IRAQI JOURNAL OF APPLIED PHYSICS
Vol. 22, No. 3, July-September 2026, pp. 453-456

Thermodynamic and Physical Analysis of the Multi-Stage Thermal Inactivation Processes of *Kluyveromyces marxianus* Inulinase

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The thermal inactivation behavior of free inulinase derived from *yeast Kluyveromyces marxianus* was examined using differential thermal analysis (DTA) over the temperature range of 30-80 °C. The thermograms revealed two distinct endothermic transitions at 54 °C and 65 °C, indicating a multistage denaturation process. The initial transition corresponds to reversible local conformational rearrangements, whereas the subsequent transition is associated with irreversible unfolding and loss of catalytic activity. The observed thermal behavior is attributed to an increase in the heat capacity of the denatured protein, resulting from enhanced exposure of hydrophobic side chains to the solvent. At the optimal temperature, the enzyme adopts a conformation that is both energetically and sterically favorable for efficient inulin hydrolysis. These findings provide insight into the thermodynamic nature of enzyme stability and inactivation, which is relevant for optimizing enzymatic processes and guiding strategies for enzyme reuse or immobilization.

Keywords: Differential thermal analysis; Thermal inactivation; Inulinase; *Kluyveromyces marxianus*
Received: 1 December 2025; Revised: 28 January 2026; Accepted: 4 February 2026; Published: 1 July 2026

1. Introduction

Free inulinase is a soluble (non-immobilized) glycoside-hydrolase that hydrolyzes inulin into fructose or fructooligosaccharides by targeting the β-2,1 bond [1]. This reaction occurs under milder conditions than acid hydrolysis, which requires a high concentration of hydrogen ions, high temperature, and the use of special acid-resistant equipment [2]. Two forms of inulinase have been identified, each with distinct properties. Firstly, exo-inulinase is capable of removing terminal fructose units, producing fructose as the primary product. Secondly, endo-inulinase can break internal bonds to generate fructooligosaccharides [3,4]. The use of inulinase for the hydrolysis of inulin-containing raw materials enables a single-step process capable of producing high-fructose syrup with fructose content ranging from 94 to 95% [5]. Consequently, converting inulin-rich biomass represents a promising alternative route for fructose production.

Free inulinase is primarily produced by various microorganisms, including yeast (e.g., *Kluyveromyces marxianus*), fungi (e.g., *Aspergillus sp.*), and bacteria (such as *Staphylococcus sp.*, *Xanthomonas sp.*, and *Pseudomonas sp.*) [6,7]. However, *Kluyveromyces marxianus* strains show promising potential for producing relatively thermostable inulinase using low-cost substrates and achieving high inulinase production rates [8]. Meanwhile, inulinase meets the requirements for Generally Recognized as Safe (GRAS) classification. Together, these properties of inulinase open new avenues for its application in pharmaceutical and industrial applications [9].

Although an increase in temperature promotes the initial enzyme activity as a result of increased kinetic energy, exceeding the optimal limit causes breaking internal bonds and denaturing the enzyme. Thermal enzyme denaturation is a process in which high heat disrupts the enzyme's three-dimensional structure, causing it to unfold [10]. This in turn leads to the irreversible loss of its specific shape and its catalytic and biological activity [11]. Hence, thermal distortion represents one of the most significant challenges to enzyme stability under operating conditions, in addition to contributing to increased production costs.

From a physicochemical perspective, a detailed understanding of the thermal inactivation mechanisms of inulinase is essential for optimizing its industrial performance. Accordingly, the objective of the present study is to investigate the thermal inactivation behavior and unfolding pathways of free inulinase derived from *Kluyveromyces marxianus*, with particular emphasis on its stability under elevated temperature conditions.

2. Methodology

The inulinase enzyme used in this study was isolated from the yeast *Kluyveromyces marxianus*. Enzyme catalytic activity was determined using a spectrophotometric method based on the quantification of reducing sugars released during the enzymatic hydrolysis of inulin. Fructose formation was specifically detected using the Seliwanoff reaction, which provides selective colorimetric determination of

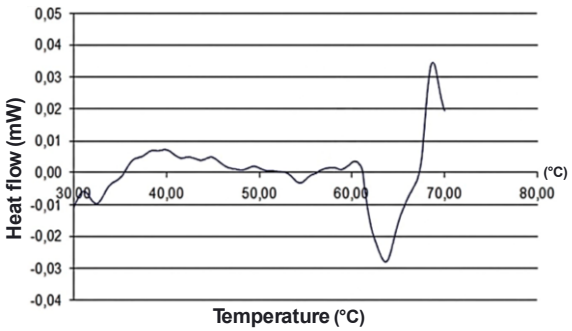
ketose sugars. Detailed procedures for the isolation, purification, and activity measurement of inulinase have been previously described [12,13].

To investigate the thermal inactivation behavior of free inulinase from *Kluyveromyces marxianus*, aqueous enzyme solutions were prepared at a molar concentration of 5×10^{-5} mol/L. Thermal experiments were conducted over the enzyme stability temperature range of 30-80 °C. Controlled heating and cooling cycles were applied at a rate of 1-1.5 °C/min to ensure quasi-equilibrium thermal conditions. Data acquisition was performed with a temporal resolution of 1 second.

Under these experimental conditions, the thermal response of the enzyme was monitored by recording the potential difference generated as a function of time. This potential difference arises from the temperature gradient between the enzyme sample and an inert reference standard, allowing detection of thermally induced structural transitions associated with enzyme unfolding and inactivation.

3. Results and Discussion

To detect the physical and chemical changes of free inulinase, the DTA was applied. The results of the DTA curve showed the presence of two heat-absorbing peaks with different areas (Fig. 1).



Feature	Temperature (°C)	Heat flow (mW)
Baseline start	30	-0.010
First maximum endothermic peak	54	-0.003
Zero-crossing after peak	57	0.000
Deep minimum endothermic peak	65	-0.028
Sharp transition onset	67	-0.005
Major exothermic peak	69	0.034
End of scan	71	0.020

Fig. (1) Differential thermal analysis curve of free inulinase isolated from *Kluyveromyces marxianus*

Based on the shape and area of the DTA curve, it was observed that the temperature corresponding to the minimum point in the first peak was 54 °C. In contrast, the second peak reached 65 °C. The first peak can be attributed to minor positional rearrangements of the protein macromolecule, whereas the second peak corresponds to its melting. These findings are consistent with the temperature characteristic of the minimum melting peak, which is close to the temperature of complete inulinase inactivation [14].

Analysis of literature data allows us to conclude that the denaturation of simple globular proteins is a cooperative translocation accompanied by a simultaneous sharp change in the physicochemical properties of the molecule and a change in a large number of amino acid residues. The increased heat capacity of the denaturing protein is a consequence of the increased contact surface of its hydrophobic side groups with a solvent. Individual enzyme domains with large molecular weights can behave similarly under thermal action, producing significant changes [15]. In context, the process of unfolding the enzyme molecule can be thought of as an intermediate state in the form of a molten globule, in which the unique packing of side groups is not preserved. In a molten globule, they gain freedom of movement and lose the energy of close contact. In this case, localized fluctuations are observed, with the decay of most of the contacts occurring long-distance along the chain [16].

Although figure (1) showed a denaturation temperature range with prominent peaks, which characterized the melting of classical periodic crystals, the transition from a regular globule structure to a chaotic helical structure of the molecule did not occur at a single point. Rather, this transition occurred in finite intervals and without disruption of thermodynamic functions (ΔH, ΔS, etc.). This in turn may explain that the process of unfolding a protein globule consists of several separate stages of conformational transitions [16,17].

According to the results obtained, the following scheme (Fig. 2) illustrates the order-disorder transition mechanism in the inulinase molecule isolated from *Kluyveromyces marxianus*, induced by increasing temperatures.

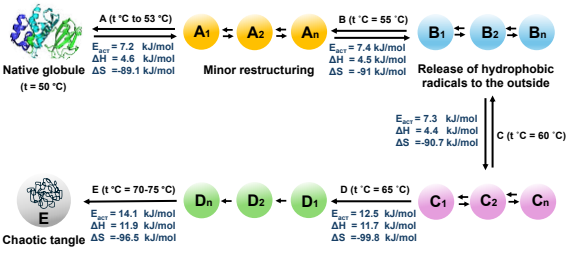


Fig. (2) Scheme of the order-disorder transition during thermal denaturation of the inulinase molecule isolated from *Kluyveromyces marxianus.*

When the optimal temperature of 50 °C is reached, the protein globule, in which the enzyme exhibits maximum catalytic activity, assumes a conformation favorable in terms of energy and steric factors for the inulin hydrolysis reaction. This means that intramolecular interactions in the enzyme are sharply weakened, and the mobility of the side chain increases

significantly. Most of the internal degrees of freedom in an enzyme molecule are associated with small-scale structural fluctuations. First of all is the movement of side groups, the release of which can lead to the enzyme's most thermodynamically stable state, known as state A.

An increase in temperature is accompanied by minor positional rearrangements of the molecular structure, and the thermodynamic parameters change slightly compared to those of state A. A 5 °C increase in temperature led to a rise in activation energy (E_{act}) of 7.4 kJ/mol, heat changes (ΔH) of 4.5 kJ/mol, and entropy (ΔS) of -91 kJ/mol. This behavior may be attributed to the breaking of a large number of hydrogen bonds and hydrophobic interactions within the protein molecule. The resulting configuration can be termed intermediate state B, which corresponds to the first endothermic peak on the DTA curve. The transition process A → B is reversible and consists of a large number of intermediate states (A_1, A_2, A_3, … A_n). The disruption of hydrogen bonds and hydrophobic interactions inherent in the native protein structure is accompanied by the formation of a new, mobile network of variable and multiple bonds [18].

The intermediate state C corresponded to the maximum between the endothermic peaks on the DTA curve and was characterized by a high degree of organization and structural rigidity. The transition C → A can also be possible, which represents the final stage of the reversible thermal denaturation of the inulinase molecule. The intermediate state C can be described as a molten globule. Its compact structure, ensured by residual hydrophobic interactions that allow free movement of the side chains, is penetrated by solvent molecules. Since the transfer of water molecules into the protein is thermodynamically favorable, it contributes to an increase in the protein's diameter. Furthermore, filling of the internal volumes of the molecule with solvent leads to a gradual reduction in polypeptide chain interactions. In this context, a further increase in temperature (60 °C) can cause the molten globule to swell and transform into a helical conformation.

The intermediate state C of the enzyme was shown at 60°C. The molten inulinase globule did not exist as a single well-defined state, but rather as a series of intermediates. Thus, the native state of inulinase is separated from other intermediate states and differs from the denatured state in the mobility of the polypeptide chains of the protein molecule. The main feature of the thermal denaturation process of a protein globule is the separation of the energy of the most stable (native) structure from the energy states at which the spectrum of the remaining, less energetically favorable enzyme conformations begins. This creates an energy gap between the most energetically expedient structure and its competitors. Moreover, this gap does not arise in a random amino acid sequence, but is generated by the selection of suitable amino acids, allowing the dense packing of the chain into a globule capable of reliably performing physiological functions.

A further increase in temperature was accompanied by destructive changes in the inulinase molecule, affecting its secondary structure. The protein molecule transitioned into the intermediate state D and lost its catalytic activity. This configuration corresponded to the second endothermic peak on the DTA curve, and was observed at 65 °C. It was also noted that there was a nonuniform increase in entropy during the C–D transition. This can be explained by the presence of a characteristic density barrier of the globule [19], where the value of ΔS sharply changed by -99.8 kJ/mol. As a result, the molten globule passes through a maximum point that separates it from the loosely denatured state [20,21].

At a temperature of 75 °C, the completion of the order–disorder transition was observed. At this stage, irreversible denaturation leads to an increase in the viscosity of the enzyme solution, since water is a poor solvent for the unfolded polypeptide chain. In addition, hydrogen bonds within the protein's peptide groups break, and new interactions form between the peptide bonds and water. The helical structure is characterized by rearranged hydrogen bonds, which provide high mobility to fragments of the polypeptide chain. The configuration of the unfolded protein macromolecule was identified as intermediate state E [22].

In the intermediate state E, the DTA curve revealed irreversible thermal denaturation of the inulinase enzyme upon cooling from 80 °C to 20 °C. Instead of the expected exothermic reaction peaks indicative of self-assembly, a monotonic change in the heat content of the system was noted. Furthermore, the studied enzyme preparations showed no catalytic activity after cooling. These results are consistent with structural thermodynamic models and with the findings of a published study [23]. It should be indicated that hydrogen bond breaking and hydration lead to a transition from the compact state to the fully denatured state.

4. Conclusion

The study revealed that the enzyme degradation process occurs in two stages. The first, at 54 °C, involves a slight structural rearrangement and is reversible due to the presence of hydrophobic residues. The second stage occurs at 65 °C and corresponds to complete enzyme degradation. This degradation is irreversible, even after cooling, as the enzyme cannot regain its activity. The study also identified an intermediate state at 55 °C prior to complete degradation, in which the enzyme partially retains its molecular structure. These findings provide insight into the thermal stability of the enzyme during use and may aid in its reuse and the application of immobilization

methods, thereby improving performance and reducing production costs.

Acknowledgments

The authors would like to acknowledge Al-Bayan University and Al-Iraqia University for providing research facilities and scientific support.

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