INFLUENCE OF CHEMICAL REAGENTS AND UV IRRADIATION ON THE ACTIVITY OF Penicillium canescens α-GALACTOSIDASE

N. V. BORZOVA, L. D. VARBANETS

Danylo Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, Kyiv; e-mail: nv_borzova@bigmir.net

Investigations of the influence of chemical and physical factors on the conformational and functional properties of enzymes make a significant contribution to the study of the mechanism of action of industrially important proteins. The aim of the work was to evaluate the effect of chemical reagents and UV irradiation on the catalytic properties of Penicillium canescens α-galactosidase. Enzyme activity was assessed with p-nitrophenyl-α-D-galactopyranoside. Studies of the functionally active glycosidase groups were carried out on the basis of inhibitory and kinetic analysis using Dixon and Luinuiver-Burke methods with help of specific chemical reagents. A significant decrease in the activity of α-galactosidase in the presence of carbodiimides, diethylpyrocarbonate, the reagents on sulfhydryl groups was shown. A UV-induced decrease in enzyme activity in the dose range of 900-7200 J/m² was noted. Based on the data obtained, the imidazole group of histidine, carboxyl groups of C-terminal amino acids and the SH-groups of cysteine are assumed to play an important role in the manifestation of the activity of P. canescens α-galactosidase.

Key words: α-galactosidase, Penicillium canescens, UV irradiation, chemical inactivation.

© 2018 N. V. Borzova, L. D. Varbanets. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
Materials and Methods

The investigation object was a preparation of \textit{P. canescens} \(\alpha\)-galactosidase. Homogeneous fractions of the enzyme were obtained by purification using the methods of gel filtration and ion exchange chromatography on TSK-gels from supernatant of the cultural fluid of micromycete as described earlier [14]. Specific activity of the enzyme preparation was 30 U/mg of protein. To obtain \(N\)-modified \(\alpha\)-galactosidase the \textit{P. canescens} culture was preliminarily grown in standard conditions for 48 h, micelium was separated by centrifugation and transferred to the medium with a standard composition which contained 15 \(\mu\)g/ml of tunicamycin. After 3 days cultivation the enzyme was isolated from the supernatant of the cultural fluid and purified according to the standard method.

Protein content in the sample was determined by the Lowry method [15], as well as recorded spectrophotometrically at 280 nm. Activity of \(\alpha\)-galactosidase was determined using synthetic \(n\)-nitrophenyl substrate [16]. One unit of enzyme activity was defined as the amount of enzyme, which hydrolyses 1 \(\mu\)mol of the substrate for 1 min under the experiment conditions.

Gel filtration of the native and UV-treated enzyme preparations was carried out on Sepharose 6B column, in 0.01 M phosphate buffer pH 7.0, the elution rate – 0.2 ml/min.

UV irradiation of the enzyme solutions (2 mg/protein in 0.01 m phosphate-citrate buffer (PCB) pH 5.2) was performed with the help of a mercury-quartz lamp of the type BUF-15, \(\lambda=254\) nm in darkness at 20 \(^\circ\)C and continuous mixing. Irradiation doses were 450, 900, 1350, 1800, 2400, 2700, 3000, 3600, 5400 \& 7200 J/m\(^2\).

UV-spectra of absorption of native and irradiated preparations of the enzyme were studied by spectrophotometer-fluorimeter DeNovix DS-11 in the range of 240-340 nm, concentration of the enzyme preparation 1.2 mg of protein/ml.

Functionally active groups of glycosidase were investigated with the help of specific chemical reagents: 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide, \(p\)-toluol sulfonic-1-cyclohexyl-3(2-morpholinoethyl)carbodiimide, diethylpyrocarbonate (DEPC), \(p\)-chloromercurybenzoate (\(p\)-ChMB), \(N\)-ethylmaleimide (Sigma-Aldrich, USA), \(H_2O_2\), potassium ferricyanide. Cations Ag\(^+\).
and Hg$^{2+}$ were used in the form of nitrate and sulfate, respectively. Chemical reagents were used in concentration $10^{-3}$-$10^{-5}$ M, the enzyme concentration was 0.2 mg of protein/ml. All the manipulations were carried out in 0.01 M PCB, pH 5.0, if it was not indicated otherwise. Aliquots were taken at regular intervals and their enzymatic activity was measured.

Lineweaver-Berk and Dixon graphic methods were used to calculate the inhibition constants [17].

Photooxidation in the presence of methylene blue ($5 \cdot 10^{-8}$ M) was also performed. Photoinactivation was carried out at 30 and 40 °C, pH 4.0 and 6.0, in a light-proof heat chamber and in an open thermostat. An incandescent lamp (200 W) with a red light filter at the distance of 15 cm from the solution surface was used as the light source. The samples, which contained a photosensibilisator, but incubated in darkness, or illuminated but without methylene blue serve as control. After the incubation time completion, the aliquots were taken and their residual activity was determined.

All investigations were repeated no more than thrice. Statistical processing of the results of experimental series was performed by the standard methods using Student’s $t$-criterion at a 5% significance level with the help of MS Excel 2007 program.

**Results and Discussion**

The inhibitory analysis of the effect of specific chemical reagents and physical factors on catalytic activity of enzymes allows intensifying certain amino acid residues in the substrate-binding centers of the enzyme molecule, as well as judging of their significance in displaying functional activity and conformation stability of the biocatalyst.

Modification of the carboxyl group of asparaginic or glutamic amino acids with the help of carbodiimides 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide methiodide and $p$-toluol sulfonic-1-cyclohexyl-3(2-morpholinoethyl)carbodiimide has shown a considerable inhibition of α-galactosidase activity under the effect of the given effectors (Fig. 1). The results obtained indicate the importance of carboxyl groups for manifestation of enzyme activity. The observed incomplete inhibition can evidence for availability of other residues of these amino acids which are important for the proceeding of enzymatic reaction, but in these conditions are inaccessible for the inhibitor. Carboxyl groups of asparaginic and glutamic acids were found in the active center of α-galactosidases referred to the families GH27, GH31, GH36, GH57, GH97 (http://www.cazy.org/).

Several methods were used to modify. These were, in particular, photooxidation in the presence of histidine residues methylene blue and inhibition DEPC which reacts specifically with histidine residues at pH 5.5–7.5.

It was shown that *P. canescens* α-galactosidase is inactivated in the light in the presence of methylene blue like *C. cladosporioides* α-galactosidase [13]. The observed photoinactivation is accelerated with the increase of temperature and pH values (Fig. 2). The last fact confirms the effect of histidine on the catalytically important group, since the rate of photooxidation of tyrosine and tryptophan residues, which can also be subject to photooxidation, does not depend on the medium pH.

The reaction with DEPC is the most selective method of chemical modification of histidine residues in proteins. The use of DEPC in studied concentrations decreased the rate of hydrolysis of a synthetic substrate, acceleration of α-galactosidase thermonactivation was also observed at 55 °C (Fig. 3, A). The given effects may be explained by the inactivation of reaction-capable residues of histidine. Interaction of the latter with DEPC results in formation of N-ethoxyformylidazole that is recorded as the increase of the absorption indices at a wavelength of 240 nm (Fig. 3, B).

The important role in displaying functional activity of numerous proteins belongs to sulfhydryl
groups of cysteine residues since they are the most reactive ones in the protein [2]. They can be effective nucleophilic agents in conditions close to physiological ones. Thus, there appears a necessity in modification of these groups at the initial stages of studying the enzyme structure. Modification of a sulfhydryl group of cysteine was performed with the help of the reaction with p-ChMB by H₂O₂ oxidation to derivatives of cysteine acid, the oxidation in soft conditions with formation of inter- and intramolecular disulphide bonds under the effect of ferricyanide, condensation with N-ethylmaleimide at pH 7.0.

The investigation results evidence for a considerable contribution of free SH-groups to displaying catalytic activity of P. canescens α-galactosidase (Table). Thus, the effective thiol inhibitors (p-ChMB and N-ethylmaleimide) and H₂O₂ inactivated the enzyme more than by 90%. The inhibition constants for ChMB, silver and mercury ions were 2.8·10⁻⁶, 1.5·10⁻⁶, and 3.0·10⁻⁷ M, respectively. It was shown before [19] that inhibition of P. canescens α-galactosidase by thiol inhibitors is of noncompetitive character, and consequently, the given groups rather make their contribution to the maintenance of the active molecule conformation that are present in the active center. Inactivation of

Fig. 2. Photoinactivation of P. canescens α-galactosidase in the presence of methylene blue

Fig. 3. A. Inactivation of α-galactosidase at treatment with DEPC (20 and 55 °C, pH 5.5). B. Dynamics of change of α-galactosidase solution absorption in the presence of 5·10⁻⁴ M DEPC at 240 nm
Inhibition of activity of P. canescens α-galactosidase in the presence of some chemical reagents (pH 5.2)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Reagent concentration, M</th>
<th>Residual activity, % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-ChMB</td>
<td>1·10^{-4}</td>
<td>10.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>1·10^{-3}</td>
<td>0</td>
</tr>
<tr>
<td>N-ethylmaleimide</td>
<td>5·10^{-3}</td>
<td>5.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>1·10^{-3}</td>
<td>8.00 ± 0.05</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>1·10^{-3}</td>
<td>5.00 ± 0.08</td>
</tr>
<tr>
<td>Potassium ferricyanide</td>
<td>5·10^{-3}</td>
<td>2.00 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>1·10^{-3}</td>
<td>10.0 ± 0.2</td>
</tr>
<tr>
<td>Ag⁺</td>
<td>1·10^{-5}</td>
<td>0</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>1·10^{-5}</td>
<td>0</td>
</tr>
</tbody>
</table>

Inhibition of activity of P. canescens α-galactosidase in the presence of some chemical reagents (pH 5.2).

Inactivation of enzymes by the silver and mercury ions is shown for a great many of the studied α-galactosidases [5-8, 13], and herewith most of them demonstrate resistance to the effect of p-ChMB and N-ethylmaleimide [6, 7].

Along with the chemical modification, which permits revealing catalytically significant residues of amino acids, the investigations of the effect of physical denaturing factors, including UV-effect, permit establishing the limits of mobility of a protein molecule and revealing the groups responsible both for the display of functional properties and for the preservation of active conformation. Protein chromophores (tryptophan, tyrosine, phenylalanine, histidine, cysteine) and their arrangement in the enzyme molecule play a leading role in the mechanism of UV-inactivation. The destruction of amino acid residues involved in the active center of the enzyme or affecting their conformation will finally result in the loss of functional activity of the given protein.

Study of the changes in catalytic activity of α-galactosidase under the effect of UV-light has shown that during the first 30 min of irradiation in a dose of 900 J/m² a true 20% decrease of activity was observed (Fig. 4). The dose is increased to 7200 J/m², a 2-fold decrease of activity was observed as compared to the control.

The process of inactivation of native enzyme is a sum of several exponents that is probably connected with quaternary structure of the enzyme molecule and availability of comparable quantities of protein sites modified and unmodified by UV-light. High resistance of N-modified P. canescens α-galactosidase to UV irradiation should be noted. We have already shown [20] that the above enzyme was glycosylated by the mixed type, and the presence of O-bound carbohydrates is critical for display-

Fig. 4. UV-induced changes of activity of native and N-modified P. canescens α-galactosidase
ing activity and stability, while the inhibition of N-glycosylation did not result in the considerable change of catalytic properties of the enzyme. The resistance of N-deglycosylated α-galactosidase to UV-action confirms once more the existing hypothesis on the important role of O-bound carbohydrates in supporting the active structure of the enzyme.

Protein inactivation is accompanied by such effects as the structure unrolling or aggregation of protein molecules. This, in its turn, can determine both a decrease and an increase of activity. As a result of the splitting of disulphide bonds the protein can lose the quaternary structure that will also lead it to denaturation. It has been already established that \textit{P. canescens} α-galactosidase is a homohexamer with the molecular mass 400 kDa [14]. The enzyme functions in the oligomer state, while separate subunits do not display activity [14], and aggregates of \textit{P. canescens} α-galactosidase cross-linked by glutaraldehyde [19] are considerably inferior in activity to the native preparation. The complete loss of activity of the monomer form as a result of dimer decay is also shown for \textit{B. coagulans} [7]. Data of gel filtration on Sepharose 6B it was demonstrated (Fig. 5) that the protein elution profiles and activity of native and irradiated sample practically coincided. Therewith one could observe a certain decrease of protein concentration and appearance of the fractures with a lower molecular mass that can be a result of simultaneous existence of two enzyme forms: active hexamer and inactive di- and monomers. But, since a rather high resistance to photodestruction was revealed, we suppose that the mechanism of UV-inactivation is rather mainly realized through the effect on aromatic amino acids and cysteine than through the break of disulfide bonds.

A decisive factor of macromolecule resistance to irradiation is, certainly, the number and arrangement of residues of aromatic amino acids, as well as the amount and accessibility of cysteine. The considerably less number of aromatic amino acids and cysteine for a protein molecule is often observed in fungal glycosidases, their resistance to UV irradiation being connected with this fact [21]. Thus, an increased resistance to the effect of UV-light in the range of 75-6040 J/m² was shown for fungal inulinase as compared with inulinases of yeast and plants [21] against a background of a lower content of tyrosine, phenylalanine and cysteine. In the case when aromatic amino acids enter the active center, or their destruction is accompanied by the rupture of the tertiary and quaternary structure of protein, a rapid photoinactivation of enzyme preparations is observed. It is shown that \textit{A. awamori} glucoamylase, containing tryptophan residue in the active center, is subject to practically full denaturation already in a dose of 1500 J/m², while the examined \textit{P. canescens} α-galactosidase is resistant to the action of twice higher doses.

It was established that the absorption spectra of the native and UV-inactivated \textit{P. canescens} α-galactosidase are similar in form (Fig. 5). It is
known that in the range of 200-230 and 270-300 nm protein absorption is mainly determined by the presence of aromatic amino acids, and in the range of 240-250 nm – of cysteine sulphydryl groups and cysteine disulfide groups. It may be noted that in our case the absorption efficiency in the range of aromatic amino acids (probably tyrosine and tryptophan, and to a less extent histidine) is higher than in the absorption range of SH-groups.

The obtained results are in line with the present concepts on the mechanism of photoinactivation of oligomer proteins, *P. canescens* α-galactosidase belonging to them. The effect of the doses to 600 J/m² does not lead to significant changes in the enzyme activity. At the doses above 900 J/m² one can observe true changes of functional activity of the enzyme, which can be caused both by the photochemical destruction of aromatic amino acids and rather by the destruction of the grouping, which reacted with the primary photoproduct, than by the group which had absorbed light. As a result of the latter reaction one can observe the enzyme inactivation as a consequence of the change of the spatial protein structure.

Thus, in accordance with our data on inhibition of *P. canescens* α-galactosidase the important role in the enzyme activity realization belongs to imidazole group of histidine and carboxylic groups of C-terminal amino acids. Cysteine SH-groups also play a foremost conformational role. It may be supposed that photodestruction of active center does not occur as a result of UV irradiation, while photomodification of aromatic and sulfur-containing amino acids results in conformational rearrangements accompanied by a decrease of catalytic activity of *P. canescens* α-galactosidase at high irradiation doses.

### ВПЛИВ ХІМІЧНИХ РЕАГЕНТІВ ТА УФ-ОПРОМІНЕННЯ НА АКТИВНІСТЬ α-ГАЛАКТОЗІДАЗИ *Penicillium canescens*

Н. В. Борзова, Л. Д. Варбанець

Інститут мікробіології і вірусології ім. Д. К. Заболотного НАН України, Київ; e-mail: nv_borzova@bigmir.net

Дослідження впливу хімічних і фізичних факторів на конформаційно-функціональні властивості ензимів вносять істотний додаток до вивчення механізму дії промислово важливих протеїнів. Метою роботи було оцінити вплив хімічних реагентів та УФ-опромінення на каталітичні властивості α-галактозидази *Penicillium canescens*. Активність ензиму визначали за допомогою *n*-нітрофеніл-α-D-галактопіранозиду. Дослідження функціонально активних груп глікозидази проводили на основі інгібіторного та кінетичного аналізу методами Діксона та Лайнуївера-Берка за допомогою специфічних хімічних реагентів. Було показано вірогідне зниження активності α-галактозидази в присутності карбодіімідів, діетилпірокарбонату, реагентів на сульфгідрильні групи. Відмічено УФ-індуковане зниження активності ензиму в діапазоні доз 900-7200 Дж/м². На основі одержаних даних передбачалося важлива роль імідазольної групи гістидину, карбоксильної групи C-кінцевих амінокислот, а також SH-групи цистеїну у проявленні активності α-галактозидази *P. canescens*.

**Ключові слова:** α-галактозидаза, *Penicillium canescens*, УФ-опромінення, хімічна інактивація.
ВЛИЯНИЕ ХИМИЧЕСКИХ РЕАГЕНТОВ И УФ-ОБЛУЧЕНИЯ НА АКТИВНОСТЬ α-ГАЛаЛКТОЗИДАЗЫ Penicillium canescens

Н. В. Борзова, Л. Д. Варбанец

Институт микробиологии и вирусологии им. Д. К. Заболотного НАН Украины, Киев;
e-mail: nv_borzova@bigmir.net

Исследования влияния химических и физических факторов на конформационно-функциональные свойства энзимов вносят весомый вклад в изучение механизма действия промышленно важных протеинов. Целью работы было оценить воздействие химических реагентов и УФ-облучения на каталитические свойства α-галактозидазы Penicillium canescens. Активность энзима определяли с помощью p-нитрофенил-α-D-галактопиранозида. Исследования функционально активных групп гликозидазы проводили на основе ингибиторного и кинетического анализа методами Диксона и Луйнуивера-Берка с помощью специфических химических реагентов. Показано достоверное снижение активности α-галактозидазы в присутствии карбодиимидов, диэтилпирокарбоната, реагентов на сульфгидрильные группы. Отмечено УФ-индукционное снижение активности энзима в диапазоне доз 900-7200 Дж/м². На основе полученных данных можно предположить важную роль имидазольной группы гистидина, карбоксильных групп C-концевых аминокислот, а также SH-группы цистеина в проявлении активности α-галактозидазы P. canescens.

Ключевые слова: α-галактозидаза, Penicillium canescens, УФ-облучение, химическая инактивация.

References

Received 05.04.2018