Kinetic and thermodynamic aspects of the process ofthermal inactivition of *kluyveromyces marxianus* inulinase

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Abstract

Background: Inulinase is widely found in microorganisms and higher plants. It catalyzes the reaction of the hydrolysis of inulin, which is present in the tubers and roots of many plants, to fructose and a small amount of glucose.

Inulin is one of several plant-based polysaccharides that contain glucose or fructose. It is used as a substrate in industrial fermentation processes and in the food industry due to its relatively cheap and abundant source for the microbiological production of high fructose juices, ethanol, acetone and butanol. Inulin-derived oligosaccharides are also used in the medical and food sectors. Inulinase which produced from the yeast K. marxianus at levels close to commercial use. This indicates that inulinase can be used in the production of fructose and fructose syrups. So our main object is to study the effect of different temperatures on the conformation of the Kluyveromyces marxianus inulinase macromolecule, to provide optimal conditions for aniline hydrolysis by inulinase.

Materials and method: Method of isolation and purification of the inulinase enzyme from Kluyveromyces marxianus, as well as the determination of the protein content and enzyme activity as mentioned in the context of the research. Experiments were carried out to study the thermal stability of enzyme. For this, an enzyme solution at a concentration of 5•10-5 mol / l was incubated in a time interval of 10-60 min at different temperatures, followed by determination of the catalytic activity.

It is known that different types of bonds and interactions participate in the formation of the molecular structure of enzymes, which are covalent bonds, hydrogen bonds, salt bridges, hydrophobic interactions, so our main goal is to study the effect of different temperatures on the formation of the enzyme molecule in order to provide optimal conditions for aniline hydrolysis. by enzyme.

Results: of this study revealed that the residual activity of the enzyme after 60 minutes of incubation at 50 °C was 10% of the initial activity at 60 °C 7%. As for the Rate Constants of thermal inactivation of Inulinase at 70 °C were 3.45.

Conclusions: Results of this study were important and useful in determining parameters of inulinase enzyme. Based on the shape of the curves of the dependence of the catalytic activity of Kluyveromyces marxianus inulinase on the time of thermal inactivation in the temperature range of 20-80 ° C.

Key words: Inulinase, Thermal inactivation, kinetics parameters.

1. Introduction

Inulinase is quite widespread in micromycetes and higher plants. It catalyzes the reaction of hydrolysis of inulin contained in tubers and rhizomes of many plants (Jerusalem artichoke, sunflower, dahlia, dandelion, etc.), to fructose and a small amount of glucose. A number of researchers have made the assumption that the action on inulin is carried out by the mechanismof a single chain, starting from the terminal link of the inulin molecule. Inulin is a plant reserve polysaccharide.The molecule consists of D-fructose and D-glucose residues and is an un branched chain of 32 - 45 fructofuranoside fragments connected by β-2,1-glycosidic bonds, to the reducing end of which D-glucopyranose is attached via a hemiacetal hydroxyl, as in the sucrose molecule (1).Inulinase belongs to the group of fructohydrolase (2,1- β-D-fructan-fructohydrolase, EC 3.2.1.7), which combines enzymes that break down fructose polymers. Inulinases are enzymes that hydrolyze β-1,2-bonds of inulin. In most cases, they are also capable of hydrolyzing sucrose. By the type of action, exo- and endoinulinases are distinguished, which, as a reaction product, form fructose in the first case and various fructooligosaccharides in the second (2).For the most complete analysis of the mechanism of thermal inactivation of enzyme molecules, it is necessary to know the kinetic-thermodynamic parameters of this process , so our main object is to study the effect of different temperatures on the conformation of the Kluyveromyces marxianus inulinase macromolecule, to provide optimal conditions for aniline hydrolysis by inulinase.

2. Material and methods

Methods for isolation and purification of inulinase enzyme from Kluyveromyces marxianus as well as determination protein content, enzyme activity as mentioned in (3).

Experiments were carried out to study the thermal stability of enzyme. For this, an enzyme solution at a concentration of $5 \cdot 10^{-5}$ mol / l was incubated in a time interval of 10-60 min at different temperatures, followed by determination of the catalytic activity.

3. Results

The dynamics of the Kluyveromyces marxianus inulinase inactivation process as shown in

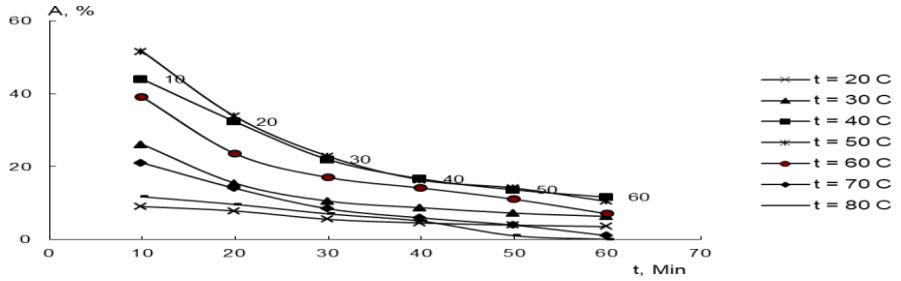

Figure (1): Dependence of the catalytic activity of inulinase Kluyveromyces marxianus from thetime of thermal inactivation

It was revealed that the residual activity of the enzyme after 60 minutes of incubation at 50 ° C was 10% of the initial one, at 60 ° C - 7%. It was found that heating the solution at 70 ° C for 60 min leads to the complete inactivation of the enzyme preparation. At 80 ° C, inulinase loses its ability to hydrolyze within 50 minutes. These results are consistent with the literature data (4). It has been shown that the rate of enzyme inactivation in solution increases with increasingtemperature; at 100 ° C, inactivation occurs almost instantly.

It is known that various types of bonds and interactions are involved in the formation of the molecular structure of enzymes: covalent, hydrogen bonds, salt bridges, hydrophobic interactions (5).Based on the shape of the curves of the dependence of the catalytic activity of Kluyveromyces marxianus inulinase on the time of thermal inactivation in the temperature range of 20-80 ° C, one can make an assumption about the partial destruction under the influence of temperature of weak electrostatic and, possibly, hydrogen bonds that maintain the conformation of the protein molecule, which is accompanied by a loss of hydrolytic activity enzyme.

The observed increase in the rate of enzyme inactivation with an increase in temperature from 60° C to 70° C for Kluyveromyces marxianus inulinase can be explained by the fact that excess heat energy causes the destruction of hydrophobic interactions that make an important contribution to the stability of proteins, resulting in a deeper unfolding of the polypeptide chain. This interpretation of the process is in good agreement with the literature data on enzyme inactivation (6,7).

Thus, the process of inulinase inactivation is complex, and its mechanisms for enzymes from different sources are not identical. One of the main kinetic characteristics of the process under study is the inactivation rate constant (k, h^{-1}), which is the loss of activity per unit of enzyme activity within an hour. This parameter was calculated using the formula:

$$k = 2.3 / t \cdot \log (E_0 / E)$$

Where E_0 is the initial activity taken as 100% .E- is the enzyme activity at time t, in% of the initial value (8).
The kinetic parameters of the inulinase thermal inactivation process are presented in table (1).

Table (1): Rate Constants of Thermal Inactivation of Inulinase

Temperature, ˚C	Inactivation rate constant,$ч^{-1}$
50	**0,32**
60	**1,67**
70	**3,45**

An analysis of the results obtained indicates a low thermal stability of Kluyveromyces marxianusinulinase, the rate constant of inactivation of which at any temperature is lower than according tothe literature data for enzymes from other sources (9,10).

When studying the mechanism of thermal inactivation of proteins, valuable information can be obtained by determining some thermodynamic parameters: enthalpy ΔH, entropy ΔS, activationenergy Eact, free energy ΔG.For this it is necessary to use the theory of absolute reaction rates (11,12).Central to this theory is the position that the rate of reaction at a given temperature depends only on the concentration of an activated complex in equilibrium with non-activated molecules. In thiscase, all activated complexes disintegrate at a rate determined by the ratio k_BT / h, in which is the Boltzmann constant (1.3305 10^{-23} J / K), h is the Planck constant (6.6267 10^{-34} J s), T is the absolute temperature (K). Thus, the reaction rate constant k was found by the equation:

$$k = k_BTK^* / h$$

Where K*- is the equilibrium constant between the activated complex and non-activatedmolecules.
Hence, the value of K* was determined as follows:

$$K^* = kh / k_BT$$

Knowing the value of the equilibrium constant, ΔG * was calculated using the formula:

$$\Delta G^* = \Delta H^* - T\Delta S^* = -RT \ln K^*$$ where

R is the gas constant (8.315 J / K mol).
To determine ΔH *, the following equation was used:

$$E_{act} = \Delta H^* + RT$$

The activation energy was found according to the Arrhenius graph from the following ratio:

$$E_{act} = 2.303\ R\ tg\alpha$$

Where α is the angle that the inclined straight line with the abscissa axis makes.Knowing E_{act}, ΔH * was calculated using the formula:

$$\Delta H^* = Eact - RTTo$$

determine ΔS *, the following equation was used:

$$\Delta S^* = (\Delta H - \Delta G) / T$$

Thermodynamic parameters of the process of thermal inactivation of Kluyveromyces marxianusinulinase are presented in table (2).

Table (2): Values of thermodynamic parameters of the inulin hydrolysis reaction catalyzed by Kluyveromycesmarxianus inulinase.

Temperature, ˚C	E_{act}, kJ / mol	ΔG, kJ / mol	ΔH, kJ / mol	ΔS,kJ / mol K
50	7,2	65,3	4,6	-89,1
55	7,4	66,1	4,5	-91,0
60	7,3	65,8	4,4	-90,7
65	12,5	67,5	11,7	-99,8
70	12,7	67,8	11,9	-98,8
75	14,1	68,0	11,9	-96,5

It was shown that the effect of high temperatures on inulinase is accompanied by an increase in E_{act}, ΔH*, ΔS* of the inulin hydrolysis reaction to fructose. Arrhenius plots for determining the activation energy are characterized by the presence of a kink, indicating a sequential catalysis reaction as seen Figure (2).

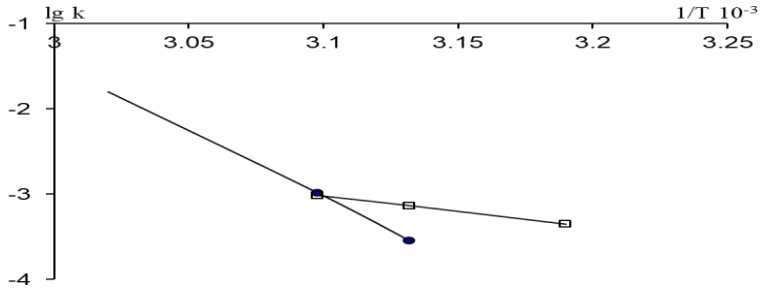

Figure (2): Arrhenius plot for Kluyveromyces marxianus inulinase

The consequence of an increase in E_{act} with increasing temperature is a decrease in the rate of thecatalysis process. Obviously, at temperatures well below the denaturation transition temperature, the protein globule undergoes significant conformational rearrangements such as small local changes, assuming a loose metastable structure, which determines steric hindrances during the formation of the enzyme-substrate complex. Hence, an increase in the energy barrier for the implementation of the catalysis reaction (E_{act}) and ΔH * follows. Analysis of the data obtained indicates that conformational changes in protein molecules, which affect their catalytic ability, occur for inulinase at 54 and 65 ° C, respectively (13).

More accurate values of the temperatures of the onset of rearrangements in the protein globule, caused by the process of thermal inactivation, can be obtained from the Arrhenius plot. The dependences of lg k on 1 / T are presented by straight lines, the intersection points of which correspond to 53 ˚C. As seen from Fig. 2, the Arrhenius graph is located with a bulge downward.The concave shape of the broken line described by the Arrhenius equation is due to the fact that large values of the activation energy are observed at high temperatures.

4. Discussion

Negative values of ΔS * for the inulin hydrolysis reaction indicate that the inulin hydrolysis reaction proceeds at a high rate and is characterized by high orderliness. An increase in ΔS * with increasing temperature is associated with the transition of inulinase molecules from an ordered globule to a chaotic coil. Small values of the change in ΔS * indicate the predominant destruction of weak bonds (hydrogen and electrostatic), leading to the smallest loss of catalytic activity.

It is known that the exposure of hydrophobic groups located in the inner space of the native molecule to the outside and an increase in conformational entropy due to the transition of a rigid native structure with a low atomic rotation freedom into a flexible structure of a disordered coil is of the greatest importance during the globule - coil transition. However, the change in entropy during denaturation is determined not only by the change in conformational entropy due to the transition of the protein macromolecule into a disordered state. A significant contribution of the opposite sign is made by a change in the structure of water adjacent to the exposed hydrophobic side chains, and, therefore, as the temperature rises, its contribution to the entropy of denaturation will decrease. As a result, the value of ΔS* denaturation will increase with increasing temperature (14).As the temperature rises in any zone of the temperature scale, the entropy component (TΔS) increases: this should weaken the strength of the spatial structure of the molecule. On the other hand, hydrophobic interactions, which play an important role in its stabilization, exhibit a complex dependence on temperature. In the range of 0-60 ˚C, as the temperature rises, the force of displacement of hydrophobic amino acid residues to the surface of the molecule increases, and their tendency to converge inside the globule also increases. With a further increase in temperature, the tendency towards convergence of hydrophobic residues weakens. The temperature dependence of the strength of hydrogen bonds is difficult to establish, it is assumed that it is insignificant. The reaction of a protein macromolecule to an increase in temperature is mainly determined by two components included in the entropy of the TΔS system:

Conformational entropy and entropy created by hydrophobic interactions. In the region of physiological temperatures, the effect of the latter on the stability of the protein is opposite, which apparently has a deep biological meaning. Since this smoothed the effect of temperature changes on the state of the protein molecule.

5. Conclusion

Results of this study were important and useful in determining parameters of inulinase enzyme. Based on the shape of the curves of the dependence of the catalytic activity of Kluyveromyces marxianus inulinase on the time of thermal inactivation in the temperature range of 20-80 ° C. one can make an assumption about the partial destruction of temperature influence on weak electrostatic and possibly on the hydrogen bonds that maintain the conformation of the protein molecule. In the region of physiological temperatures, its effect on the stability of the protein is Opposite, which apparently has a deep biological meaning. Since this smoothed the effect of temperature changes on the state of the protein molecule.

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الجوانب الحركية والحرارية لعملية التثبيط الحراري لأنزيم الانولينيز من KLUYVEROMYCES MARXIANUS

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الملخص

خلفية عن الموضوع : يوجد انزيم الانيولينيز على نطاق واسع في الكائنات الدقيقة والنباتات العليا. إنه يحفز تفاعل التحلل المائي للأنولين الموجود في الدرنات والجذور للعديد من النباتات، إلى الفركتوز وكمية صغيرة من الجلوكوز.الإنولين هو أحد السكريات العديدة ذات الأصل النباتي التي تحتوي على الجلوكوز أو الفركتوز. يتم استخدامه كركيزة في عمليات التخمير الصناعية وفي الصناعات الغذائية نظرًا لمصدره الرخيص نسبيًا والوفير للإنتاج الميكروبيولوجي للعصائر عالية الفركتوز والإيثانول والأسيتون - البيوتانول. كما أن السكريات قليلة التعدد المشتقة من الإنولين تجد تطبيقها في القطاع الطبي والغذائي. يتم إنتاج الانيولينيز من الخمائر K. marxianus بمستويات تقترب من المنفعة التجارية. هذا يدل على امكانية استخدام الانيولينيز في إنتاج شراب الفركتوز والفركتوز. لذا فإن هدفنا الرئيسي هو دراسة تأثير درجات الحرارة المختلفة على تشكيل الجزيء الكبير Kluyveromyces marxianus inulinase ، لتوفير الظروف المثلى لتحلل الأنيلين بواسطة الانيولينيز.

المواد وطريقة العمل :طريقة عزل وتنقية إنزيم inulinase من Kluyveromyces marxianus وكذلك تحديد محتوى البروتين ونشاط الإنزيم كما هو مذكور في سياق البحث.أما التجارب التي اجريت لدراسة الثبات الحراري للإنزيم, اذ تم تحضين محلول الانزيم بتركيز $5 \cdot 10^{-5}$ مول / لتر في فترة زمنية تتراوح من 10- 60 دقيقة عند درجات حرارة مختلفة ، متبوعًا بتحديد النشاط الحفزي وللحصول على التحليل الأكثر اكتمالا لآلية التعطيل الحراري لجزيئات الإنزيم كان لابد من الضروري معرفة المعلمات الديناميكية الحرارية و الحركية لهذه العملية.

من المتعارف عليه أن أنواعًا مختلفة من الروابط والتفاعلات تشارك في تكوين التركيب الجزيئي للأنزيمات وهي الاواصرالتساهمية , الاواصر الهيدروجينية ، الجسور الملحية ، التفاعلات الكارهة للماء,لذلك فإن هدفنا الرئيسي هو دراسة تأثير درجات الحرارة المختلفة على تشكيل جزيئة الانزيم من اجل توفير الظروف المثلى للتحلل المائي الأنيلين بواسطة الانزيم.

النتائج: تم الكشف عن أن النشاط المتبقي للإنزيم بعد 60 دقيقة من الحضانة عند 50 درجة مئوية كان 10٪ من النشاط الأولي عند 60 درجة مئوية 7٪. اما معدل ثابت التثبيط الحراري عند درجة 70 مئوي 3.45 .

الاستنتاجات :النتائج التي تم الحصول عليها مهمة ومفيدة في تحديد المعاملات الحركية والحرارية لإنزيم الانيولينيز. بناءً على شكل منحنيات اعتماد النشاط التحفيزي للانزيم على وقت التعطيل الحراري في نطاق درجة حرارة 80 -20 درجة مئوية .

الكلمات المفتاحية : الانيولينيز ، التثبيط الحراري ، المعاملات الحركية.