

DENATURATION OF NATIVE AND DEGLYCOSYLATED α -GALACTOSIDASES FROM *PENICILLIUM CANESCENS* BY GUANIDINE HYDROCHLORIDE

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The aim of this work was the study of native and deglycosylated α -galactosidases from *Penicillium canescens* under denaturing conditions caused by guanidine hydrochloride. Calculation of kinetics and constants of enzymes inactivation was carried out on using experimental kinetic curves of enzyme denaturation. We observed significant differences in the kinetics of inactivation of native and deglycosylated α -galactosidases from *P. canescens* caused by guanidine hydrochloride. Native enzyme was stable within the selected range of guanidine hydrochloride concentrations (from 0.1 to 3.0 M), retaining no less than 50% of the initial enzyme activity for 3 days. Deglycosylated enzyme preparations were less stable and they lost their activity within 5–30 minutes, when they were treated with guanidine hydrochloride in concentrations above 1 M. Dissociation rate constant of native and deglycosylated forms of the enzyme differed by 10 to 100 folds. It was shown that subunit interactions play a major role in the process of inactivation of the enzyme, and the carbohydrate component is essential for stabilizing of subunit bonds and maintaining conformational stability of the enzyme under denaturing conditions of chemical agents.

Key words: α -galactosidase, *Penicillium canescens*, guanidine hydrochloride, denaturation.

Investigation of the mechanism of inactivation and reactivation of enzymes is very interesting from a theoretical and practical point of view. The processes of folding and unfolding of the molecule, and hence the catalytic properties of the enzyme are largely dependent on the conditions of the reaction medium. The interest in these processes is also associated with the appearance of work concerning genetic engineering, gene cloning, folding and the acquisition of the catalytically active conformation of recombinant and modified proteins.

It is known that a carbohydrate component of extracellular enzymes may have a significant effect on their stability and activity [1, 2]. The amount of protein glycosylation sites of the molecule may vary substantially depending on the way of producer growing. So, the study concerning the stability of various forms of enzymes that were obtained as a result of modifications of the carbohydrate component of the protein molecule can determine the

role of carbohydrate in maintaining of active conformation and quaternary structure of the protein. One of the ways of such modification is the growing of enzyme producers in the presence of inhibitors of glycosylation: tunicamycin, 2-deoxy-D-glucose, α -factor, and others [3–6].

We have previously obtained α -galactosidase (EC 3.2.1.22) from *Penicillium canescens* and shown that this enzyme is a glycoprotein with a molecular weight of 400 kDa, in which the carbohydrate content is 16% (m / m) [7]. The carbohydrate component includes mannose, D-glucosamine, arabinose and rhamnose.

The aim of this work was to explore how the inhibition of N- and O-glycosylation affects the stability of α -galactosidase from *P. canescens* under condition of chemical denaturation, to investigate the kinetics of enzyme denaturation using guanidine hydrochloride and find opportunities for renaturation of enzymes with different content of carbohydrate component.

Materials and Methods

It was used native and modified preparations of α -galactosidase from *Penicillium canescens* that were purified from culture liquid of the producer by precipitation with 90% ammonium sulfate. The preparations were purified to homogeneity by gelfiltration on TSK Toyoperl HW-55 and Sepharose 6B, and using ionexchange chromatography on DEAE-Toyoperl 650M [7]. Treatment of enzymes was carried out with guanidine hydrochloride (AppliChem GmbH, Germany).

Effect of inhibitors on the secretion of α -galactosidases was studied in this manner. Micromycetes culture was pregrown for 48 hours then mycelium was separated by centrifugation and transferred to medium of standard composition containing the desired inhibitor. The cultures were grown in the presence of 100 and 150 $\mu\text{g}/\text{ml}$ of 2-deoxy-D-glucose or 15 $\mu\text{g}/\text{ml}$ of tunicamycin. Then the cultures were grown for 3 days. α -Galactosidase activity was determined by the rate of degradation of *p*-nitrophenyl substrate.

The degree of substrate hydrolysis was defined by the colorimetric method as it was shown in [8]. One unit of activity corresponded the amount of enzyme that catalyzes the hydrolysis of 1 μM substrate for 1 min under standard test conditions (37 °C, pH 5.2).

Inactivation of the enzyme with guanidine hydrochloride was carried out by the following procedure. To a solution containing 1.5 U/ml of α -galactosidase in 0.1 M phosphate-citrate buffer (PCB, pH 5.2) we added guanidine hydrochloride in different concentrations (from 0.1 to 4 M) and at the enzyme activity was registered at regular intervals.

To perform reactivation the solutions of enzyme treated with guanidine hydrochloride were dialyzed against 0.1 M PCB, pH 5.2. Changes in the activity of the native and modified enzyme were recorded over several days.

The study of the kinetics of inactivation and calculation of correspondent constants were performed according to [9].

All experiments were carried out at least in 3–5 repetitions. Statistical processing was performed by standard methods with the definition of Student's *t* test at the 5% significance level.

Results and Discussion

We used three α -galactosidase preparations from *Penicillium canescens*: the native preparation isolated from the culture medium under standard conditions and two deglycosylated samples obtained by cultivation of the producer in the presence of tunicamycin and 2-deoxy-D-glucose.

The total carbohydrate content was reduced from 16.5% (native enzyme) to 11.7% (N-modified) and 8.2% (O-modified). After appropriate purification, we obtained enzymes that were different by quantitative content of neutral monosaccharides (Fig. 1).

Our further investigations concerned the role of carbohydrate component in supporting of active conformation of the enzyme under influence of guanidine hydrochloride and the kinetics of the protein denaturation. There are data about denaturation of different proteins in the presence of guanidine hydrochloride or urea [10]. This process can take place in one irreversible stage or include some reversible.

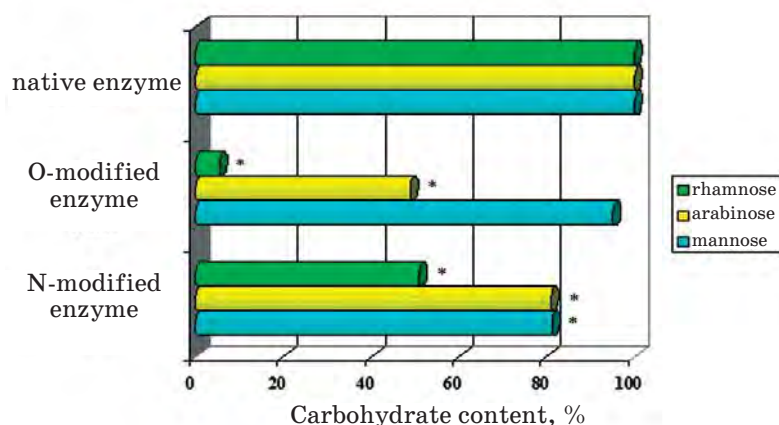


Fig. 1. Changing the content of neutral monosaccharides in N- and O-modified α -galactosidases from *Penicillium canescens* compared to the native enzyme (glycosylation %)

Here and after * — $P \leq 0.05$

After removing of the denaturant the spontaneous recovery of activity sometimes takes place. The investigated preparations of the enzyme in 0.1 M PCB (pH 5.2) possessed 100% of activity under the room temperature for 7 days. We chose these conditions as initial to carry out denaturation of α -galactosidase from *P. canescens* by guanidine hydrochloride. When guanidine hydrochloride was added to different preparations of α -galactosidase the gradual decrease of enzyme activity was observed (Fig. 2).

However, there were significant differences in kinetics of native and deglycosylated preparations. First of all, the degree of denaturing action of guanidine hydrochloride depended on enzyme preparation. The native enzyme was more resistant to different concentrations of guanidine hydrochloride (from 0.1 to 3.0 M) keeping no less than 50% of the initial activity for 3 days. Deglycosylated preparations were less resistant to high concentrations of the denaturant. These preparations lost brashly their activity

at denaturant concentration more than 1M over 5–30 min. At lower concentrations the native preparation decreased its activity over the first 150 min, then we observed the increase of activity followed by the decrease and then the rate was not high. Probably, in this case there was a change of some conformation states of the enzyme when it was switching to a less stable form. The decrease of activity was 20–30%, so we can suggest that partial unfolding of the molecule does not touch the active center of the enzyme.

There are data concerning the presence of lag-periods during thermal inactivation of different proteins in cases when the intermediate form of the enzyme appears, whose activity is comparable to the initial one [11].

The analysis of inactivation curves of α -galactosidase from *P. canescens* under influence of guanidine hydrochloride at 20 °C let us suggest that at all used concentrations of guanidine hydrochloride the dependence activity on the time can be described by two exhibitors. These kinetics curves are similar

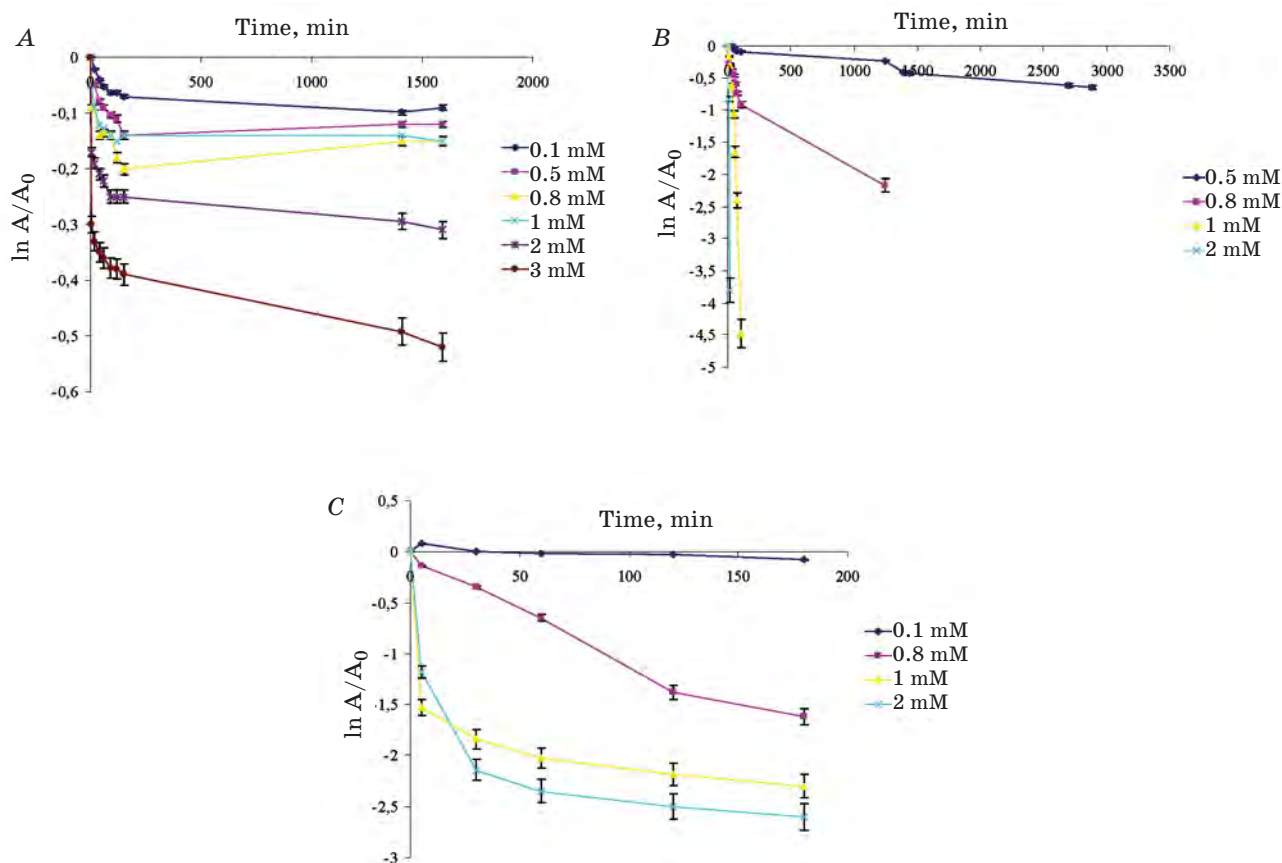


Fig. 2. Kinetics curves of guanidine hydrochloride inactivation of native (A) and deglycosylated (B — O-deglycosylated; C — N-deglycosylated) α -galactosidases from *P. canescens* in semi-logarithmic coordinates: pH 5.2, 20 °C, $n = 5$

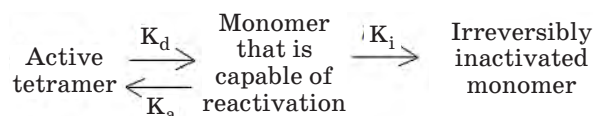
to the curves of thermal inactivation of α -galactosidase at 55–65 °C [12]. It proves the oligomeric structure of this enzyme. So, dissociative character of the enzyme denaturation takes place in case of guanidine hydrochloride: dissociation of tetramer into less active monomers with their following irreversible inactivation.

Inactivation of deglycosylated α -galactosidases from *P. canescens* takes place at the higher rate. To achieve the total loss of activity it needed from 5 to 120 min depending on the preparation and guanidine hydrochloride concentration. When the denaturant concentration was more than 1M the inactivation of these enzymes was observed over first 5 min of incubation.

Our attempts to carry out reactivation of modified enzymes had no effect. Reactivation was only observed for native α -galactosidase in case of removing of guanidine hydrochloride over first 3 hours, to the point of fracture

(Fig. 3). It proves the presence of monomers or dimers that are capable of reversible denaturation. At the later stages the observed denaturation was irreversible. Partially deglycosylated enzymes were not capable of renaturation.

So, denaturation of the native α -galactosidase from *P. canescens* by guanidine hydrochloride can be described by the scheme that was similar for previously described thermal inactivation [12]:



Calculation of kinetics constants of inactivation of α -galactosidase by guanidine hydrochloride was carried out according to Poltorak and Chukhray [9], who investigated in detail the character of inactivation of oligomeric proteins.

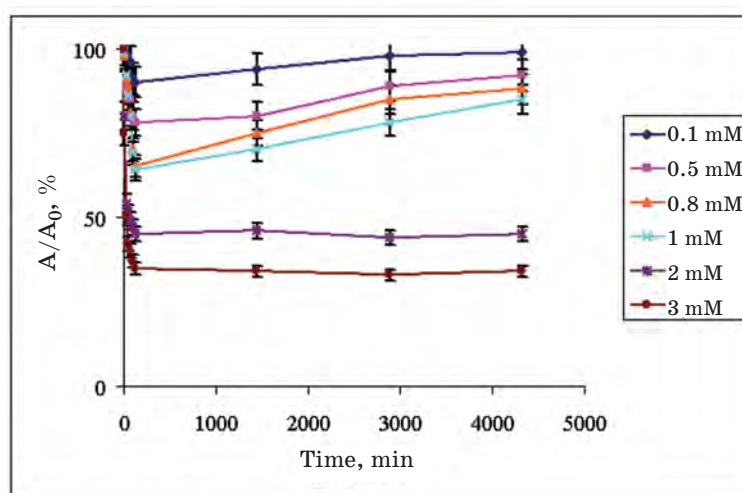


Fig. 3. Reactivation of the native α -galactosidase from *P. canescens* after removing of guanidine hydrochloride: pH 5.2, 20 °C, $n = 3$

Dissociation rate constants for α -galactosidases that were treated with guanidine hydrochloride (min^{-1})

| Enzyme from <i>P. canescens</i> | Guanidine hydrochloride concentration, M | | | |
|---------------------------------|--|---------------------|---------------------|---------------------|
| | 0.25 | 0.8 | 1 | 2 |
| Native | $6.3 \cdot 10^{-5}$ | $8.2 \cdot 10^{-5}$ | $8.5 \cdot 10^{-5}$ | $1.9 \cdot 10^{-4}$ |
| O-modified | $5.4 \cdot 10^{-4}$ | $1.3 \cdot 10^{-3}$ | – | – |
| N-modified | $4.4 \cdot 10^{-4}$ | $6.3 \cdot 10^{-3}$ | – | – |

Note: « \rightarrow » dissociation occurs very quickly.

The constants of dissociation rate of native and deglycosylated preparations were differed 10–100 (Table). We also calculated other kinetics parameters of native α -galactosidase from *P. canescens*. When concentration of guanidine hydrochloride was 0.5 M, they were the following $k_d = 7.3 \cdot 10^{-5} \text{ min}^{-1}$, $k_a = 7.4 \cdot 10^{-5} \text{ min}^{-1}$, $K_d = 3.8 \text{ }\mu\text{M}$; when 1.0 M – $k_d = 8.5 \cdot 10^{-5} \text{ min}^{-1}$, $k_a = 7.4 \cdot 10^{-6} \text{ min}^{-1}$, $K_d = 29 \text{ }\mu\text{M}$; when 3.0 M – $k_d = 2.3 \cdot 10^{-3} \text{ min}^{-1}$, $k_a = 1.8 \cdot 10^{-4} \text{ min}^{-1}$, $K_d = 92 \text{ }\mu\text{M}$. Inactivation rate constants for deglycosylated enzymes at 1.0 M of guanidine hydrochloride were $4 \cdot 10^{-2} \text{ min}^{-1}$ (O-deglycosylated enzyme) and $7.1 \cdot 10^{-3} \text{ min}^{-1}$ (N-deglycosylated form), and at 2.0 M of guanidine hydrochloride — $9.7 \cdot 10^{-2}$ and $7.5 \cdot 10^{-3} \text{ min}^{-1}$ respectively.

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**ДЕНАТУРАЦІЯ НАТИВНИХ
ТА ДЕГЛІКОЗИЛЬОВАНИХ
 α -ГАЛАКТОЗИДАЗ *Penicillium canescens*
ГУАНІДИНХЛОРИДОМ**

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Метою роботи було порівняльне дослідження нативних та деглікозильованих α -галактозидаз *Penicillium canescens* за умов денатуруючого впливу гуанідинхлориду. Розрахунок кінетики та констант інактивації проводили на підставі аналізу експериментальних кінетичних кривих денатурації ензимів. Виявлено значні відмінності в кінетиці інактивації нативної та деглікозильованої α -галактозидази *P. canescens* за дії гуанідинхлориду. Нативний ензим був стійким у всьому дослідженому діапазоні концентрацій гуанідинхлориду (від 0,1 до 3,0 М), зберігаючи не менш ніж 50% від вихідної активності протягом 72 год. Деглікозильовані препарати менш стійкі й за концентрацій гуанідинхлориду вище за 1 М були стрімко інактивовані протягом 5–30 хв. Константи швидкості дисоціації нативних та деглікозильованих ензимів відрізнялися на 1–2 порядки. Показано, що субєдиничні взаємодії відіграють головну роль у процесі інактивації ензиму, а вуглеводний компонент суттєво впливає на забезпечення міцності зв'язків та конформаційної стійкості до хімічної денатурації.

Ключові слова: α -галактозидаза, *Penicillium canescens*, гуанідинхлорид, денатурація.

**ДЕНАТУРАЦІЯ НАТИВНИХ
И ДЕГЛІКОЗИЛИРОВАННЫХ
 α -ГАЛАКТОЗИДАЗ *Penicillium canescens*
ГУАНИДИНХЛОРИДОМ**

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Целью работы было сравнительное изучение нативной и дегликозилированных α -галактозидаз *Penicillium canescens* в условиях денатурирующего воздействия гуанидинхлорида. Расчет кинетики и констант инактивации проводили на основании анализа экспериментальных кинетических кривых денатурации энзимов. Отмечены значительные отличия в кинетике инактивации нативной и дегликозилированной α -галактозидаз *P. canescens* под действием гуанидинхлорида. Нативный энзим был устойчив во всем исследованном диапазоне концентраций гуанидинхлорида (от 0,1 до 3 М), сохраняя не менее 50% от исходной активности в течение 72 ч. Дегликозилированные препараты менее устойчивы и при концентрациях гуанидинхлорида выше 1 М были инактивированы в течение 5–30 мин. Константы скорости диссоциации нативных и дегликозилированных энзимов отличались на 1–2 порядка. Показано, что субъединичные взаимодействия играют основную роль в процессе инактивации энзима, а углеводный компонент оказывает существенное влияние на прочность связей и конформационную устойчивость к химической денатурации.

Ключевые слова: α -галактозидаза, *Penicillium canescens*, гуанидинхлорид, денатурация.