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Homa Torabizadeh, Mehran Habibi-Rezaei, Mohammad Safari, Ali Akbar Moosavi-Movahedi, Ahmad Sharifizadeh, Homa Azizian & Massoud Amanlou

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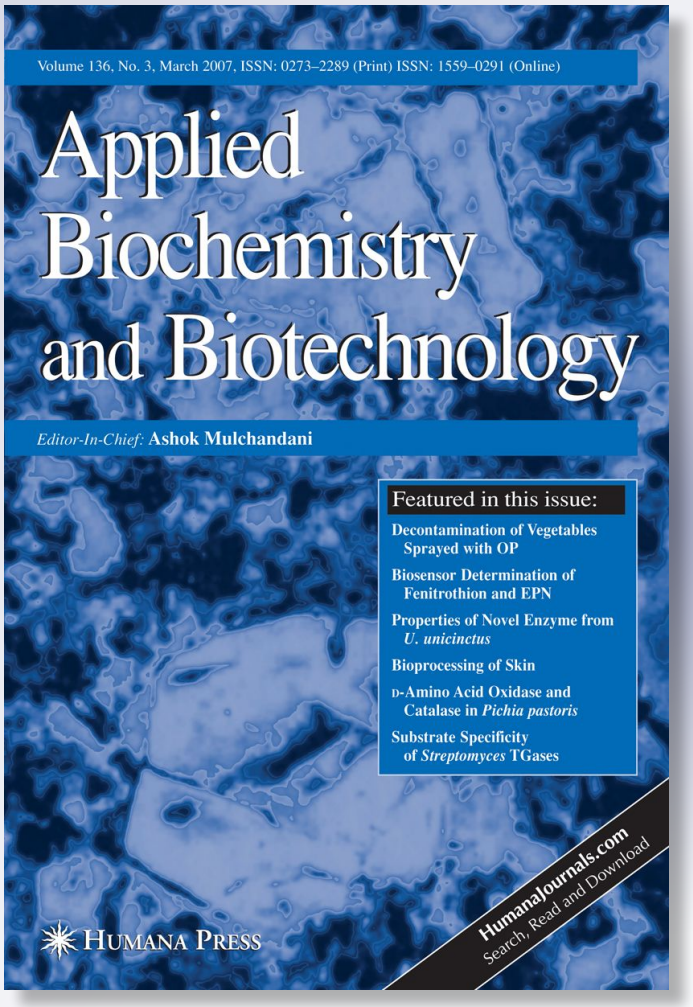

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Endo-inulinase Stabilization by Pyridoxal Phosphate Modification: A Kinetics, Thermodynamics, and Simulation Approach

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Abstract The structural and storage and functional thermostabilization of endo-inulinase (EC 3.2.1.7) through semi-rational modification of surface accessible lysine residues by pyridoxal-5′-phosphate (PLP) and ascorbate reduction have been explored. Improved stability was observed on modifications in the absence or presence of inulin, which indicates storage or functional thermostabilization, respectively. Comparisons have been made between non-modified and modified enzyme by the determination of *T*m as an indicator of structural stability, temperature-dependent half-lives (*t*1/2), energy barrier of the inactivation process, and thermodynamic parameters (ΔH^*, ΔG^*, and ΔS^*) in a storage thermostability approach. These parameters coincided well with the observed stabilization of the engineered enzyme. Moreover, relative activities with sucrose and inulin were determined for non-modified and modified endo-inulinases at different temperatures. A comparison of the sucrose-to-inulin ratios of the initial rate of hydrolysis as an indicator of substrate specificity revealed about twofold improvement in inulinase versus sucrose activity by enzyme modification. Molecular dynamics simulations and molecular docking approaches were employed to explain the observed structural and functional thermostabilization of endo-inulinase upon modification. We hypothesize the establishment of

H. Torabizadeh · M. Safari
Department of Food Science and Engineering, Faculty of Biosystem Engineering, University of Tehran, Tehran, Iran

H. Torabizadeh
e-mail: htoraby@ut.ac.ir

M. Habibi-Rezaei (✉)
School of Biology, College of Science, University of Tehran, Tehran, Iran
e-mail: mhabibi@khayam.ut.ac.ir

A. A. Moosavi-Movahedi · A. Sharifizadeh
Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran

H. Azizian · M. Amanlou
Department of Medicinal Chemistry, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

intramolecular interactions between the covalently attached PLP–Lys381 and Arg526 and Ser376 residues as a representative of modification-originated intramolecular contacts in the modified enzyme.

Keywords Endo-inulinase · Pyridoxal 5′-phosphate · Molecular dynamics · Chemical modification · Thermostability

Abbreviations

PLP	Pyridoxal-5′-phosphate
T_m	Transition midpoint temperature
$t_{1/2}$	Half-life
$Ea_{(in)}$	Activation energy of denaturation/inactivation
ΔH^*	Enthalpy of inactivation
ΔG^*	Gibbs free energy of inactivation
ΔS^*	Entropy of inactivation
HFS	High-fructose syrup
ASA	Accessible surface area (in angstrom squared)
DNS	Dinitrosalicylic acid
DSC	Differential scanning calorimetry
MD	Molecular dynamics
SPC	Simple point charges
RMSD	Root mean square deviation

Introduction

Inulinases are comprised of endo-inulinase (EC 3.2.1.7) and exo-inulinase (EC 3.2.1.80). Inulinases belong to the glycoside hydrolase family GH32 [1, 2] based on amino acid sequence comparisons and the presence of conserved amino acid domains. They act as β-fructan fructanohydrolases and hydrolyze inulin to produce high-fructose syrup (HFS) and fructo-oligosaccharide. These products are important ingredients in the food and pharmaceutical industries. The structure of inulin consists of a linear polyfructan with $\beta 2 \rightarrow 1$ linkages between fructose residues and a terminal sucrose moiety [3]. Inulin is a storage polysaccharide and is accumulated in the underground tubers of many plants, including the Jerusalem artichoke (*Helianthus tuberosus*), chicory (*Cichorium intybus*), dahlia (*Dahlia pinnata*), and dandelion (*Taraxacum officinale*) [4]. Because of solubility limitation and microbial contamination of inulin, the industrial hydrolysis of inulin is carried out at ≥50 °C, which is necessary to obtain an appropriate hydrolysis rate. At this temperature, most of the inulinases lose their activity after a few hours. Therefore, there is a growing interest in introducing inulinases with improved thermostability [5]. The thermal stability of enzymes is an important parameter in enzymatic processes as it determines the limits for use and reuse of the enzyme, and therefore affects the cost. In our previous work, we reported the semi-rational modification of endo-inulinase by using pyridoxal 5′-phosphate (PLP) [6] as a specific modifier of lysine residues [2]. We have reported that lysine residues are more accessible at the surface of the C-terminal domain compared to the N-terminal domain of the inulinase based on the calculated accessible surface areas (ASA) of 123.1 and 74.1 $Å^2$, respectively [6]. Even though, the PLP modifications have brought about enzyme inactivation in several studies [7–16], we reported a case in which not only the enzyme activity was retained but also thermostability was improved. In this reaction, a Schiff base is formed between the ε-amino group of lysine as a nucleophile part and the

aldehyde group of PLP [17–19]. The selectivity of the reaction, the spectral properties of the modified product, and the reversibility of the reaction are among the advantages of this chemical modification strategy [6, 19]. Although, sodium borohydride is routinely used as a Schiff base-reducing agent, a safe and efficient reduction method using ascorbic acid as a novel Schiff base-reducing agent has been used. In this study, the knowledge of modeling, kinetics, and thermodynamics have been brought together to explain the endo-inulinase stabilization process by using PLP and ascorbate as the modification and reduction agents, respectively.

Materials and Methods

Materials

Ascorbic acid was purchased from Acros Organics (Morris Plains, NJ, USA). Endo-inulinase (27 U/mg), inulin (chicory inulin), and pyridoxal 5′-phosphate were obtained from Fluka (Switzerland) and dinitrosalicylic acid (DNS) and other chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA).

PLP Modification of Endo-inulinase

Endo-inulinase modification was performed by using PLP and ascorbate as the modifier and reducing agents, respectively [6]. In brief, endo-inulinase at 0.1 mg ml^{-1} was modified by using PLP at 31.25 μM in 50 mM sodium phosphate buffer (pH 7.5) for at least 30 min then the enzyme–PLP complex was stabilized by reduction with ascorbic acid using 100 μl of freshly prepared solution of ascorbic acid (1 mM) in 50 mM phosphate buffer pH 7.0. To remove the excess ascorbate and PLP, overnight dialysis was performed against 50 mM sodium acetate buffer of pH 6.0 at 4 °C.

Enzyme Assay

Endo-inulinase assay was carried out through the assessment of the librated reducing sugar by using DNS. In brief, the assay mixture (55 μg ml^{-1} enzyme and 0.36 mg ml^{-1} inulin in 50 mM sodium acetate buffer of pH 5.5–6) was incubated at 37 °C for 15 min. The same concentration of sucrose was used for determining invertase activity instead of inulinase activity of the enzyme species. Next, the reaction was terminated by adding an equal volume of DNS reagent followed by incubating at 90 °C for 10 min. The absorbance was measured at 575 nm using a Camspec M550 spectrophotometer in cells with 1 cm path length. One unit inulinase or invertase is defined as the amount of enzyme that liberated 1 μmol fructose (or glucose) per minute under assay conditions [20, 21].

Structural Thermostability Analysis of Endo-inulinase

Comparative structural thermostability analysis of non-modified and modified endo-inulinases was carried out using differential scanning calorimetry (DSC) measurements with a Model 6100 Nano II differential scanning calorimeter (Calorimetry Sciences Corp., USA) at a heating rate of 2 °C min^{-1} between 10 and 85 °C, under an extra constant pressure of 2 atm. The sample protein was used at 2.0 mg ml^{-1} in 50 mM phosphate buffer of pH 7.5, after degassing. The same buffer was used as a reference to perform the baseline

run. For data collection and estimation of T_m values, the standard CpCalc software package and data acquisition program, DSC Run, were used [22].

Storage and Functional Thermostability Analysis of Endo-inulinase

The storage and functional thermostability of the non-modified and modified endo-inulinases was carried out both in the absence and in the presence of inulin as the enzyme substrate respectively with residual activity assessment at four representative temperatures: 25, 45, 50, and 60 °C. For the storage thermostability analysis, both the non-modified and modified enzymes were incubated in the absence of substrate at similar conditions, except at 25, 45, 50, and 60 °C. Samples were withdrawn at appropriate periodic intervals from incubated enzymes and were assayed at room temperature. For the functional thermostability analysis, non-modified and modified species were incubated at 25, 45, 50, and 60 °C in the presence of 0.36 mg ml^{-1} inulin in 50 mM sodium acetate buffer of pH 5.5 for 90 min. The final concentrations of enzyme species were used at 6×10^{-3} μg ml^{-1} to ensure the linearity of the reaction as the function of substrate concentration. Samples were withdrawn at intervals, inactivated for the enzyme activity at 95 °C, and then assayed for liberated reducing sugar.

In continuation, based on the data captured by storage thermostability study, the inactivation rate constants (k_{in}) were determined and the apparent half-lives ($t_{1/2}$) were estimated by using Eq. 1.

$$t_{1/2} = \ln 2/k_{in} \tag{1}$$

Half-life is a unit of time and is defined as the time required for loss of half of the original activity [23, 24]. The data were fitted to first-order plots and were analyzed as described earlier [25]. The thermodynamics of the irreversible thermo-inactivation of endo-inulinase was determined by rearranging the Eyring's absolute rate equation, derived from the transition state theory [26].

$$k_{in} = (k_B T/h)e^{(-\Delta H^*/RT)}.e^{(\Delta S^*/R)} \tag{2}$$

Where, h represents the Planck's constant (6.63×10^{-34} J s), k_B is the Boltzman's constant (1.38×10^{-23} J K^{-1}), R is the gas constant (8.314 J.K^{-1} mol^{-1}), N is the Avogadro number ($6.02\times1{,}023$ mol^{-1}), and T represents the absolute temperature. ΔH^*, ΔG^*, and ΔS^* represent enthalpy, free energy, and entropy of inactivation respectively and are introduced in Eqs. 3–5.

$$\Delta H^* = \mathrm{Ea}_{(in)} - RT \tag{3}$$

$$\Delta G^* = -RT \ln(k_{in}.h)/(k_B.T) \tag{4}$$

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T \tag{5}$$

The energy barriers of thermoinactivation, $\mathrm{Ea}_{(in)}$, pertaining to the non-modified and modified endo-inulinases were estimated by applying the Arrhenius equation (Eq. 6) and plot [27].

$$\ln k_{in} = -\mathrm{Ea}_{(in)}/RT \tag{6}$$

The thermal unfolding processes of endo-inulinase were observed to be irreversible. The irreversible inactivation is generally shown as $N \leftrightarrow U^* \rightarrow I$, where N represents the native

enzyme, *U** is the unfolded inactive enzyme with a tendency to reversibly refold on cooling, and *I* is the inactive enzyme formed after prolonged exposure to heat that cannot be recovered on cooling. It is shown that the proposed model can be considered in one particular case of that proposed by Lumry and Eyring [22]. Under such conditions, the thermodynamic parameters can, therefore, be calculated using $Ea_{(in)}$ [13, 21, 28, 29].

Substrate Specificity and S/I ratio

Sucrose-to-inulin (S/I) ratios were determined for non-modified and modified endo-inulinases at different temperatures. In brief, to determine the invertase activity or inulinase activity of enzyme species, 0.36 mg ml^{-1} of sucrose or inulin in 50 mM sodium acetate buffer pH 5.5–6 was incubated at 25, 45, 50, and 60 °C for 15 min, respectively. Then, reaction was terminated and assessed for liberated reducing sugar as mentioned in the "Enzyme Assay" section.

Molecular Dynamics Simulation

The crystal structure of the native endo-inulinase from *Aspergillus niger* has not yet been elucidated, whereas the crystal structure of exo-inulinase from *Aspergillus awamori* has been reported [2]. To determine the identity and similarity between amino acid sequences of exo-inulinase from *A. awamori* (EC 3.2.1.80, with 537 amino acid residues) and endo-inulinase from *A. niger* (EC 3.2.1.7 with 494 amino acid residues), binary sequence alignment was performed using the FASTA program version 3 (35.04) that is located on the ExPasy server. The results revealed that the sequence identity between the two proteins was 31.6% and the similarity was 62.9–64.5% over aligned residues. Because, the similarity value between endo-inulinases and exo-inulinases was greater than 50%, for exo-inulinase, the three-dimensional crystal structure coordinates were used to determine the molecular dynamics (MD) simulation and docking studies of endo-inulinase [6]. MD simulations were performed using the GROMACS program (version 4.0.3) with the GROMACS force field [30]. The starting geometries for the simulation were prepared from the initial coordinates of protein that were extracted from the modeled structure of inulinase in the protein data bank (PDB, entry 1Y9M). The protein consisted of 537 residues. Modified lysine was created as a new residue in the GROMACS program database. For this purpose, a PLP–lysine parameter was created by the PRODRG server [31], which is used to generate topologies for ligand–protein complexes. The parameters were then transferred to the GROMACS-related libraries. The MD simulation protocol is as follows: The protein was first placed in a simulation cubic box of a suitable size and solvated with a 37,578 simple point charge (SPC) water model and 20 Na^+ counter ions to neutralize the entire negative charge. The water molecules and ions were subjected to energy minimization, while the inulinase was kept fixed in its initial configuration. Subsequently, the water and ions were allowed to evolve using MD simulation for 50 ps with a step time of 1 fs, again keeping the structure of the inulinase fixed. Next, the entire system was minimized using the steepest descent of 1,000 steps followed by conjugate gradients of 9,000 steps. In order to obtain equilibrium geometry at 300 K and 1 atm, the system was heated at a weak temperature (τ=0.1 ps) and pressure (τ=0.5 ps) coupling by taking advantage of the Berendsen algorithms [32]. The heating time for MD simulation at 100 and 200 K was 100 ps with a nonbounded cutoff of 14 Å. The MD simulation was further carried out for 2 ns at 300 K. We used LINCS to constrain the bond length [33]. MD simulations were carried out by particle mesh Ewald

method [34]. The dynamic behavior and structural changes of the protein were analyzed by calculation of the root mean square deviation (RMSD).

Docking Analysis

The intramolecular residues interacting with PLP were examined by using the LIGPLOT program. The program generates Lys–PLP interactions from three-dimensional coordinates in a 1Y9M PDB file (PDB ID, 1Y9M). The LIGPLOT algorithm was described by Wallace et al. and Turnay et al. in 2002 [29, 35]. Simulations were performed for both the non-modified and modified enzyme species.

Results

Structural, Storage, and Functional Thermostability Analysis

We examined the thermodynamic and molecular simulation approaches to explain the structural, storage, and functional thermostabilization of endo-inulinase after the application of PLP modification followed by ascorbate reduction. According to our previous report [6], Figure 1 presents the heat capacity scans of the non-modified and PLP modified without reducing (En-PLP), and PLP modified with reducing by ascorbic acid (En-PLP-As). The results revealed that the thermal denaturation of endo-inulinase was an irreversible process. The T_m of modified enzyme (En-PLP-As) was 72.2 °C, whereas it was 64.1 °C for the non-modified and 64.9 °C for PLP-treated (without reducing) sample (En-PLP). Accordingly, the T_m of the enzyme is shown to have an 8.1 °C increase upon modification and emphasizes on the necessity of the reducing step. Therefore, the structural thermo-stabilization of the endo-inulinase has been achieved by using this modification strategy consisting of PLP treatment and ascorbate reduction. The

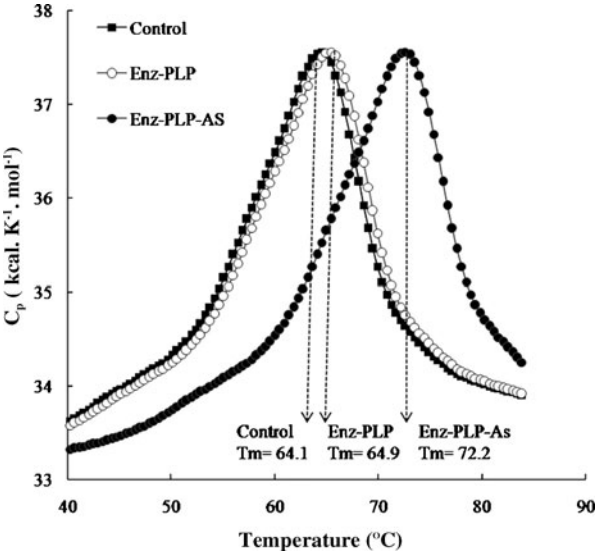

Fig. 1 Heat capacity scans of non-modified endo-inulinase (control) and modified without (Enz-PLP) and with reduction (Enz-PLP-As) by ascorbic acid. The standard CpCalc software package and data acquisition program, DSC Run, were employed for data analysis and evaluation of T_m values, (n=3)

comparative study on storage and functional thermo-stability of the enzyme species including non-modified and modified endo-inulinases were performed in the absence and the presence of substrates respectively at four representative temperatures 25, 45, 50, and 60 °C for at least 90 min (Fig. 2). Figure 3 reveals the natural logarithm of residual activities as a function of the incubation time in the absence (Fig. 3a) and presence (Fig. 3b) of inulin as the enzyme substrate. Thermo-inactivation of the enzyme species (non-modified and modified) up to 150 min in the absence of the substrate was further analyzed to determine the inactivation rate constants or k_{in}. The half-lives corresponding to each sample were calculated using Eq. 1 and were plotted versus temperature (Fig. 4). The results indicated an improved thermostability of the modified compared to the non-modified enzyme species. An $Ea_{(in)}$ is thus proposed to be an important indicator of the thermostability of an enzyme. This parameter was obtained by applying an Arrhenius plot to non-modified and modified samples (Fig. 5). The energy barrier of the thermal inactivation process was thereafter used to calculate the thermodynamic parameters of non-modified and modified endo-inulinase inactivation using Eqs. 3–5. The results are presented in Table 1. These results indicated that modification brings about increases in $Ea_{(in)}$, ΔH^*, and ΔG^* and decreases in ΔS^* values.

Invertase/Inulinase Ratio in Modified Versus Non-Modified Endo-inulinases

Inulinases also have well-known invertase activity associated with the inulinase activity, so, they are described by the ratio of the activity on sucrose versus inulin in terms of the S/I ratio. The ratios of the initial rate of hydrolysis of sucrose and inulin (S/I) as an indicator of substrate specificity for non-modified and modified endo-inulinases were determined at different temperatures (25, 45, 50, and 60 °C), and the result has been summarized in Table 2. This ratio does not show significant changes with increasing hydrolysis time.

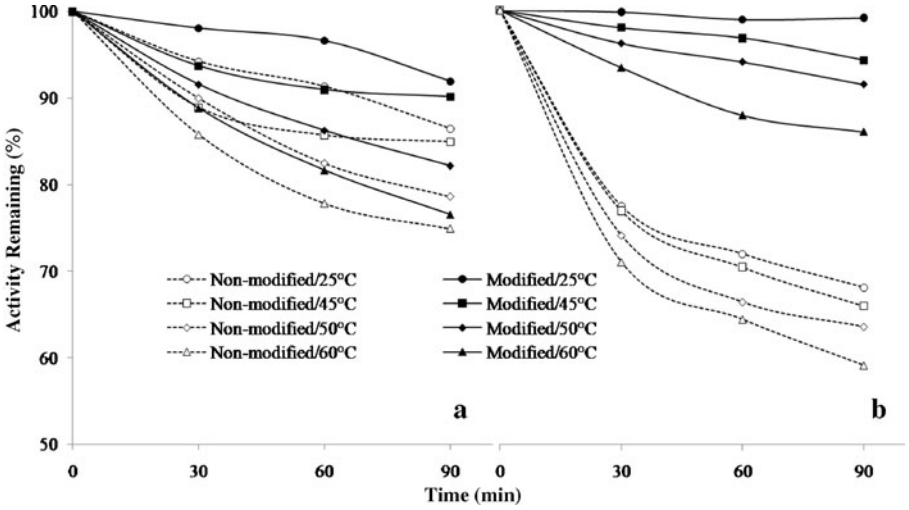

Fig. 2 Thermostability monitoring of the non-modified and modified endo-inulinases as a function of time in the absence (**a**) and presence (**b**) of inulin as the enzyme substrate. For the latter, enzyme at 6×10^{-3} μg ml^{-1} was incubated with the substrate for up to 90 min at 25, 45, 50, and 60 °C. The liberated sugars were determined in samples, which were withdrawn at appropriate intervals then inactivated at 95 °C. All the data presented in this study are average values±standard deviation (SD) of three experiments

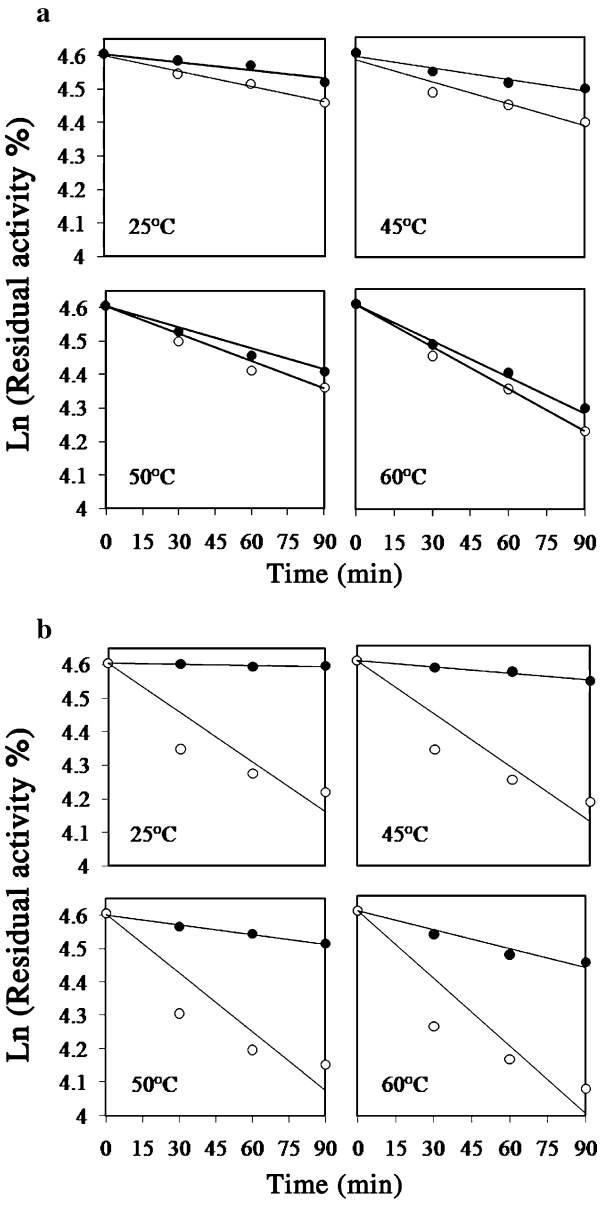

Fig. 3 The natural logarithm of residual activities of the non-modified (*empty circle*) and modified (*filled circle*) endo-inulinases as a function of the incubation time in the absence (**a**) and presence (**b**) of inulin as the enzyme substrate. All the data presented in this study are average values±SD of three experiments

Molecular Dynamics Simulation and Docking Analysis

Molecular dynamics has enabled us to simulate the PLP-modified species of the enzyme to determine that modification created additional intramolecular contacts, which are thought be responsible for improving the thermostability and therefore the resistance as it relates to the thermoinactivation of the endo-inulinase. Figure 6 shows the simulated structure and focuses on lysine residue (Lys381) at the linker closer to the C-domain, which is prone to modification. The RMSD value as a factor of the equilibrated modified lysine inulinase and

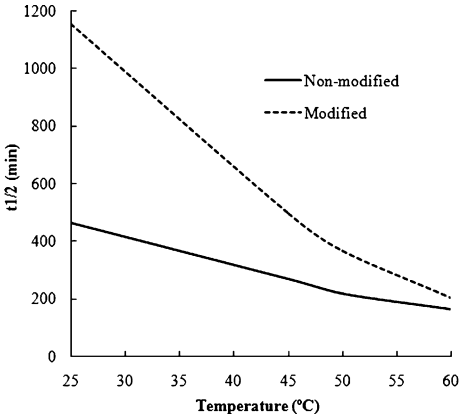

Fig. 4 Presentation of the endo-inulinase half-lives ($t_{1/2}$) as a function of temperature (in degrees Celsius)

non-modified lysine is shown inset in Fig. 6. It is evident that after 300 ps, both the structures reached an equilibrated state. The modified molecule was analyzed against the non-modified one by using the LIGPLOT program to examine the newly established intramolecular hydrogen bonds after the PLP modification process at the portion of Lys381 (Fig. 7). Modification made it possible to create additional intramolecular contacts between covalently attached PLP–Lys381 with Arg526 (two possible hydrogen bonds between the phosphate of PLP and the guanidinium group of Arg526) and with Ser376, as illustrated in Figs. 6 and 7. Also, additional intramolecular hydrophobic interactions are formed due to the modification process, in which Pro383 is involved. It seems that the modification process involved an interaction that enhanced the rigidity of the enzyme molecule.

Discussion

We are reporting the chemical modification of endo-inulinase to improve the thermostability of the enzyme through covalent attachment of the PLP molecules to the accessible lysine residues followed by ascorbate reduction [6]. Although most reports on the PLP modification of enzymes have resulted in enzyme inactivation due to the engagement of essential residues at the active site or non-desired structural alterations [14, 18], our

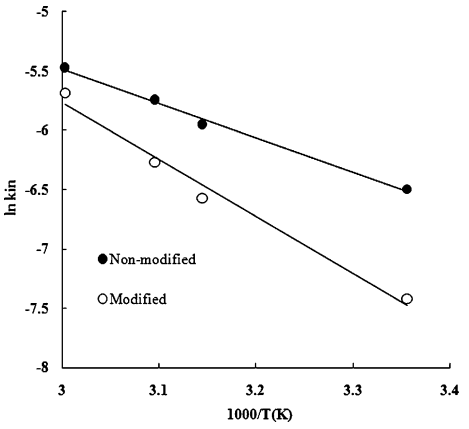

Fig. 5 Arrhenius analysis of the endo-inulinase inactivation rate constant (k_{in}) as a function of absolute temperature (in Kelvin). Data are average values of three repeats (n=3)

Table 1 Thermodynamic parameters for irreversible inactivation of non-modified and modified endo-inulinase

	$Ea_{(in)}$ (kJ mol^{-1})	ΔH^* (kJ mol^{-1})	ΔG^* (kJ mol^{-1})	ΔS^* (kJ mol^{-1} K^{-1})
Non-modified	24.19	21.42	46.06	−0.227
Modified	39.88	37.11	46.62	−0.182

The amounts of ΔH^*, ΔG^*, and ΔS^* are calculated at 60 °C and $Ea_{(in)}$ is resulted from Arrhenius plot

Ea_{in} activation energy of denaturation/inactivation, ΔH^* enthalpy of inactivation, ΔG^* Gibbs free energy of inactivation, ΔS^* entropy of inactivation

observations have demonstrated that the PLP modification of endo-inulinase not only result in no activity loss but also brings about thermostability. Moreover, modification improves the inulinase versus invertase activity, in which the S/I ratio is decreased upon modification process. The relative activities with sucrose and inulin were determined for non-modified and modified endo-inulinases at different temperatures (25, 45, 50, and 60 °C) and the result has been presented in Table 2. For all cases, the S/I ratio was relatively low (1.78–2.86 and 1.07–1.33 for non-modified and modified enzyme species, respectively) that is indicative of a true fructanase. As summarized in Table 2, the resultant S/I values were increased twofold by modification of endo-inulinase, which means that substrate specificity in the modified enzyme had improved.

Modified endo-inulinase has an industrial potential for inulo-oligosaccharides and HFS production for use in food and pharmaceutical products. Thus, the industrial process for HFS and fructo-oligosaccharide production from inulin could be carried out at the higher temperatures necessary to achieve the appropriate hydrolysis rate. The DSC results (Fig. 1) revealed that PLP modification of endo-inulinase increases the T_m of the enzyme from 64.1 to 72.2 °C (8.1 °C increase in T_m). The thermal unfolding processes of the enzyme samples were achieved to be irreversible, as no transition peaks were obtained in the second run of heating. Therefore, under such conditions, the thermodynamic parameters can be calculated using $Ea_{(in)}$ [13, 21, 28, 29]. The results of the DSC analysis showed good agreement with the observed 4% increase in α-helical contents revealed by circular dichroism spectropolarimetry (Table 2) (details not shown) [6]. Protein thermostability has been reported to correlate with the larger fraction of residues in the α-helical conformation (Table 3) [36].

Monitoring of the residual activity versus time in the absence and presence of substrate (Figs. 2 and 3) indicate the achievement of a modification-based thermostabilization of the enzyme. Although, enzyme modification resulted in improved thermostability both in the absence and in the presence of the substrate, but this important property was observed to be more distinguishable between modified versus non-modified enzyme species in the

Table 2 Estimated invertase to inulinase activities (S/I ratio) for non-modified and modified endo-inulinases as a function of the temperature

	Invertase/inulinase (S/I)			
	25 °C	45 °C	50 °C	60 °C
Non-modified	1.78	1.54	2.36	2.86
Modified	1.07	0.83	1.15	1.33
Non-modified/modified	1.66	1.85	2.05	2.15

All the data presented in this study are average values±standard deviation of three experiments

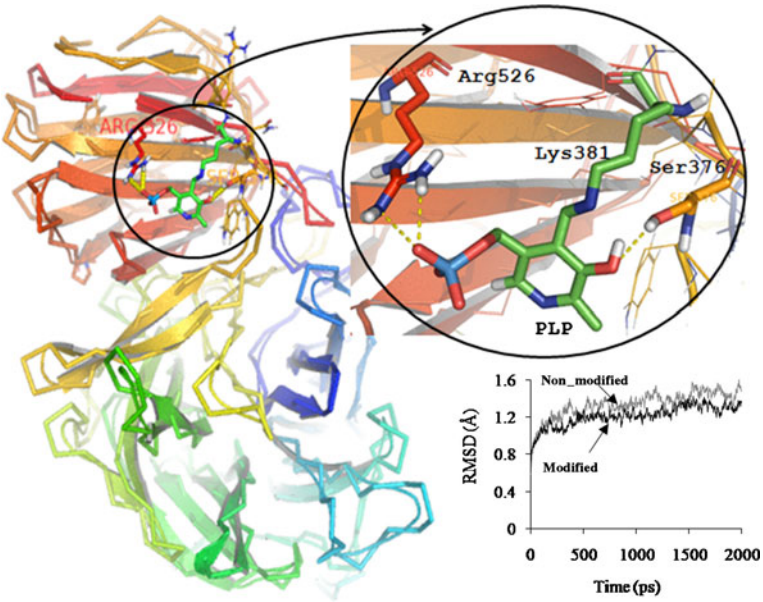

Fig. 6 The simulated structure of PLP-modified endo-inulinase on Lys^{381} at the link closer to the C-domain. Molecular dynamics simulations were performed by using the GROMACS program (version 4.0.3). For more details, please see the "Materials and Methods" section. In the modified enzyme, additional intramolecular contacts between covalently attached PLP–Lys^{381} with guanidinium group of Arg^{526} and at the hydroxyl group of Ser^{376} are formed. *Inset* to the figure presents the RMSD versus time plot for the non-modified and PLP-modified endo-inulinases during 2 ns of MD simulation

presence of the substrate during the study of functional or operational stability at all representative temperatures of 25, 45, 50, and 60 °C (Figs. 2 and 3). Further analysis of the storage stability, including the storage half-life of the endo-inulinase ($t_{1/2}$) as a function of

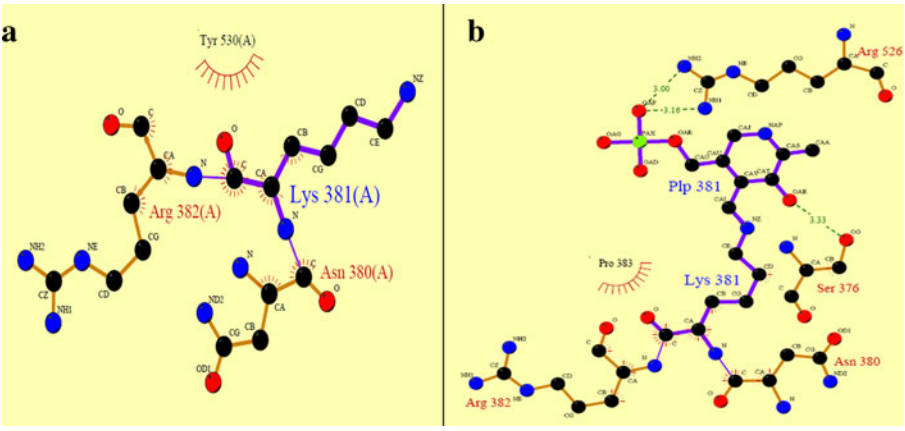

Fig. 7 Docking analysis of intramolecular interactions in a two-dimensional representation of simulated endo-inulinase structure [based on *A. awamori* exo-inulinase (PDB ID, 1Y9M)], before (**a**) and after (**b**) PLP-modification at the Lys^{381} using the LIGPLOT program. New hydrogen bonds are formed between the phosphate group of PLP and Arg^{526} and Ser^{376}. The *dashed lines* indicate hydrogen bonds and the *values* indicate hydrogen bond lengths (in angstrom)

Table 3 The result of the secondary structure analysis of endo-inulinase, before and after modification [6]

	α-Helix (%)	β-Sheet (%)	Turns (%)	Random (%)
Control	13.60	42.60	14.50	29.30
En-PLP	13.80	44.50	13.10	28.60
Endo-PLP-As	17.60	31.80	20.20	30.40

the temperature (Fig. 4), also points towards the success of the modification-based thermostabilization of the enzyme. The folds of stabilization were affected above 25 °C and were decreased by increasing the incubation temperature. As a result, more than 2.5-fold stabilization was achieved when the product of modification was operated at 25 °C (the representative half-lives of the non-modified and modified species were determined to be 460 min and 1,155 min, respectively). The $Ea_{(in)}$ as an important indicator of thermostability (Fig. 5) and the ΔH^* as the amount of heat required for enzyme inactivation were increased through the modification process of the enzyme (Table 1). The large positive enthalpic term is associated with a more stable enzyme because greater amounts of energy are required for the inactivation process to take place. However, the smaller the change in the free Gibbs energy of endo-inulinase inactivation (ΔG^*), the more readily the enzyme was inactivated; this may be interpreted as being a less stable enzyme [29]. There is an assumption that this type of modification strategy increases the enzyme thermal stability. The thermostabilization of the enzymes is mostly accompanied by a decrease in ΔS^* via the charge neutralization of amino acid residues of the enzyme molecules. The reason for further decreases in ΔS^* (19.8%) of chemically modified endo-inulinase could be due to the elimination of the repulsion between negatively and/or positively charged groups, thus decreasing the flexibility of the external loop, thereby stabilizing the modified enzyme. The neutralization of excessive negative or positive charges on the surface of enzymes by chemical modification has been shown to increase the thermostability of enzymes. Another reason for the increasing thermostability of endo-inulinase after PLP modification could be the formation of new electrostatic interactions between the phosphate group of bound pyridoxal phosphate and the positively charged groups, which impart rigidity to the enzyme molecule, thus lowering the ΔS^* [27].

Molecular dynamics and simulation approaches were used to elucidate the newly established interactions that explain the thermostabilization of the engineered enzyme. The simulated structure of the PLP-modified enzyme at the linker closer to the C-domain, which is prone to modification (Fig. 6) and the constructed LIGPLOT (Fig. 7), presents newly established intramolecular interactions after modification at Lys381. This modification results in two possible hydrogen bonds between phosphates covalently attached to PLP–Lys381 and the guanidinium group of Arg526 residue as well as between the hydroxyl group of PLP with Ser376. The hydrophobic groups of amino acid residues become closer through the formation of these intramolecular interactions. In conclusion, the structural, storage, and functional thermostabilization of PLP-modified/ascorbate-reduced endo-inulinase has been documented. In conclusion, the substrate specificity, structural, and storage/functional thermostability of the enzyme were efficiently improved upon PLP modification strategy, which has a potential application in the inulinase-based technology for the production of HFS and fructo-oligosaccharides. The mechanism of thermostabilization was assumed to be involved with the establishment of intramolecular interactions between covalently attached PLP–

Lys381 and both the Arg526 and Ser376 residues as representative modification-originated intramolecular contacts in the modified enzyme.

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