



Differential scanning calorimetry (DSC) as a tool for studying thermal properties of a crude cellulase cocktail

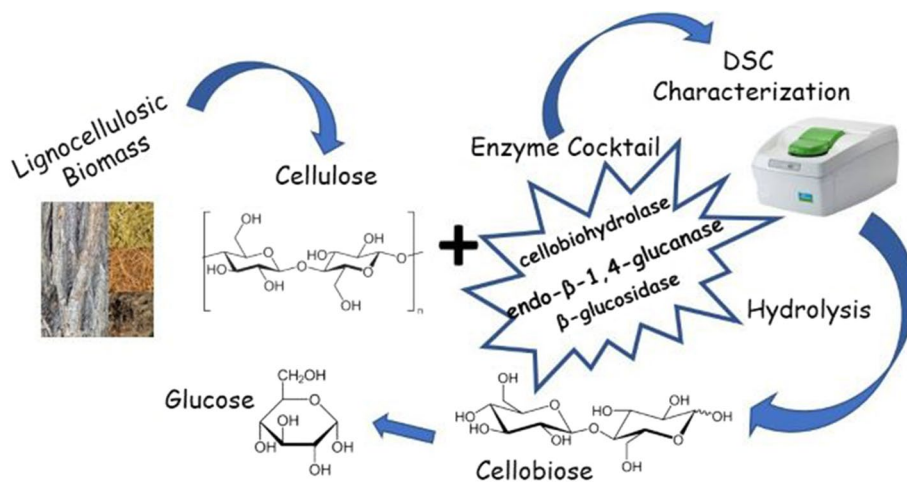
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Abstract

Differential scanning calorimetry (DSC) was used as an efficient and rapid tool in studying the conformational transitions between the folded and unfolded structures of cellulolytic enzymes. The thermal properties of two crude hydrolytic enzyme cocktails containing extracellular cellulases from *Trichoderma longibrachiatum* DIBAF-10 were analyzed and compared with three commercial cellulase preparations. Differences in the thermal behavior of fungal cellulases in the liquid phase, freeze-dried state, liquid formulations in sodium citrate buffer (pH 4.8), and contact with cellulose, carboxymethyl cellulose, and cellobiose were evaluated. DSC profiles of cellulases from the DIBAF-10 strain provided important thermodynamic information about the thermal stability of the included proteins. Crude enzyme cocktails underwent a reproducible and irreversible exothermic aggregation phenomenon at 52.45 ± 0.90 °C like commercial β -glucosidase. Freeze-dried and resuspended in a sodium citrate buffer, cellulases from *T. longibrachiatum* showed an endothermic peak dependent on buffer and enzyme concentration. In the enzyme-substrates systems, a shift of the same peak was recorded for all substrates tested. The thermal analysis of freeze-dried cellulase samples in the range of 20–150 °C gave information on the denaturation process. In conclusion, we demonstrated that DSC is a cost-effective tool for obtaining "conformational fingerprinting" of crude fungal cellulase preparations.

Graphical abstract



Keywords Differential scanning calorimetry · Cellulase activity · Cellulase stability · *Trichoderma longibrachiatum* · Thermal stability

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Introduction

The thermodynamic properties of proteins are important parameters that reveal information regarding their structure and function. The stability of the protein tertiary structure is essential to understanding their physiological functions related to amino acid sequence, depending on the thermodynamic environment. Therefore, the thermodynamic parameters of protein denaturation as a function of temperature are important to elucidate the mechanisms of protein folding and stabilization (Matsurua et al. 2015). Usually, the protein aggregation process co-occurs with their irreversible thermal denaturation and conformational unfolding. It is accompanied by an exothermal effect that results in the formation of a precipitate (Guzzi et al. 2008). For this reason, studying thermal properties is essential to better characterize and understand the interactions between enzymes, their substrates, and their temperature dependence.

Among the different techniques available, differential scanning calorimetry (DSC) is a powerful tool that can be used directly to investigate the thermodynamic parameters which characterize proteins. In addition, DSC has been identified as the primary technique for studying the thermal stability of proteins (Haifeng et al. 2008; Rialdi et al. 1995; La Rosa et al. 2005; Bugyi et al. 2005; Michnik et al. 2005, 2007). During the unfolding process, solvent molecules around the exposed non-polar side chains of protein reorganize their structures and cause heat capacity changes that DSC can measure. Such data can be used to understand how aggregates can be formed from non-native protein conformations. In depth, the transition between the folded and unfolded structure is recorded as an endothermic peak at the transition temperature. At this point, DSC measures the excess heat capacity of protein solution that occurs at the transition temperature, and the area under the transition peak (ΔH_m) correlates with the molecules' content with ordered secondary structure. These data are widely used to measure protein stability (Cooper 1999; Neudecker et al. 2012). However, DSC can provide temperature stability studies of proteins only if performed under conditions of reversibility (Freire 1995; Freire et al. 1978; Privalov 1979). To carry out thermal stability experiments, buffers and pH values are away from physiological conditions to reach the folding/unfolding reversibility of the protein (Nidetzky et al. 1994; Kumar et al. 2016; Liu et al. 2016).

The conversion of cellulose to soluble sugars by enzymatic hydrolysis is a crucial step in producing biofuels from lignocellulosic biomass (Yang et al. 2011). The thermo-stability of the involved enzyme is essential during saccharification reactions because steam is always used to make the substrates more suitable for enzymatic hydrolysis (Olajuyigbe et al. 2016).

In the natural world, cellulases, a complex group of enzymes, play a central role in the degrading process of insoluble cellulose to soluble sugars. These enzymes can be used in various industrial fields, including biomass refining, textile, laundry, food, agriculture, and paper.

Fungi, bacteria, and actinomycetes produce a complex enzymatic mix of cellulases in free and/or cell surface-bound form and in various compositions, different from each other.

In the commercial market, cellulases are mainly produced by filamentous fungi, such as *Trichoderma reesei* (Nidetzky et al. 1994), as a multienzyme extracellular complex that includes endo- β -1,4-glucanase [EC 3.2.1.4], cellobiohydrolase [EC 3.2.1.91] and β -glucosidase [EC 3.2.1.21] (Yeoman et al. 2010). Since thermo-chemical pretreatments, like a steam explosion or acidic/alkaline processing, are an important step in converting lignocellulosic substrates into fermentable sugars (Galbe and Zacchi 2007), enzymes involved in lignocellulose hydrolysis should operate at high temperatures for prolonged periods and withstand the extreme condition of biorefinery processes. For this reason, cellulases with high thermal stability or optimum activity at elevated temperatures have gained increasing attention (Luziatelli et al. 2014; Haki et al. 2003; Jensen et al. 2018).

Trichoderma longibrachiatum DIBAF-10, a strain isolated from poplar wood chips for its ability to secrete a cocktail of hydrolytic enzymes active on different lignocellulosic substrates, has been characterized for its ability to produce a cellulase cocktail which showed maximum activity at 60 °C. In contrast, the optimal temperature of activity of *Trichoderma* cellulases is usually 40–50 °C.

The T_m of a protein in different buffers and at various enzymatic activity concentrations can determine the formulation that offers greater stability to the protein (Durowoju et al. 2017). In this work, the thermal properties of the crude enzymes from *T. longibrachiatum* DIBAF-10 strain were investigated by DSC in liquid form, freeze-dried, and resuspended in sodium citrate buffer after the freeze-drying process. The enzyme activity was measured by colorimetric assay. Data were compared with a commercial cellulase preparation to verify if DSC can be used as a rapid tool to identify the molecular structure of crude cellulase preparations from DIBAF-10. Moreover, we studied the dependence of denaturation temperature T_m of crude cellulase in relation to the concentration of the sodium citrate buffer and the enzyme concentration.

Developing an efficient enzymatic preparation for converting cellulosic biomass to sugars depends on the knowledge of the interactions of cellulase component enzymes acting on specific substrates. To obtain important information for understanding cellulases kinetics, we observed the thermal behavior of cellulases on different substrates such as cellulose, carboxymethyl cellulose, and cellobiose. In this case, the DSC techniques can be applied to follow up on the

conformational variations that may occur in the enzyme-substrates complex during the cellulose hydrolysis reaction.

Experimental section

Materials

All the chemicals were of analytical grade, purchased from Sigma-Aldrich, and used as received. In detail, the commercial enzyme cocktails used are Accelerase® 1500 from DuPont, β -glucosidases, and Cellulase mix from Novozyme. All enzymes were stored at 278 K until use. The sodium citrate buffer at pH 4.8 was prepared using bidistilled water at different concentrations (15, 30, and 50 mM).

Cellulase production

For cellulase production, *T. longibrachiatum* DIBAF-10 strain was grown in 500 mL Erlenmeyer flasks containing 250 mL of followed production medium (g L^{-1}): NaNO_3 , 3; KCl, 0.5; KH_2PO_4 , 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; CaCl_2 , 0.1; yeast extract, 5; Avicel PH-101, 5. The initial pH of the media was adjusted to pH 7.0 before sterilization (394 K for 15 min). The cultures were incubated at 30 °C on a rotary shaker (180 rpm). After 12 days of growth, the mycelium was separated from the culture broth by centrifugation (12,000 rpm for 10 min), and the supernatant was collected for filter paper activity assay and DSC measurements. Part of the crude enzyme sample was freeze-dried and used for DSC measurements in dry form or resuspended at 5, 10, and 20 mg mL^{-1} in citrate buffer solution at various concentrations (15, 30, and 50 mM).

Enzymatic assay

Filter paper activity (FPase) for total cellulase activity was determined using Whatman filter paper no.1 (1 \times 6 cm strip, 50 mg) as substrate according to the methods described by Ghose (1987). One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 μmol of reducing-sugar equivalents per mL per min.

DSC measurements

Measurements were carried out with a Perkin-Elmer DSC Pyris 8500 equipped with a cooling module. Purified nitrogen was the purge gas (20 mL min^{-1}). The temperature and energy calibrations were performed using a pure indium standard. The baseline was obtained with an empty hermetically sealed stainless-steel pan for freeze-dried samples or filled with buffer for liquid samples before each measurement. Before calorimetric measurement, cellulase

in lyophilized form was dissolved in various concentrations of sodium citrate buffer, as described above. Liquid samples of crude and commercial enzyme (20 \pm 1 mg) were weighed into stainless steel pan and then hermetically sealed. The enzyme concentration used in the experiments was 5, 10, and 20 mg mL^{-1} . The buffer solution was used as a reference. Preliminary tests were performed by heating the samples, from 25 to 85 °C, at 1, 2, and 5 $^\circ\text{C min}^{-1}$ to define the proper test conditions. All the samples were then heated, from 25 to 85 °C, at 2 $^\circ\text{C min}^{-1}$. The heating rate was 5, 7, and 10 $^\circ\text{C min}^{-1}$ in the 25–150 °C. For the crude enzyme in dry form, 4 \pm 1 mg was examined, and an empty pan was used as a reference.

Measurements of cellulase in contact with different substrates were carried out using 0.5 mL of the enzyme solution at 10 mg mL^{-1} in citrate buffer 50 mM and 0.5 mL of substrates solutions at the following concentration: cellobiose 15 mM, CMC, and Avicel 2% in citrate buffer 50 mM. After mixing, an aliquot of 20 \pm 1 mg of the reaction solution was weighed into stainless steel pan and then hermetically sealed. The heating rate was 2 $^\circ\text{C min}^{-1}$ in the range of 25–80 °C. The citrate buffer pH 4.80 and the substrate solutions in citrate buffer pH 4.8 without the enzyme were used as a reference for the sole enzyme and the substrates-enzyme curves, respectively.

The tests were carried out in triplicate for each enzyme mix. Data processing was performed with PYRYS software.

Results and discussion

During the growth on a medium containing Avicel (microcrystalline cellulose) as the sole carbon source, *T. longibrachiatum* DIBAF-10 strain produced cellulase-degrading enzymes in secreted form, with total cellulase activity of 0.19 \pm 0.004 UI mL^{-1} , measured by FPase enzymatic assay. The thermal behavior of crude cellulase produced by this microorganism was monitored in relation to the commercial cellulase preparations.

The DSC curves of crude enzyme cocktail (Fig. 1), commercial β -glucosidase (Fig. 2), and Accelerase® 1500 from Du Pont and Cellulase mix from Novozymes (Fig. 3) showed similar exothermic peaks and comparable ΔH values (Table 1). This is due to the same conformational change leading to the aggregation of proteins and their subsequent precipitation. As reported in the literature (Privalov et al. 1974; Murray et al. 1985; Farkas et al. 1996), the presence of exothermic peaks in the DSC curves of crude cellulases is explained by the fact that the transition is a net value due to from combination of endothermic contributions, such as the disruption of hydrogen bonds and denaturation, and exothermic ones, such as the break-up of hydrophobic interactions that causes aggregation. In the case of the crude sample

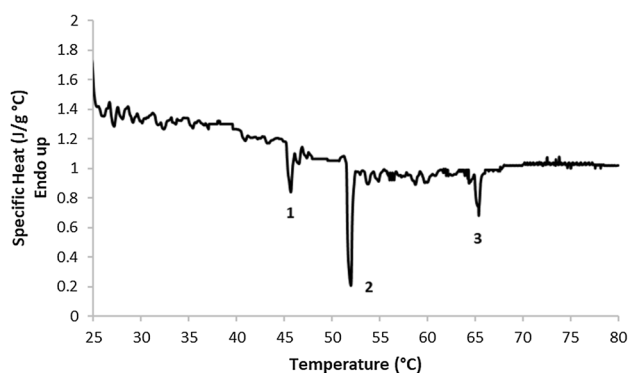


Fig. 1 DSC profile of crude cellulase preparation (scan rate: $2\text{ }^{\circ}\text{C min}^{-1}$). The values of temperatures 1, 2, and 3 are reported in Table 1

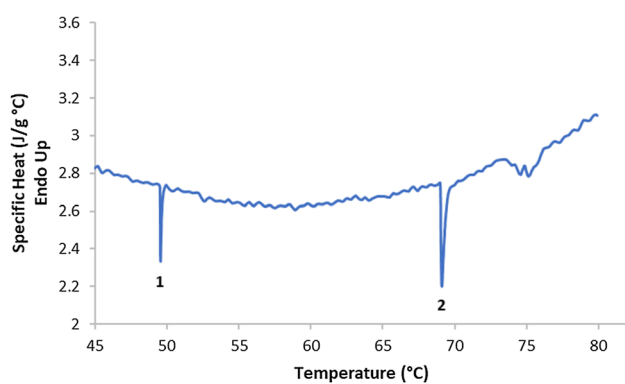


Fig. 2 DSC profiles of commercial β -glucosidase (scan rate: $2\text{ }^{\circ}\text{C min}^{-1}$). The values of temperatures 1 and 2 are reported in Table 1

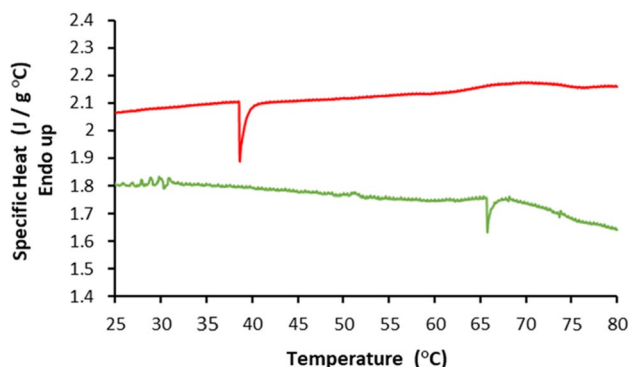


Fig. 3 DSC profiles of commercial Cellulase mix from Novozymes (red line) and Accelerase® 1500 from Du Pont (green line); scan rate: $2\text{ }^{\circ}\text{C min}^{-1}$

thermally analyzed, the signature of protein unfolding was probably altered by aggregate formation. The presence of different peaks suggested that more conformational transitions of proteins are involved in both the crude enzyme

Table 1 Peak temperatures and ΔH values measured for crude cellulase sample and commercial β -glucosidase, Accelerase, and Novozymes

	Temperature ($^{\circ}\text{C}$)	ΔH (mJ g^{-1})
Crude cellulase sample-peak 1	45.68 ± 0.90	-191.15 ± 0.41
Crude cellulase sample-peak 2	51.95 ± 0.50	-446.10 ± 0.76
Crude cellulase sample-peak 3	65.25 ± 0.17	-119.25 ± 1.87
Commercial β -glucosidase-peak 1	49.55 ± 0.49	-534.31 ± 0.56
Commercial β -glucosidase-peak 2	69.09 ± 0.21	-153.41 ± 0.14
Accelerase® 1500	65.77 ± 0.86	-114.12 ± 1.31
Cellulase mix Novozymes	38.65 ± 0.66	-101.10 ± 0.47

Values are means \pm standard error for three determinations

and commercial preparations. As reported in the literature (Rosales-Calderon et al. 2014), β -glucosidase undergoes an irreversible structural transformation from its native conformation to a denatured form from 50 to 60 $^{\circ}\text{C}$. The β -glucosidase in the crude cellulase preparation may suffer a similar conformation change.

Accelerase 1500 from Du Pont contains endoglucanases, exoglucanases, and high levels of β -glucosidase, as reported from the technical sheet; it enables a complete conversion of cellobiose to glucose. In Fig. 3, the peak at $65.77 \pm 0.86\text{ }^{\circ}\text{C}$ was well-defined and near the second peak observed in the β -glucosidase sample. In the same figure, the thermogram of Cellulase mix from Novozymes showed a peak at $38.65 \pm 0.66\text{ }^{\circ}\text{C}$.

The thermograms of commercial enzymatic preparations had a more regular signal than the crude enzymatic cocktail. This is due to the high complexity of the untreated sample.

When aggregation is present, analyzing thermostability in equilibrium thermodynamics is not applicable. A solvent modification, the addition of polar co-solvents, or a change in the pH or ionic strength might enhance or usefully modify the enzyme activity by affecting the equilibrium thermodynamics, the uncatalyzed reaction kinetics, or the enzyme specificity (Harris et al. 2014). For this reason, DSC measurements were carried out by changing the experimental conditions. Part of the enzyme cocktail was freeze-dried and thermally analyzed in dry form and resuspended in sodium citrate buffer at various concentrations and pH 4.8. The choice of the buffer was made according to the enzymatic assay procedure for the filter paper assay.

Regarding the freeze-dried enzyme samples, the DSC curves, obtained at a different heating rate, showed various endothermic transitions at a temperature higher than 65 $^{\circ}\text{C}$, as reported in Fig. 4. A denaturation peak with the same shape was present in all the curves at a temperature of about 118 $^{\circ}\text{C}$. The results indicated that in the lyophilized form, cellulases complex from *T. longibrachiatum* DIBAF-10 was

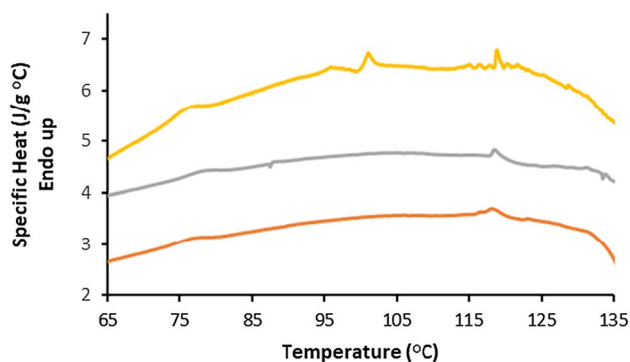


Fig. 4 DSC heating curves of freeze-dried cellulase at a different heating rate: 5 °C min⁻¹, yellow line; 7 °C min⁻¹, grey line; and 10 °C min⁻¹, orange line

more stable than in liquid form and denatured at a higher temperature. Table 2 reported the ΔH values related to the denaturation of the freeze-dried cellulases obtained at different scanning rates. DSC experiments at three different scan rates assess that the denaturation of freeze-dried cellulase was not under kinetic control because as the scan rate changed, the thermodynamic events and their corresponding energies were similar.

The reversibility of denaturation is usually checked by re-heating the sample: if heat absorption is not observed for the preheated sample, the process is considered irreversible. Moreover, the process is under kinetic control, and if the DSC profiles are also protein concentration-dependent, the kinetic mechanism includes a bimolecular step (Lyubarev et al. 2000).

DSC measurements were carried out at different protein concentrations to verify this hypothesis.

Figure 5 shows the DSC curves of freeze-dried enzymatic preparations resuspended at a concentration of 5, 10, and 20 mg mL⁻¹ in 15 mM sodium citrate buffer.

In the thermogram at 5 mg mL⁻¹, with a heating rate of 2 °C min⁻¹, an endothermic peak was recorded at 57.00 °C, corresponding to the unfolding of the proteins. The transition occurred over a narrow temperature range, suggesting the highly cooperative transition from

Table 2 Denaturation temperature (T_m) and ΔH of denaturation (ΔH_m) were measured for freeze-dried cellulase at different heating rates

	T_m (°C)	ΔH_m (mJ g ⁻¹)
Heating rate (°C min ⁻¹)		
5	118.80 ± 0.01	231.20 ± 0.10
7	118.49 ± 0.03	310.12 ± 0.11
10	118.21 ± 0.04	385.10 ± 0.14

Values are means ± standard error for three determinations

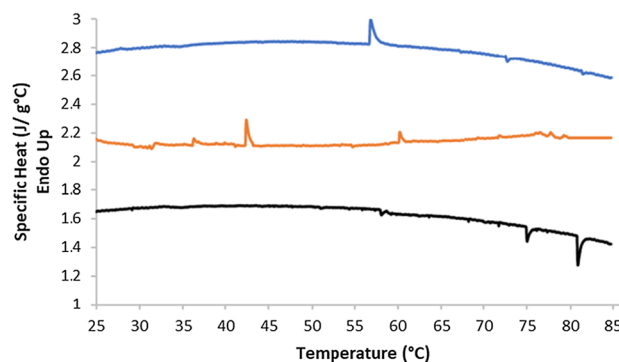


Fig. 5 DSC heating curves of freeze-dried cellulase resuspended in citrate buffer 15 mM and pH 4.8, at different concentrations: 5 mg mL⁻¹, blue line; 10 mg mL⁻¹, orange line; 20 mg mL⁻¹, black line. Heating rate 2 °C min⁻¹

native to denatured state (Lyubarev et al. 2000), and it depended on the heating rate. In particular, at 1 °C min⁻¹, the denaturation temperature was shifted to 48.52 °C, and at 5 °C min⁻¹, a glass transition temperature was observed at 63.20 °C. The first peak was followed by two exothermic and minor events at 72 °C and 81 °C, indicating the aggregation phenomenon. Comparative analysis of the DSC profiles after successive heating and cooling cycles (from 25 to 85 °C and then to 25 °C) suggested that the transition was not reversible.

The variation of the denaturation temperature with the protein concentration indicated intermolecular cooperativity. Moreover, the denaturation temperature value was affected by the protein concentration in the range of 5–20 mg mL⁻¹. Three endothermic peaks (36.38, 42.54, and 60.32 °C) were observed at 10 mg mL⁻¹, while only exothermic peaks were present at 20 mg mL⁻¹.

The increase of cellulase concentration in the aqueous solution caused changes in the protein–protein interactions and lead to the formation of aggregates followed by precipitation. This process is exothermic and at high enzyme concentration prevailed on the endothermic denaturation.

To analyze the effect of buffer solution, DSC tests on crude cellulase (5 mg mL⁻¹) in 15, 30, and 50 mM sodium citrate buffer, pH 4.8, were carried out. Curves in Fig. 6 indicated that the denaturation temperature values decreased with the increasing buffer concentration: the sodium citrate accelerated the unfolding process of the cellulases.

A set of tests was carried out to evaluate the effect of enzyme–substrate interactions.

The complex enzyme–substrate promotes the hydrolysis reaction and reduces activation energy. The enzyme brings substrates together to obtain an optimal orientation and can cause conformational changes in the substrate molecular structures detectable by calorimetric analysis.

Fig. 6 DSC profiles of crude cellulase (5 mg mL^{-1}): thermal denaturation in 15 (solid line), 30 (point-dotted line), 50 mM (dotted line) sodium citrate buffer, pH 4.8. The scan rate was $2 \text{ }^\circ\text{C min}^{-1}$

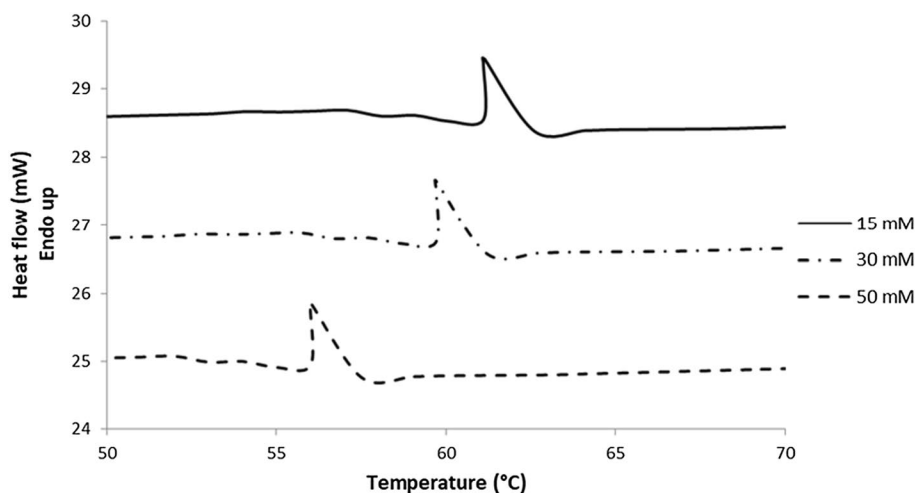


Figure 7 reports the DSC curves of the crude cellulase mix in the presence of microcrystalline cellulose, carboxymethyl cellulose, and cellobiose.

In the case of microcrystalline cellulose and carboxymethyl cellulose, there were different endothermic peaks in the same temperature range as those recorded for crude enzyme suspension. When the enzyme was in contact with a substrate, the first peak at $36.38 \text{ }^\circ\text{C}$ shifted to a lower temperature; otherwise, the second, at $42.54 \text{ }^\circ\text{C}$, and the third, at $60.32 \text{ }^\circ\text{C}$, moved to higher temperatures. In the presence of cellobiose, the structural unit of cellulose molecules, and the main product in enzymatic hydrolysis of cellulose, there was one exothermic peak at $65.92 \text{ }^\circ\text{C}$, corresponding

to the β -glucosidase precipitation. The other peaks disappeared probably because the cellobiose strongly inhibited the endoglucanase activity and influenced the unfolding process (Zhao et al. 2004).

Conclusions

In this work, the crude cellulase from *T. longibrachiatum* DIBAF-10 was studied by DSC. Our results demonstrated that a similar conformational change occurs in both crude and commercial cellulase cocktails and provided important thermodynamic information about the denaturation and the

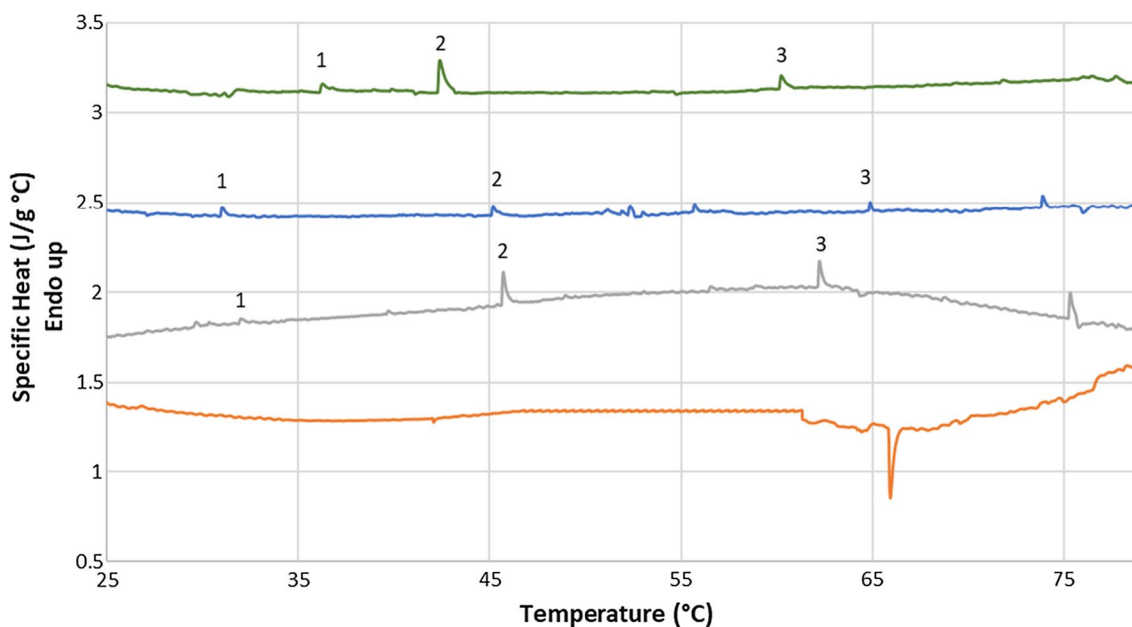


Fig. 7 DSC thermograms of the crude enzyme preparation in citrate buffer solution at 10 mg mL^{-1} (green line) and the presence of different substrates: microcrystalline cellulose (blue line), carboxymethyl cellulose (grey line), and cellobiose (orange line); heating rate $2 \text{ }^\circ\text{C min}^{-1}$

unfolding of these hydrolytic enzymes. DSC was a valuable tool for obtaining information on the structural conformation of the crude enzyme cellulase preparations from *T. longibrachiatum* DIBAF-10 without a purification step and using freeze-dried samples. This technique enables to monitor the thermal behavior that depends on the structural changes of the enzyme with or without the presence of a substrate.

The data obtained could be used as a preliminary study for further and complementary analysis to develop a procedure to monitor key production steps of fungal cellulases by DSC.

Author contributions All authors contributed to the study's conception and design. All authors read and approved the final manuscript.

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Data availability Data are available on request from the corresponding author.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethics approval This study does not involve any human or animal testing.

Consent to participate All authors have approved the manuscript and agree with its submission to *Applied Biochemistry and Biotechnology Journal*.

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