enzymes under technologically applicable conditions is considered only poorly.

The goal of the present work is experimental comparison between the kinetics of denaturation of cellulosolytic enzymes under heating in dry and dissolved forms, as well as the investigation of denaturation under mechanical action on enzymatic preparations.

2. MATERIALS AND METHODS

2.1. Reagents and materials. Cellulosolytic enzymatic preparation "Celloviridin" (trade mark "Cellolux 2000") (JSC Sibbiofarm, Novosibirsk, Russia); chromatographic paper Whatman No. 1. Acetate buffer with pH 4.7.

Cellulosolytic preparation is a mixture of enzymes formed by drying the culture fluid of *Trichoderma viride* fungi. The preparation includes xylanase – 8000 units of activity (UA) per gram, cellulase – 2000 UA/g, β-glucanase – up to 1500 UA/g, glucoamylase – 20 UA/g.

2.2. Denaturation of enzymes in the dry state. A weighed portion (10 g) of the enzymatic preparation was kept at a temperature of 70-110 ^{o}C. After definite time intervals, samples were taken to determine the cellulosotylic activity.

2.3. Denaturation of enzymes in solution. Aqueous solutions of the enzymatic preparation (2 mg/ml) were kept at a temperature of 55-70 ^{o}C. Samples were taken after definite time intervals in order to determine the cellulosotylic activity.

2.4. Mechanical denaturation of enzymes. The weighed portion of the enzymatic preparation «Celloviridin» (50 g) was placed in the ball attritor mill (similar to the «01-HD Lab Attritor» mill manufactured by the Union Process company, USA) (the mass of the balls was 2 kg) and subjected to mechanical action of different intensities (0.5-1.25 W/g). After definite time intervals, samples (300 mg) were taken to determine the cellulosotylic activity.

2.5. Determination of cellulosolytic activity. In order to determine cellulosolytic activity of the enzymes in the initial preparation and in the preparation subjected to thermal treatment, we performed enzymatic hydrolysis of chlomatographic paper and subsequent determination of the total amount of reducible carbohydrates (calculated for glucose).

To the weighed portions (100 mg) of filtering paper Whatman No 1, we added 4.0 ml of the solutions of enzymes with concentration 1.0 mg/ml. The reaction mixture was incubated for 1 hour at a temperature of 50^{o}C. Then the mixture was kept for 15 minutes at 95 ^{o}C for complete inactivation of enzymes, then cooled to room temperature. The undissolved residue of filtering paper was removed by centrifuging for 10 minutes at 7000 r/p/m/; the supernatant liquid was used to determine the total amount of reducing carbohydrates.

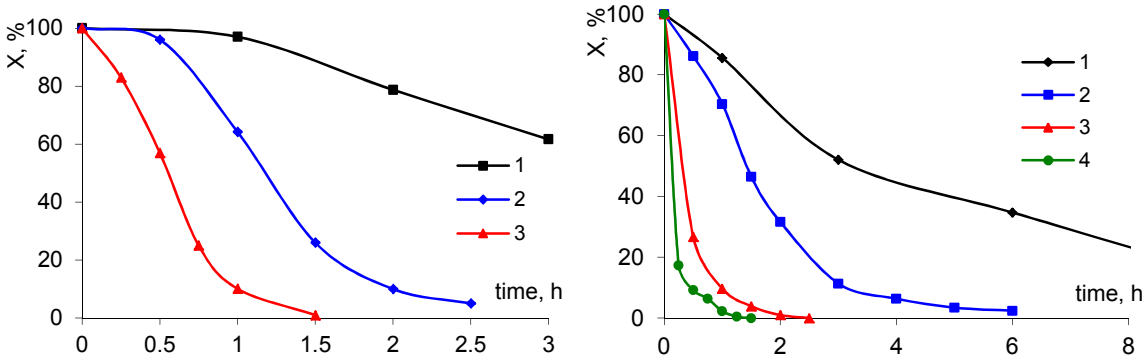
2.6. Determination of the total amount of reducing carbohydrates was carried out through the reduction of potassium ferricyanide $K_3[Fe(CN)_6]$. For this purpose, to 1.0 ml of carbohydrate solutions with concentrations 30 to 150 mg/l we added 3.0 ml of the 0.06 % solution of $K_3[Fe(CN)_6]$, mixed and kept at 100 ^{o}C for 10 minutes. After cooling, the solutions were examined by means of photometry with the UV/VIS «UNICO» spectrophotometer (USA) at the wavelength of 420 nm, with respect to distilled water. Standard solutions of glucose with concentrations 30.0-150 mg/l were used to plot the calibration curves.

We accepted the number of milligrams of glucose obtained through hydrolysis of chromatographic paper by one milligram of the enzymatic preparation during 1 hour as the unit of activity of the preparation.

3. RESULTS AND DISCUSSION

3.1. Thermal denaturation of enzymes.

In order to compare the stability of enzymes during heating in solution and in the solid phase, we made experiments in which the cellulosolytic preparation «Celloviridin» was kept at a temperature of 50-110 °C. The results are demonstrated in Fig. 1.



a *b*

Fig. 1. Denaturation of cellulosolutic enzymes: a – in solution (*1* – 55 °C, *2* – 60 °C, *3* – 65 °C, X – residual activity of the enzymatic preparation, %), b – in the solid phase (*1* – 80 °C, *2* – 90 °C, *3* – 100 °C, *4* – 110 °C)

The solutions of enzymatic preparations possess a narrow stability zone (Fig. 1-a). Under the most frequently used conditions of enzymatic hydrolysis of lignocellulose materials 50-55 °C – the activity of enzymes decreases only slightly. Temperature rise to 60 °C causes rapid denaturation of enzymes (2.5-3 hours), while at a temperature above 65 °C denaturation occurs almost instantly.

Enzymatic preparations in the solid phase possess higher stability to heating (Fig. 1-b). At temperatures below 70 °C, a decrease in the activity is insignificant; only at temperatures above 100 °C denaturation occurs almost instantly.

It is known from literature (Weijers and Van't Riet 1992a, 1992 b) that the overall process of enzyme denaturation (1) is described within the framework of the first-order kinetic equations. In this case, one may use equations (2) and (3) to determine the thermodynamic parameters of the process.

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

Here N is the native form of enzymes, D is denaturated form of enzymes.

$$-\ln X = kt \qquad (2)$$

Here X is the transformation degree, k is the effective rate constant of the reaction, t is reaction time.

Semi-logarithmic coordinates were used to determine the kinetic parameters of the process

$$\frac{E_a}{R} * \frac{1}{T} + \ln A = -\ln k \qquad (3)$$

Here *Ea* is effective activation energy, *T* is temperature at which the process takes place, *k* is the effective rate constants determined from (2), *A* is the preexponential factor, *R* is the gas constant.

The kinetic curves of denaturation processes are shown in Fig. 2; calculated effective rate constants and activation energies are listed in Table 1.

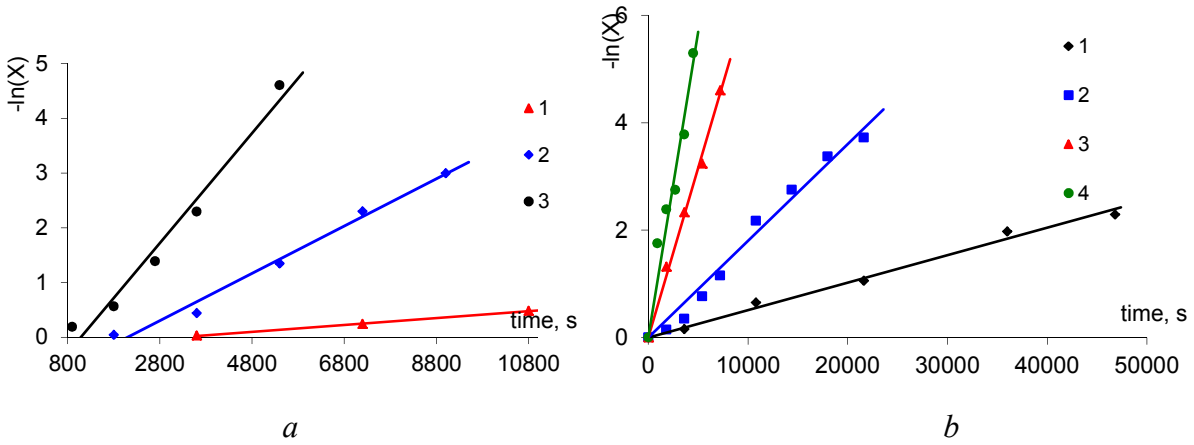


Fig. 2. Kinetics of denaturation processes in semi-logarithmic coordinates: a – in solution (*1* – 55 °C, *2* – 60 °C, *3* – 65 °C, X – residual activity of the enzymatic preparation, %), b – in the solid phase (*1* – 80 °C, *2* – 90 °C, *3* – 100 °C, *4* – 110 °C)

One can see in the data presented here that the activation energy of enzyme denaturation in solution is twice as high as that in the solid phase. This fact can be explained as follows: enzymes in solution are solvated with many water molecules, so that in order to change the tertiary structure that provides denaturation, is it necessary to spend more energy than in the solid phase where enzyme molecules are not solvated. It is also possible that the dissolved enzymes passing into the inactive state undergo more essential changes of the tertiary configuration than they do in the solid phase.

The following conclusions may be made from the presented experimental results. The solutions of the cellulosolytic enzyme preparation «Celloviridin» are stable at temperatures below 55 °C and quickly get denaturated at temperatures above 65 °C.

During heating in the solid phase, enzymes exhibit higher stability and denaturate rapidly only at temperatures above 100 °C. For the processes taking place at elevated temperature, as well as during mechanical treatment which may involve local temperature rise (Avvakumov et al. 2001), it is reasonable to use a more stable dry form of the enzymes.

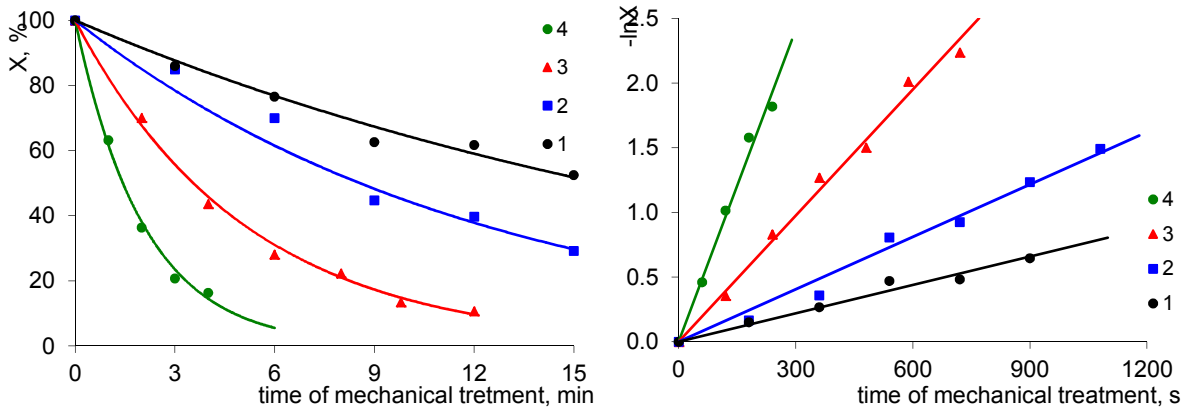
3.2. Mechanical denaturation of enzymes.

In order to study mechanical denaturation of cellulosolytic enzymes, the «Celloviridin» preparation was subjected to mechanical treatment in the attritor. Temperature inside the reactor was controlled by passing thermostated water into the cooling jacket of the reactor; short treatment time did not allow substantial heating of the milling bodies due to the transformation of mechanical energy into heat. The results are presented in Fig. 3. The intensity of treatment was measured in W/g*s, while the input mechanical energy was measured in W/g.

ISSN 1313-2539, Published at: http://www.science-journals.eu

Table 1. Kinetic parameters of enzyme denaturation in solution and in the solid phase.

	Temperature, °C	k, 1/s	Activation energy, kJ/mol
In solution	55	$6.5*10^{-5}$	250
	60	$4.0*10^{-4}$	
	65	$1.0*10^{-3}$	
In the solid phase	80	$5.5*10^{-5}$	118
	90	$1.8*10^{-4}$	
	100	$6.3*10^{-4}$	
	110	$1.1*10^{-3}$	



a *b*

Fig. 3. *a* – kinetics of mechanical denaturation of cellulosolytic enzymes, *b* – kinetics of enzyme denaturation in semi-logarithmic coordinates; *1* – 0.5 W/g, *2* – 0.75 W/g, *3* – 1.0 W/g, *4* – 1.25 W/g.

One can see that the overall process of mechanical denaturation is described within the framework of the first-order kinetic equations; the rate of enzyme denaturation under mechanical action is strongly dependent on the applied energy rating. For instance, for energy rating less than 0.5 W/g, enzymes are stable for a long time, while for energy rating more than 1.25 W/g they get denaturated almost instantly. The dependence of the effective rate constant of the mechanical denaturation of enzymes on mechanical energy rating is described by the exponential law (Fig. 4).

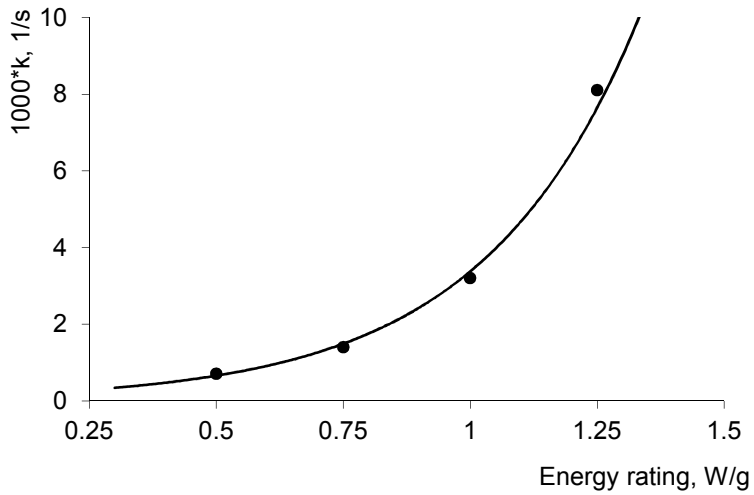


Figure 4. Dependence of the effective rate constant of mechanical denaturation of enzymes on mechanical energy rating.

A very weak dependence of denaturation rate on reactor temperature was observed. Variation of temperature from 30 ^{o}C to 80 ^{o}C for the same energy rating does not cause an increase or decrease in reaction rate, which points to the non-thermal mechanism of the mechanical denaturation of enzymes.

The results presented here allow us to conclude that the mechanical treatment of systems containing enzymatic preparations may have a technologically possible intensity. To prevent mechanical denaturation in biotechnological processes, it is necessary to apply treatment in the devices with low energy rating (below 0.5 W/g). Treatment in the devices with higher energy rating (more than 1.0 W/g) is possible only for a short time.

4. CONCLUSIONS

1. Solutions of cellulosolytic preparation «Celloviridin» are stable only at temperatures below 55 ^{o}C and rapidly get denaturated at temperatures above 65 ^{o}C.

2. During heating in the solid phase, enzymes exhibit higher stability and rapidly get denaturated at temperatures above 100 ^{o}C.

3. For the processes taking place at elevated temperature, and for the processes involving short-term local temperature rise, for example mechanical treatment, it is reasonable to use the dry form of enzymes that is more stable.

4. Under mechanical treatment of cellulosolytic enzymes in the solid phase, the rate of denaturation is strongly dependent on the energy rating of the reactor and weakly dependent on temperature. To prevent mechanical denaturation of proteins, it is necessary to carry out mechanical treatment in the devices with energy rating below 1.25 W/g.

ACKNOWLEDGEMENTS:

The investigation was supported by BRHE Y4-C-08-04, Ministry of Education RF RPN 2.2.2.2.3.1029, PharmaMed RUXO-008-NO-06, Government of Novosibirsk under Project No. 10-06,

Regional Social Foundation for Support of Science in Russia and Federal Program "Research and development on priority directions of scientific-technological complex of Russia".

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