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Semi-rational chemical modification of endoinulinase by pyridoxal 5′-phosphate and ascorbic acid

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ABSTRACT

It is important to improve the quality of the enzyme inulinase used in industrial applications without allowing the treatment to have any adverse effects on enzyme activity. We achieved preferential chemical modification of the non-catalytic domain of endoinulinase (EC 3.2.1.7) to enhance the thermostability of the enzyme. We used pyridoxal 5′-phosphate (PLP) to modify the more accessible lysine residues at the surface of endoinulinase and then performed a necessary step of reduction with ascorbate. Endoinulinase was incubated in the presence of PLP at various concentrations; this step was followed by reduction of the resulting Schiff base and dialysis. The effects of different PLP concentrations and incubation times on enzyme modification were evaluated. Enzyme deactivation was observed immediately after treatment, even at low PLP concentrations, while reactivation was observed for samples treated with low PLP concentrations after a period of time. Structural analysis revealed that the α-helix content increased from 13.60% to 17.60% after applying the modification strategy; consequently, enzyme stabilization was achieved. The melting temperature (T_m) of the modified enzyme increased from 64.1 °C to 72.2 °C, and a comparative study of thermal stability at 25 °C, 45 °C, and 50 °C for 150 min confirmed that the enzyme was stabilized because of increase in its half-life ($t_{1/2}$) after PLP modification/ascorbate reduction. The modification process was optimized to achieve the optimum mole ratio for the PLP/endoinulinase (1.37). Excess moles of the modifier are thought to be responsible for enzyme deactivation through unwanted/nonspecific and noncovalent interactions, and the optimization ensured that there was no excess modifier after the desired covalent reaction was complete.

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1. Introduction

The use of inulinases is the most promising approach for producing high-fructose syrup (HFS) and fructo-oligosaccharides from inulin. Hydrolysis of inulin by inulinases yields a syrup with more than 95% D-fructose in a single-step process, whereas amylolysis of starch – a conventional method for fructose production – yields a maximum of only about 45% D-fructose after a multistep enzymatic process [1–3]. The production methods and properties of inulinases have been reviewed [4–6]. Inulin is a fructan that serves as a storage polysaccharide in the plant families *Compositae* and *Gramineae* and is accumulated in the underground parts of several plants [7]. Its structure consists of a linear polyfructan with (β2 → 1) linkages between fructose residues and a terminal sucrose moiety [8]. Solubility limitation and ample chance of microbial contamination of inulin at room temperature are the major problems associated with industrial application of inulinases for the production of fructose. Thus, the industrial production of HFS and fructo-oligosaccharides from inulin is carried out at temperatures higher than 50 °C, which is necessary to ensure an appropriate hydrolysis rate. In most cases, inulinases lose their activity after a few hours at this temperature. Therefore, there is a growing interest in producing inulinases with improved thermostability [9]. Exoinulinase (β-D-fructan fructohydrolase; EC 3.2.1.80, or exo-β-D-fructosidase), hydrolyzes β2 → 1 and β2 → 6 linkages at the nonreducing terminal in fructans to release fructose. Endoinulinase (EC 3.2.1.7) hydrolyzes fructans and produces oligofructose [10]. The results of amino acid sequence comparisons and the presence of conserved amino acid domains have shown that inulinases belong to the glycoside hydrolase family GH32 [11]. GH32, together with the glycoside hydrolase family GH68, belongs to the

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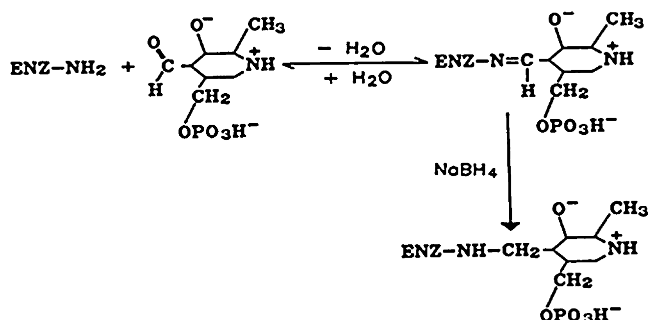

Fig. 1. Reversible reaction between PLP and the ε-amino group of the lysine residue, leading to the formation of a Schiff base (aldimine), and consequent reduction to form a stable phosphopyridoxyl-lysyl group.

enzyme clan GH-J; all enzymes in this group have the 5-bladed β-propeller fold [12]. The catalytic activity of the GH32 enzymes is based on a mechanism that involves overall retention of the configuration at the anomeric carbon atom of the substrate. The tertiary structure of exoinulinase consists of two domains: the N-terminal catalytic domain with a 5-bladed β-propeller fold and the C-terminal domain folded into a β-sandwich-like structure [13]. The similarities in sequences of the GH32 members and their activities on fructo-oligosaccharides or fructo-polysaccharides possibly reflect an evolutionary relationship among them [14]. Most of the currently employed methods for chemical modification of enzymes are based on reactions with limited selectivity and efficiency. So, more selective strategies are required, such as combination of chemical modification with molecular modeling techniques. The emphasis on a specific functional group in a protein provides a robust method for ensuring rational chemical modification. However, exploitation of the existing differences in reactivities between functional groups of the same type in different environments within a given protein provides semi-rational chemical modification. Pyridoxal phosphate (PLP) is useful for the modification of lysine residues because of the selectivity and reversibility of the reaction and the spectral properties of the modified residue. This reaction is illustrated in Fig. 1 [15,16]. During this reaction, a Schiff base is formed between the aldehyde group of PLP and the ε-amino group of lysine [17]. We used pyridoxal 5′-phosphate for chemical modification of lysine residues that are easily accessible at the surface of the C-terminal domain but less accessible in the N-terminal domain of the endoinulinase molecule. The objective of this research was to study the preferential domain modification of endoinulinase through PLP binding to lysine residues followed by reduction with ascorbic acid. Our method resulted in improved thermostability of this enzyme with no adverse effect on enzyme activity.

2. Materials and methods

2.1. Chemicals

Inulinase (27 U/mg), inulin (chicory inulin), and pyridoxal 5′-phosphate were purchased from Fluka (Switzerland); ascorbic acid, from Acros Organics (New Jersey, USA); sodium borohydride, from Merck (Germany). Dinitrosalicylic acid (DNS) and other chemicals, were obtained from Sigma Chemical Company (St. Louis, MO, USA).

2.2. Binary alignment

The SWISSPROT sequence database (accession numbers: endoinulinase, Q8J229_ASPNG; exoinulinase, Q96TU3-ASPAW) on the ExPASy server was used. A binary alignment of exo- and endoinulinases was constructed using FASTA version 3 (35.04) at the EBI website. The default parameters for open gap (−10) and gap extension (−2) were used. The amino acid comparison matrix was BLOSUM 5 [18].

2.3. Accessible surface area analysis

To select an appropriate strategy for chemical modification of endoinulinase, the accessible surface area was calculated using the GETAREA 1.1 program (water probe size, 1.4 Å) [19,20] on the basis of the X-ray crystal structure of exoinulinase (PDB code: 1Y9M). The highly accessible residues were determined in both the N-terminal and the C-terminal domains.

2.4. Protein estimation and enzyme assay

The protein content was estimated by the Bradford method [21], using bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) as a standard. Inulinase activity was assayed by measuring the amount of reducing sugars released from inulin using the dinitrosalicylic acid (DNS) method [22]. The assay mixture of inulinase contained 50 μl of appropriately diluted enzyme, 100 μl of 0.2% inulin, and 400 μl of 50 mM sodium acetate buffer (pH 5.5–6). The mixture was incubated at 37–38 °C for 5–30 min. The reaction was stopped by adding 550 μl DNS reagent and incubating the mixture at 90 °C for 10 min. The absorbance was read at 575 nm using a Camspec M550 spectrophotometer in cells with 1-cm path length. One unit inulinase (IU) was defined as the amount of enzyme that liberated 1 μmol of fructose per minute under the assay conditions [23]. To ensure the reproducibility of the results, each assay was repeated three times.

2.5. Enzyme treatment by PLP

A stock solution of 1 mM PLP in 50 mM sodium phosphate buffer (pH 7.5) was freshly prepared before use. Endoinulinase (50 μl; 0.11 mg ml^{-1}) was incubated in the presence of 450 μl PLP (concentration range, 0–1 mM) for an appropriate time in the range of 0–60 min at 25 °C in the dark. After addition of PLP, aliquots were removed every 5 min and analyzed immediately for enzyme activity. UV–vis difference spectra were obtained for the PLP-treated and untreated samples in the wavelength range of 250–490 nm by using Camspec M550 spectrophotometer and a 1-cm quartz cell. The applied mole ratios of PLP/enzyme have been presented in Table 1.

2.6. Reduction of enzyme–PLP complex by sodium borohydride or ascorbic acid

The enzyme–PLP complex was stabilized by reduction using sodium borohydride ($NaBH_4$) or ascorbic acid. A freshly prepared solution of sodium borohydride (30 mM) in 1 mM sodium hydroxide (pH 8.5) was added in sevenfold excess with respect to the pyridoxal 5′-phosphate [24,25]. The solution was left at 4 °C for 15 min in the dark. Then, the pH of the solution was adjusted to

Table 1
Concentration equivalents of the moles of PLP per mole of endoinulinase.

PLP concentration (μM)	3.9	7.8	15.6	31.25	62.5	125	250	500	1000
PLP/endoinulinase	0.17	0.34	0.68	1.37	2.73	5.47	10.93	21.87	43.73

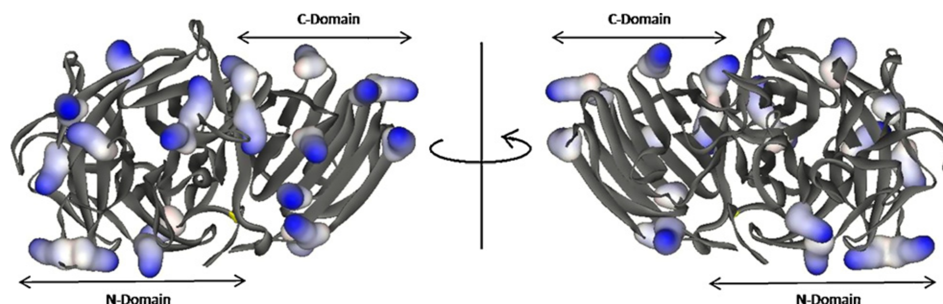

Fig. 2. Ribbon representation of the exoinulinase from *Aspergillus awamori* (*PDB* code; 1y4w). The location of lysine residues in the N- and C-domains are presented as bulky shapes.

6.0 by adding 0.1 M acetic acid to stop the reduction. Overnight dialysis was performed (MWCO 12 kDa) against a 50 mM sodium acetate buffer (pH 6.0) at 4 °C. Ascorbic acid (1 mM in 50 mM phosphate buffer; pH 7.0) was used as an alternative to Schiff base. At a given time after enzyme incubation with PLP, 100 μl of 1 mM ascorbic acid was added to the cocktail, and the mixture was kept in the dark for 15 min at 25 °C. Excess ascorbate was then removed by dialysis against 50 mM sodium acetate (pH 6.0) at 4 °C overnight.

2.7. Intrinsic tryptophan fluorescence analysis

Fluorescence measurements were performed using a Hitachi F-4500 spectrofluorometer. The emission spectra were obtained for samples at a concentration of 1 mg ml^{-1}, by excitation at 280 nm followed by wavelength scanning in the range of 300–500 nm.

2.8. Circular dichroism spectropolarimetry

Circular dichroism (CD) spectra were obtained between 190 and 260 nm, at the far-UV region using a Jasco J-810 spectropolarimeter (Jasco, Japan) at a scanning rate of 50 nm min^{-1} at 25 °C. The results were expressed as molar ellipticity $[\theta]$ (° cm^2 dmol^{-1}) on the basis of mean amino acid residue weight (MRW). The molar ellipticity was determined as $[\theta]_\lambda = (\theta \times 100\text{MRW})/(c \cdot l)$, where c is the protein concentration in mg ml^{-1}, l is the light path length in centimeters, and θ is the measured ellipticity in degrees at a given wavelength [26,27]. The instrument was calibrated with (+)-10-camphorsulfonic acid, assuming $[\theta]_{291} = 7820$ ° cm^2 dmol^{-1}, and with standard non-hygroscopic ammonium (+)-10-camphorsulfonate, assuming $[\theta]_{290.5} = 7910$ ° cm^2 dmol^{-1}. Samples were prepared in sodium phosphate buffer (pH 7.5). The CD measurement was performed using a quartz cuvette with a 1-mm path length, at a final protein concentration of 2.0 mg ml^{-1}. For each sample, two spectra were recorded, averaged, and corrected for buffer background. Deconvolution of the data was performed to estimate the α-helix and β-sheet contents of the samples using a Jasco model JWSSE-480 original software program.

2.9. Differential scanning calorimetry

Calorimetric measurements were performed in a Model 6100 Nano II differential scanning calorimeter (Calorimetry Sciences Corp., USA) at a heating rate of 2 °C per minute between 10 °C and 85 °C, under an extra constant pressure of 2 atm. For each experiment, 600 μl of non-modified sample (control), PLP-modified sample (En–PLP), and PLP-modified and reduced by ascorbic acid (En–PLP–As) were prepared at 2.0 mg ml^{-1} in 50 mM phosphate buffer (pH 7.5). The samples were degassed for 30 min before each calorimetric measurement. The same buffer was used for loading into the reference cell and for performing the baseline run that preceded each sample run. The standard CpCalc software package and the data acquisition program DSC Run were used in data collection and estimation of melting temperature. T_m represents the temperature (K) at 50% of the peak area of the unfolding transition [28].

2.10. Kinetics of thermal inactivation

The kinetics of thermal inactivation of endoinulinase before and after modification were studied by incubating the enzyme at different temperatures (25 °C, 45 °C, and 50 °C). The samples were withdrawn during enzyme treatment at periodic intervals and cooled in ice prior to the assay by the DNS method as described above. The residual activity was expressed as a percentage of initial activity. From a semi-logarithmic plot of residual activity versus time, the inactivation rate constants (k_d) were calculated, and apparent half-lives were estimated using Eq. (1). The half-life of an enzyme is the time at which its residual activity reaches 50% [29,30].

$$t_{1.2} = \frac{\ln 2}{k_d} \tag{1}$$

3. Results and discussion

3.1. Accessible surface area estimation

The use of chemical modification overcomes the limitations associated with redesigning the enzyme structure and function to allow alteration of proteinogenic amino acid side chains. In this study, to ensure preferential modification of the C-domain of endoinulinase, we employed chemical modification based on preferential accessibility analysis as a semi-rational strategy, instead of exclusive (rational) or nonspecific (nonrational) modification. Inulinases play an important role in the hydrolysis of inulin for commercial applications. They consist of exo- and endo-acting enzymes, which constitute an important class of enzymes used in the production of fructose and fructo-oligosaccharides, and are extensively used in the pharmaceutical and food industries [31]. The crystal form of the native exoinulinase from *Aspergillus awamori* was resolved a few years ago at a resolution of 1.55 Å in the monoclinic form [13]. The crystal structure of endoinulinase from *Aspergillus niger*, however, has not yet been elucidated. The exoinulinase folds into two domains (Fig. 2). The N-terminal domain, comprising 372 amino acid residues, bears a catalytic site, and the C-terminal domain comprises 156 amino acid residues (Arg_{382} to Asn_{537}) arranged in two β-sheets that are assembled into a sandwich-like structure. Because the currently known three-dimensional X-ray crystal structures of all members of the GH32 family have the same basic features with respect to their secondary structures [13], homology models will serve as the best structural reference for understanding

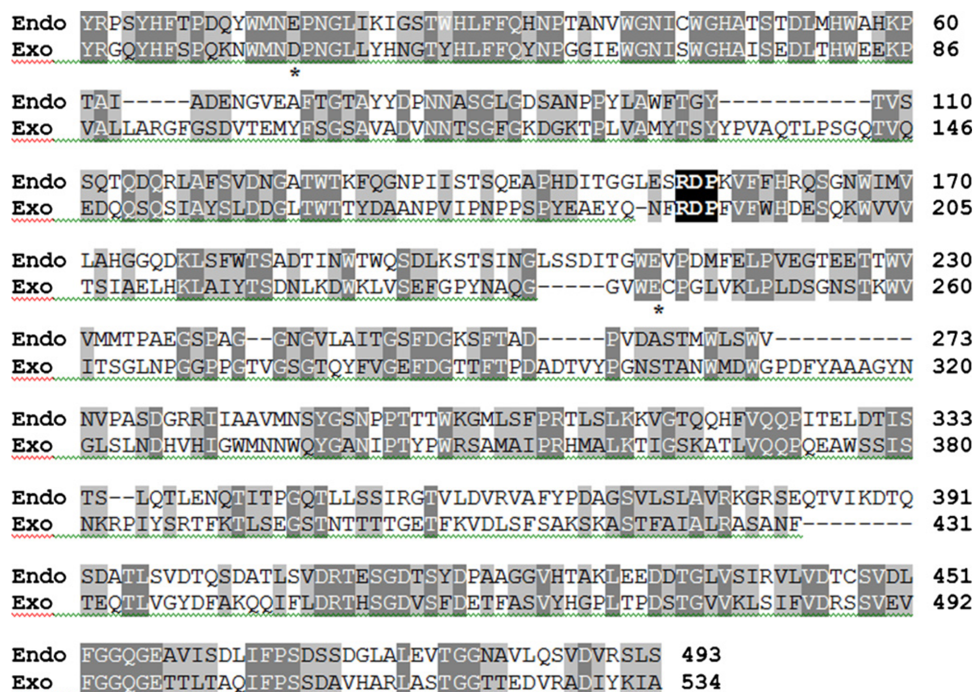

Fig. 3. Sequence alignment of *Aspergillus niger* endoinulinase and *Aspergillus awamori* exoinulinase. The amino acid similarity between the primary structures as calculated by the program FASTA is shown in two different shades of gray. The darker color corresponds to higher levels of sequence similarity. Catalytic residues are denoted by "*" and the conserved Arg-Asp-Pro (RDP) motif (which is assumed to be involved in substrate binding through hydrogen bond interactions with hydroxyl groups 3 and 4 of fructose [12]) is highlighted by a black box.

structure/function relationships in the inulinase family until the three-dimensional structure of endoinulinase from *A. niger* is determined and refined at atomic resolution. Endoinulinase from *A. niger* comprises 494 amino acid residues with a molecular mass of 53,470 Da [32]. Fig. 3 shows the optimal binary sequence alignment of endoinulinase from *A. niger* and exoinulinase from *A. awamori*. The sequence identity between the two proteins was 31.6%, and the similarity was 62.9–64.5% over 537 aligned residues (determined using the FASTA program on the Expasy server) [17]. Therefore, in this study, the solvent-accessible surface area (SASA) for exoinulinase with known Cartesian coordinates can be generalized to the endoinulinase with acceptable confidence.

Table 2 shows generalized SASA data of two structural domains of *A. awamori* exoinulinase. The total number of lysine residues in the exoinulinase structure is 21—13 and 7 residues in the N-terminal and C-terminal domains, respectively, and 1 in the short polypeptide linker. The calculated SASA determined using the GETAREA 1.1 program indicated that 3 out of 13 (in the N-terminal domain) and 7 out of 7 (in C-terminal domain) lysine residues were

Table 2
Solvent-accessible surface area (SASA) data of two structural domains and linker petide of *A. awamori* exoinulinase. (PDB code: 1Y9M) (probe size 1.4 Å).

Residue	N-terminal domain		C-terminal domain		Short linker	
	Total no.	Mean SASA (Å^2)	Total no.	Mean SASA (Å^2)	Total no.	Mean SASA (Å^2)
Phe	15	17.2	12	8.11	0	0
Asn	22	38.57	3	59.26	1	105.42
Tyr	23	33.39	4	15.70	0	0
Asp	21	59.78	10	36.37	0	0
Gln	18	35.79	5	51.87	0	0
Pro	28	34.31	4	36.20	0	0
Arg	5	23.42	7	85.52	0	0
Gly	36	21.65	11	18.03	0	0
His	10	23.61	3	66.54	0	0
Ser	27	29.71	19	43.67	3	64.17
Lys*	**13**	**74.09**	**7**	**123.13**	**1**	**190.06**
Trp	15	25.06	1	76.58	1	49.43
Met	10	4.89	0	0	0	0
Leu	25	23.57	9	6.31	0	0
Thr	27	39.38	22	38.99	0	0
Ile	14	11.93	7	12.31	1	12.05
Glu	13	71.93	7	64.07	1	12.59
Ala	27	21.29	14	23.49	1	34.70
Cys	1	11.91	0	0	0	0
Val	22	21.42	11	13.86	0	0
Total	372	30.85	156	36.73	9	66.31

The Lys residue has been highlighted for its SASA values, specially in C-terminal domain.

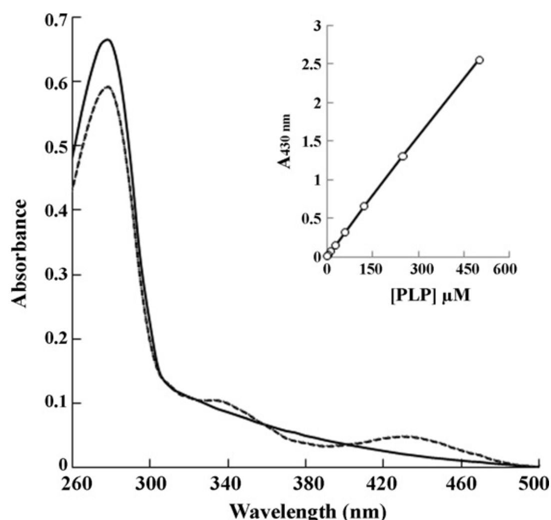

Fig. 4. Spectroscopic analysis of PLP modification of endoinulinase. UV–vis spectra of non-modified (—) and PLP-modified (- - -) endoinulinase show two representative maxima at 340 and 430 nm for the modified sample. The spectra were obtained after 30 min of incubation of the enzyme with PLP. Inset to figure shows the absorbance at 430 nm as a linear function of concentrations of PLP-treated enzyme.

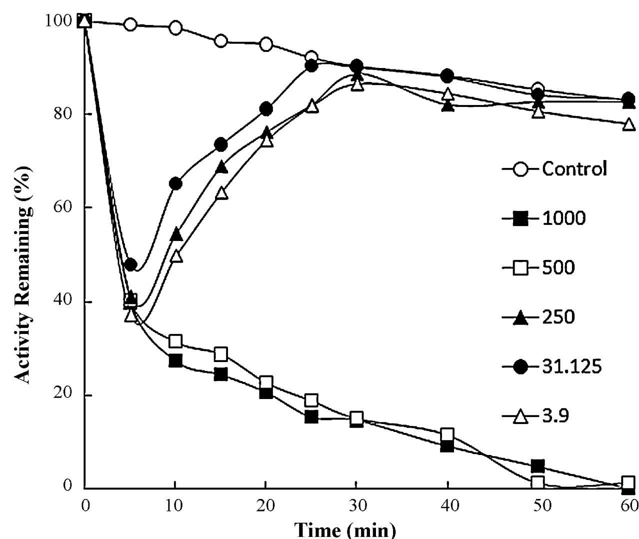

Fig. 5. The remaining activity of endoinulinase as a function of incubation time for different concentrations of PLP. Treatment of the enzyme with PLP was carried out for the indicated time in 50 mM sodium phosphate buffer (pH 7.5) at 25 °C. The data presented are average values ± SD of three experiments.

accessible. Furthermore, distinct distribution of the mean SASA (Å^2) of lysine residues in the N- and C-domains have been estimated at 74.09 and 123.13, respectively, with the absence of lysine residues in the enzyme active site [18] (Table 2). This finding led us to select lysine residues as targets for semi-rational chemical modification of the endoinulinase, preferentially at the C-domain.

3.2. Monitoring of enzyme–PLP interactions

PLP reacts primarily with the ε-amino groups of the lysine residues through the formation of a Schiff base. This reaction proceeds with an observable time dependency for a range of protein:modifier concentration ratios. The spectrum of endoinulinase and PLP reaction products in 50 mM sodium phosphate buffer (pH 7.5) exhibits peaks at 340 and 430 nm ($A_{340}/A_{430} \approx 2.1$), in addition to the protein representative peak at 280 nm (Fig. 4). The absorption maxima of PLP–protein complexes are typical at 430 nm and are attributed to protonated Schiff base complexes [23,25,32]. A second smaller band at 340 nm may represent an unsubstituted aldimine [25]. The absorption maximum in the 430 nm region was shown to be constant after the reaction was allowed to occur for an appropriate length of time (about 30 min). Moreover, it was shown to have a good linear correlation with the PLP concentration used for the chemical modification of endoinulinase.

3.3. Effect of PLP concentration and incubation time

Wavelength absorption of endoinulinase at 280 nm as a function of PLP molar concentration shows a rapid decline, which is followed by an almost steady trend of change. However, a maximum was observed at 31.2 μM of PLP. These observations indicate the presence of specific protein conformations due to covalent interactions between PLP and the lysine residues in endoinulinase. The observed maximum will be discussed in detail later. Fig. 5 shows endoinulinase activity as a function of the incubation time for different concentrations of PLP. Treatment of the enzyme with PLP in 50 mM sodium phosphate buffer (pH 7.5) at 25 °C led to a sharp decline in activity at all concentrations. Similar observations were previously reported by Shapiro et al. [24]. However, at concentrations lower than 250 μM, this decline was reversed and the activity was restored to the original level. Activity retention indicates that exposed lysine residues are not essential for the activity of endoinulinase, like in the case of exoinulinase. When the modifier concentration was adjusted to provide 1.37 mol equivalents of PLP per mole of endoinulinase (enzyme treated with 31.25 μM PLP), the activity retention was more efficient than when higher mole equivalents were used. The progressive reactivation was observed to occur in 30 min. We infer that rapid, noncovalent, and nonspecific interactions occur between PLP and protein (even at the active site) upon protein treatment with PLP, which brings about primary enzyme deactivation. Stoichiometric and covalent interactions occur, with observable time dependency, between PLP and the protein in the second step. However, the extra moles of the modifier remain and noncovalent/nonspecific interactions continue to occur at higher concentrations. This is most probably responsible for keeping the enzyme in the deactivated state at concentrations of PLP higher than 31.25 μM which causes to retain the noncovalent interactions between PLP and enzyme, particularly at the active site of the enzyme or the portions where efficiently affect there (Fig. 5). The inhibitory effect of PLP on enzymes is reported to be reversible because of Schiff base (aldimine) formation in the reaction between PLP and lysyl residues [24]. Goyal and Katiyar showed that such enzyme inhibition was completely reversed by prolonged dialysis [33]. To obtain a stable analog of enzyme–PLP, the Schiff base can be reduced to produce stable phosphopyridoxyl-lysyl residues on the enzyme. Sodium borohydride is routinely used to reduce Schiff bases [24,25,33]. Figs. 5 and 6 show the effect of treating the enzyme with PLP and reducing the product of the enzyme–PLP reaction with sodium borohydride (30 mM in 1 mM sodium hydroxide; pH 8.5) and ascorbic acid (1 mM in 50 mM phosphate buffer; pH 7.0) on the structure and activity of endoinulinase. Non-distributed chemical modifications alter the covalent structure of endoinulinase, which is in turn followed by alteration of the entire conformation, but occur preferably at the more susceptible C-terminal domain. Therefore, on the basis of "structure and function relationship", conformational alterations affect the functional properties of endoinulinase, including activity and stability, especially at the C-domain.

3.4. Intrinsic fluorescence study

Fluorescence spectra analyses confirm the different effects of the two examined reduction strategies on the product of

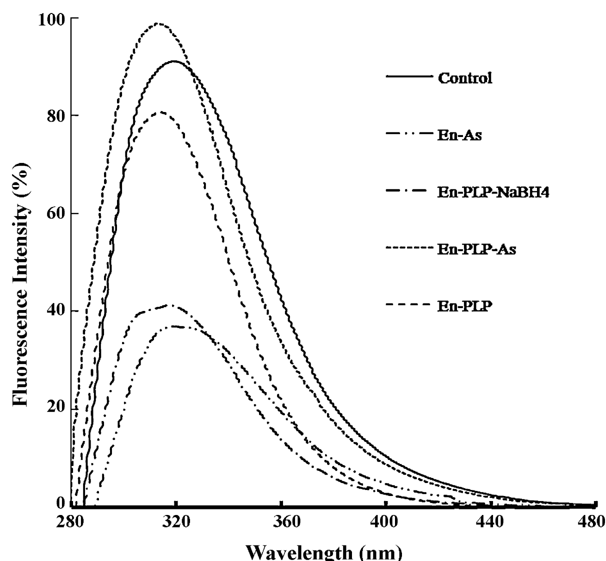

Fig. 6. Effect of PLP modification and reduction of Schiff base using sodium borohydrate or ascorbic acid. Freshly prepared sodium borohydrate ($NaBH_4$) at 30 mM in 1 mM sodium hydroxide (pH 8.5) and ascorbic acid at 1 mM in 50 mM phosphate buffer, pH 7.0 were used. Fluorescence emission spectra were obtained after excitation at 280 nm.

enzyme–PLP treatment. Sodium borohydride and ascorbic acid reduction strategies bring about less and more tryptophan-based fluorescence emissions, respectively, than strategies involving no reduction (Fig. 6). The activity of PLP-treated enzyme was significantly improved in the ascorbic acid method. However, sodium borohydride reduction caused greater inactivation (Table 3).

3.5. Circular dichroism study

Fig. 7 shows the CD spectra in the far-UV region of non-modified endoinulinase (as control), PLP-modified endoinulinase (En–PLP), and PLP-modified endoinulinase reduced with ascorbic acid (En–PLP–As), at 25 °C. There are obvious differences between the CD spectra of the samples; analysis of the spectra using the JWSSE-480 program for the estimation of the secondary structure of proteins indicates slight differences between the α-helix percentages of the control and En–PLP samples (∼14%). However, PLP modification on its own brings about small increases in the percentage of α-helix. Turning the presence of reversible aldimine linkages in En–PLP to irreversible reduced linkages in En–PLP–As enhanced the stable presence of the α-helix conformation and increased helix percentage to ∼18% (Table 4). In correlation with enhanced α-helicity (Fig. 7) and observed conformational effects (Fig. 6), thermal stabilization of endoinulinase was achieved through PLP modification followed by Schiff base reduction by using ascorbic acid, and no adverse effects were observed on enzyme activity (Table 3).

Table 3
Remaining activity analysis of the non-modified (control) and modified without (En–PLP) and with reduction by ascorbate (En–PLP–As) or sodium borohydrate (En–PLP–$NaBH_4$). En–As represents the effect of reduction of the enzyme by asorbate.

Treatment	Remaining activity (μmol/min)
Control	0.588
En–PLP	0.385
En–PLP–As	0.614
En–As	0.293
En–PLP–$NaBH_4$	0.096

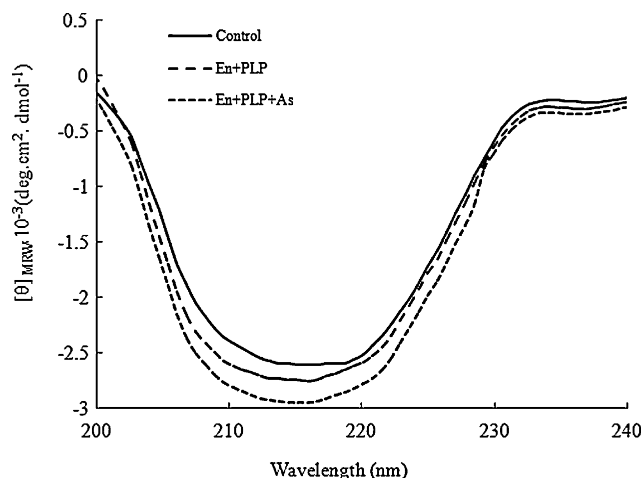

Fig. 7. Circular dichroism spectra in the far-UV region of non-modified endoinulinase (as control), PLP-modified endoinulinase (En–PLP), and PLP-modified enzyme reduced with ascorbic acid (En–PLP–As) at 25 °C.

Table 4
Secondary structure percentage of endoinulinase (control), PLP-modified (En–PLP), and reduced PLP-modified enzyme by ascorbic acid (En–PLP–As).

Structure	Control (°/o)	En–PLP (%)	En–PLP–As (%)
α-Helix	13.60	13.80	17.60
β-Sheet	42.60	44.50	31.80
Turn	14.50	13.10	20.20
Random	29.30	28.60	30.40

3.6. Thermostability of native and PLP-modified enzyme

Resistance to thermal inactivation of the enzyme is an important characteristic of biocatalysts used in industrial processes. Fig. 8 presents a comparison of the thermal inactivation of the native and modified samples (En–PLP, En–PLP–As, and En–PLP–$NaBH_4$). The formation of reversible Schiff base linkages between enzyme and PLP molecules brought about enzyme inactivation probably due to the possible nonspecific/undesired interactions between free PLP molecules and the enzyme. Ascorbic acid reduction followed by

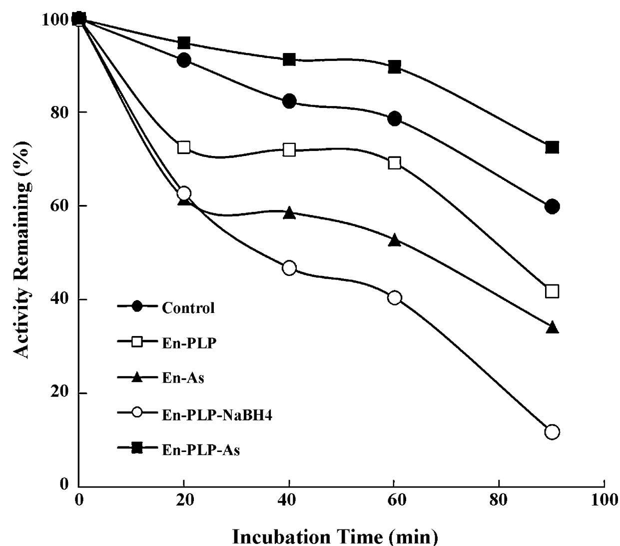

Fig. 8. Comparative stability at 50 °C. We compared the non-modified (control) and modified samples, which comprised the non-reduced samples (En–PLP) and samples reduced with ascorbic acid (En–PLP–As) and sodium borohydrate (En–PLP–$NaBH_4$). The data presented are average values ± SD of 3 experiments.

Table 5
Thermal analysis of endoinulinase with and without modification at various temperatures; 25, 45 and 50 °C.

	k_d (×10^3) (min^{-1})			$t_{1/2}$ (min)		
	25 °C	45 °C	50 °C	25 °C	45 °C	50 °C
Endoinulinase	1.5	2.6	3.2	462	267	217
En–PLP	6.2	7.2	9	112	96	77
En–PLP–As[a]	0.6	1.4	1.9	1155	495	365
En–PLP–$NaBH_4$[b]	9.4	12.7	15.3	74	55	45

[a] En–PLP–As: endoinulinase + 0.03 mM PLP + 1 mN ascorbic acid.
[b] En–PLP–$NaBH_4$: endoinulinase + 0.03 mM PLP + 30 mN sodium borohydride.

dialysis not only resulted in activity retention at mole ratios lower than 10.93, but also caused considerable stabilization of endoinulinase at 50 °C (Fig. 8). In contrast, the borohydride reduction strategy (30 mM in 1 mM sodium hydroxide) resulted in further destabilization. The kinetics of thermal inactivation of endoinulinase with and without modification were studied by incubating the enzyme at various temperatures: 25 °C, 45 °C, and 50 °C. The inactivation rate constants (k_d) for samples were calculated from the semi-logarithmic plot of the remaining activity as a function of time. Similarly, the half-lives ($t_{1/2}$) of samples were estimated by using the rate constants and Eq. (1) [29,30]. According to the data summarized in Table 5, the native enzyme was thermally stabilized when the modification/reduction strategy was employed. The k_d and $t_{1/2}$ values decreased and increased about twofold, respectively, when endoinulinase was modified by PLP and then reduced with ascorbic acid.

3.7. Differential scanning calorimetry

Fig. 9 shows heat-capacity scans of the non-modified endoinulinase (control) and PLP-modified endoinulinase without (En–PLP) and with reduction (En–PLP–As) by ascorbic acid. PLP modification of endoinulinase followed by reduction of the modification product increases the T_m of the enzyme from 64.1 °C to 72.2 °C (∼8.1 °C increase in T_m), which is in good accordance with the observed increase in the α-helix percentages determined by CD spectroscopy (Fig. 7 and Table 4). Protein thermostability has been reported to be correlated with the larger fraction of residues in the α-helix conformation [34]. The results of fluorescence, CD, and DSC experiments provide strong evidence that PLP-modified endoinulinase undergoes a significant conformational change when it is reduced by ascorbic acid.

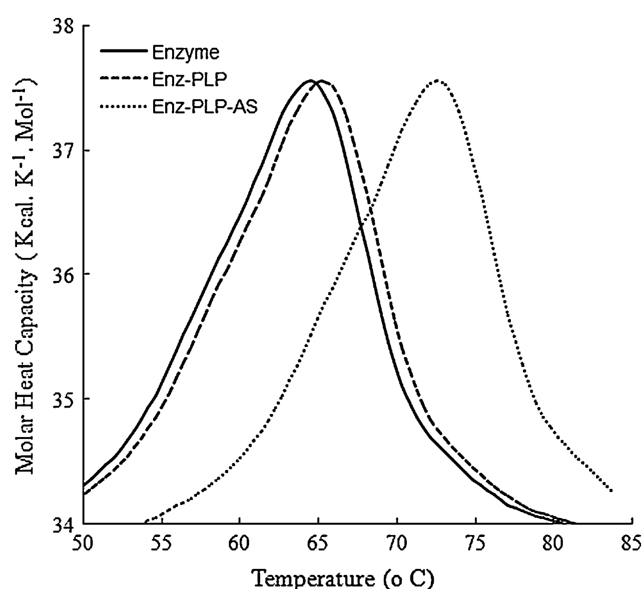

Fig. 9. Heat-capacity scans of the non-modified endoinulinase (control) and modified endoinulinase without (En–PLP) and with reduction (En–PLP–As) by ascorbic acid. The standard CpCalc software package and the data acquisition program DSC Run were employed for data analysis and evaluation of T_m values.

4. Conclusion

We used a novel Schiff base reduction strategy for the preferential modification of surface-accessible lysine residues of endoinulinase by PLP. Aldimine linkages were reduced using ascorbic acid, which resulted in activity retention and, most importantly, enhanced the thermostability of the enzyme. The modification was observed to be a function of time and modifier concentration. In most studies, enzyme modification by PLP resulted in inactivation [16,17,24,25,33,35] because of the presence and involvement of the modification target at the enzyme active site [24,26] or because of conformational effects [36] in catalytic domain. Treatment of endoinulinase with PLP led to rapid inactivation, and at a PLP concentration equivalent to the optimum mole ratios of the modifier per mole of protein (1.37), reactivation was achieved at an observable rate. This may be because nonspecific/noncovalent interactions are possibly replaced by specific/covalent interactions during the time course of treatment with PLP. Nonspecific/noncovalent interactions cause endoinulinase inactivation. However, in the absence of lysine residues at the endoinulinase active site, reactivation was achieved at appropriate (or less) mole ratios of the modifier to the protein. Increase in the number of solvent-accessible lysine residues at the C-domain (non-catalytic domain) of endoinulinase resulted in thermal stability of the enzyme, and the activity was restored (or increased). Thus, the discussed modification strategy, as a semi-rational protein domain engineering method, could be used to provide enzymes that are more suitable for industrial applications.

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