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Stability and solubility studies of native and activated *Aspergillus awamori* feruloyl esterase

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ABSTRACT

Feruloyl esterases can break the ester linkage between ferulic acid and the attached sugar of feruloyl polysaccharides, releasing ferulic acid and oligosaccharides from plant cell. The activity of native and activated feruloyl esterase (FAE-II) from *Aspergillus awamori*, which catalyzes the sugarcane bagasse hydrolysis, was studied using two analytical techniques (HPLC and UV–vis spectrophotometry). The activation of native feruloyl esterase (FAE-II), by its binding with calcium ion, resulted in an increase of V_{max} of two times, and a higher sensitivity of the enzyme to product and guanidine hydrochloride denaturation, with almost no change in the K_m for ferulic acid. The kinetic and thermodynamic parameters associated with the activation of FAE-II enzyme by the interaction with calcium ions were calculated and explained to show how calcium ions are involved. Also, the denaturation effect of guanidine hydrochloride (GdnHCl) towards FAE-II feruloyl esterase was studied using far UV-circular dichroism spectra. In addition, the densities and solubility of the activated FAE-II enzyme by calcium ions were determined and discussed under different conditions of temperatures and pH medium.

1. Introduction

Over the past three decades, the chemistry and biochemistry of feruloyl esterases have attracted considerable attention, due to their catalytic activity. Generally, feruloyl esterases benefit microorganisms, industry, and biochemists [1–3]. The prospect of broad applications of feruloyl esterases has fueled much interest in these enzymes, as shown by the increasing number of feruloyl esterases discovered in microbial organisms in recent years [4–6]. Feruloyl esterases (EC 3.1.1.73) are a subclass of the carboxylic acid esterases (EC 3.1.1) that play a key physiological role in the degradation of the intricate structure of plant cell wall by hydrolysing the ferulate ester groups involved in the cross-linking between hemicelluloses and between hemicellulose and lignin [7–9]. This enzyme belongs to the family of hydrolases, specifically those acting on carboxylic ester bonds. The systematic name of this enzyme class is other names in common use such as ferulic acid esterase, hydroxycinnamoyl esterase, hemicellulase, accessory enzymes, and FAE-III, cinnamoyl ester hydrolase, FAEA, cinnAE, FAE-I and FAE-II [1–5].

The objective of the present study is to evaluate the efficacy of feruloyl esterase (FAE-II) enzyme recovered from *Aspergillus awamori* in the presence and absence of calcium ions. Also, the denaturation effect of guanidine hydrochloride (GdnHCl) towards FAE-II feruloyl esterase was studied using far UV-circular dichroism spectra. In addition, we focused our attention on studying the densities and solubilities of the native FAE-II enzyme in the presence of calcium metal ion at different pH medium under various temperatures (298.15–328.15 K).

2. Experimental

2.1. Feruloyl esterase (FAE-II) enzyme activities

Native *A. awamori* FAE-II used in this study was purified from the fermentation broth of *A. awamori* strain IFO4033 grew in a culture medium of crude wheat straw in our laboratory [10]. The activity of *A. awamori* feruloyl esterase FAE-II was assayed by the analysis of free ferulic acid (FA) released from the hydrolysis of sugarcane bagasse as substrate in water solution using HPLC analytical technique and confirmed by UV–vis spectrophotometry [2,10]. The activity of FAE-II enzyme was determined in the presence and absence of a suspected chemical denaturant guanidine hydrochloride (1.5 mol dm^{-3} GdnHCl in 50 mmol dm^{-3} phosphate buffer (pH ~ 5.5) containing 0.15 mol dm^{-3} NaCl), at 55 °C with varying concentrations of sugarcane bagasse substrate (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 mg sugarcane bagasse per ml water) in the presence and absence of Ca^{2+} ions (0.03–0.5 M). Thermal inactivation was evaluated by measuring the relative activity of

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native feruloyl esterase enzyme (native FAE-II) and activated one by 0.2 mol dm^{-3} calcium ion (activated FAE-II) at different temperatures (288, 298, 308, 318, 328, 338, 348 and 358 K) in water solutions (50 mmol dm^{-3} phosphate buffer (pH ∼ 5.5) containing 0.15 mol dm^{-3} NaCl). The Lineweaver–Burk double reciprocal plot obtained from the rearrangement of the Michaelis–Menten equation [11] was employed to give the best precision for estimating K_m and V_{max}. The catalytic stability of *A. awamori* feruloyl esterase (FAE-II) enzyme was measured by progressive inactivation of the enzyme at various temperatures (298, 308, 318, 328, 338, 348 and 358 K). Applying the thermodynamic equations [11–16], we determine: the energy of inactivation (E_{ai}), half-life of inactivation ($t_{1/2}$), ΔG^* (free energy of activation), ΔH^* (enthalpy of activation), ΔS^* (entropy of activation), ΔG^*_{E-S} (free energy of substrate binding) and ΔG^*_{E-T} (transition state formation) parameters. All experiments were performed in duplicates and the analytical measurements at least in triplicates. Excel program was used to check the statistical analysis of the experimental data. After regression analysis, correlation coefficient of that data was found to range between 0.9742 and 0.9935.

2.2. Far UV-circular dichroism spectra

Circular dichroism (CD) measurements were carried out at 25 °C between 180 and 260 nm (0.2 nm/min) using 1-mm quartz cells in a Jasco 710-spectrophotometer. The cell temperature was controlled to within ±0.1 °C by circulating water via a water bath through a cell jacket. Protein samples (native FAE-II, and activated FAE-II with or without GdnHCl) were prepared in 50 mmol dm^{-3} phosphate buffer (pH ∼ 5.5) containing 0.15 mol dm^{-3} NaCl to a working concentration of 50 μg dm^{-3}. The spectra obtained were averages of 3 scans and baseline corrected by subtracting the reference spectrum. The spectra were smoothed via an internal algorithm in the Jasco software package, J-700 for Windows.

2.3. Solubility measurements

Solubilities of FAE-II protein in the presence of calcium metal ion at three pH medium (4.5, 5.5 and 6.5) under various temperatures (298.15, 308.15, 318.15 and 328.15 K) were obtained from density measurements [17–19]. Each sample vial containing a fixed amount of solvent (water or aqueous electrolyte solution) was added a weighted amount of FAE-II enzyme powder to provide a series of mixtures with increasing composition of protein mass. At least 10 samples were prepared and each vial was then sealed with a Teflon coated screw cap, and the cap was sealed with Parafilm to produce airtight and watertight seal. The vials were completely immersed in a low temperature shaker equipped with a water bath (BT-350R, Yih-Der, Taiwan). The bath temperature was controlled to desired temperature (298.15 ± 0.01, 308.15 ± 0.01, 318.15 ± 0.01, and 328.15 ± 0.01 K) for 36–48 h, with a constant shaken rate. The water bath was housed in a constant temperature chamber, also maintained at (298.15, 308.15, 318.15 and 328.15 K). The weighed samples were prepared such that six to seven vials should eventually result in unsaturated solutions, with the remaining vials at saturated state. After 36–48 h the shaker was stopped, the supernatant of each solution was removed through syringe and filtered by 0.47 μm disposal filter (Millipore, Millex-GS) before subjecting to density measurements. The densities of the solutions were measured by a high precision vibrating tube digital densitometer (Model 4500, Anton Paar, Austria), with an uncertainty of ±5 × 10^{-5} g cm^{-3}, and temperature was controlled to within ±0.02 K. The densitometer has a built-in thermostat for maintaining the desired temperatures within a temperature range of 273.15–363.15 K. The instrument was calibrated with air and degassed distilled water at 293.15 K.

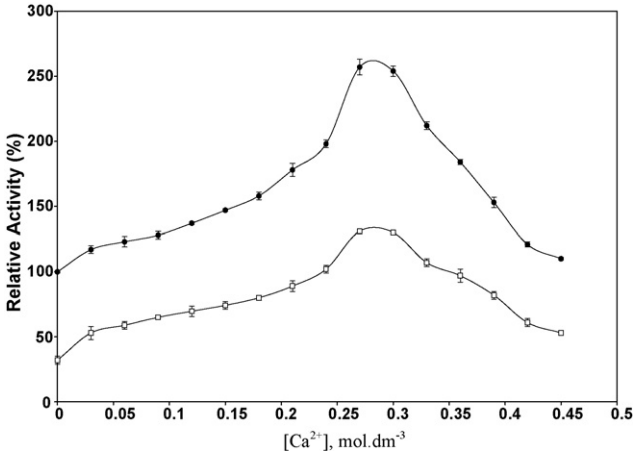

Fig. 1. Effect of calcium ions [Ca^{2+}] on the relative activity of feruloyl esterase for the sugarcane bagasse hydrolyzed reaction in water solution (activation of native feruloyl esterase). (□) With chemical denaturant, GdnHCl = 0.5 mol dm^{-3}; (■) without chemical denaturant.

3. Results and discussion

Experimental data obtained for the effect of calcium ions [Ca^{2+}] on the relative activity of feruloyl esterase for sugarcane bagasse hydrolyzed reaction at 328 K and pH5.5 in water solution in the presence and absence of GdnHCl as enzyme chemical denaturant were represented graphically in Fig. 1. The experimental findings for the thermal stability of native *A. awamori* feruloyl esterase (FAE-II) and activated *A. awamori* feruloyl esterase (FAE-II) by calcium ions were represented graphically in Fig. 2. From the Lineweaver–Burk double reciprocal and Eadie–Hofstee plots we determined the kinetic parameters (Table 1) which include maximal velocity, V_m (μmol/min), catalytic constant, K_{Cat} (min^{-1}), Michaelis constant, K_m (mM), and K_{Cat}/K_m constants.

From the first order plots of thermal inactivation of the enzymes (Fig. 3), Arrhenius (Fig. 4) plots of thermal inactivation of the enzymes (native and activated FAE-II), and Arrhenius plots of thermal activation of the enzymes (Fig. 5), the thermodynamic parameters: ΔH^* (enthalpy of activation), ΔG^* (free energy of activation, ΔS^* (entropy of activation), E_a (energy of activation), E_{ai} (energy of inactivation), ΔG^*_{E-S} (free energy of substrate binding), ΔG^*_{E-T} (free energy of transition state formation), related to that biochemical process (sugarcane bagasse hydrolysis) were

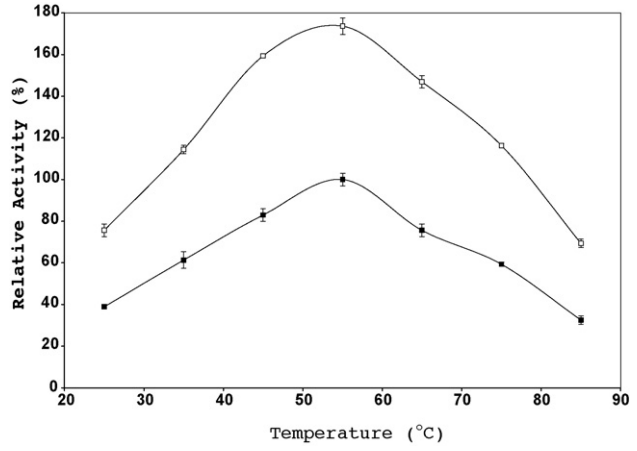

Fig. 2. Temperature stability of native and activated feruloyl esterase enzyme for the sugarcane bagasse hydrolyzed reaction at different temperatures in water solution. (■) Native enzyme; (□) activated enzyme with calcium ions [Ca^{2+}] = 0.3 mol dm^{-3}.

Table 1
Kinetic parameters of native feruloyl esterase (FAE-II) and activated feruloyl esterase.

	V_{max} (μmol min^{-1})	K_{Cat} (min^{-1})	K_m (mM)	K_{Cat}/K_m (min^{-1}/mM)
Without GdnHCl[a]				
Native FAE-II	22.2 ± 0.46	4.44 ± 0.46	2.50 ± 0.15	1.78 ± 0.14
Activated FAE-II	54.05 ± 0.26	10.81 ± 0.46	2.33 ± 0.10	4.64 ± 0.31
With GdnHCl[a]				
Native FAE-II	10.44 ± 0.42	2.09 ± 0.46	2.49 ± 0.10	0.84 ± 0.09
Activated FAE-II	25.25 ± 0.24	5.05 ± 0.46	2.33 ± 0.06	2.17 ± 0.20

[a] Guanidine hydrochloride (1.5 M in 50 mM phosphate buffer (pH 5.5) containing 0.15 M NaCl). The V_{max}, K_{Cat}, and K_m values were determined from Lineweaver–Burk plots. The data given are average values ± standard deviations (S.D.) of $n = 3$ independent experiments.

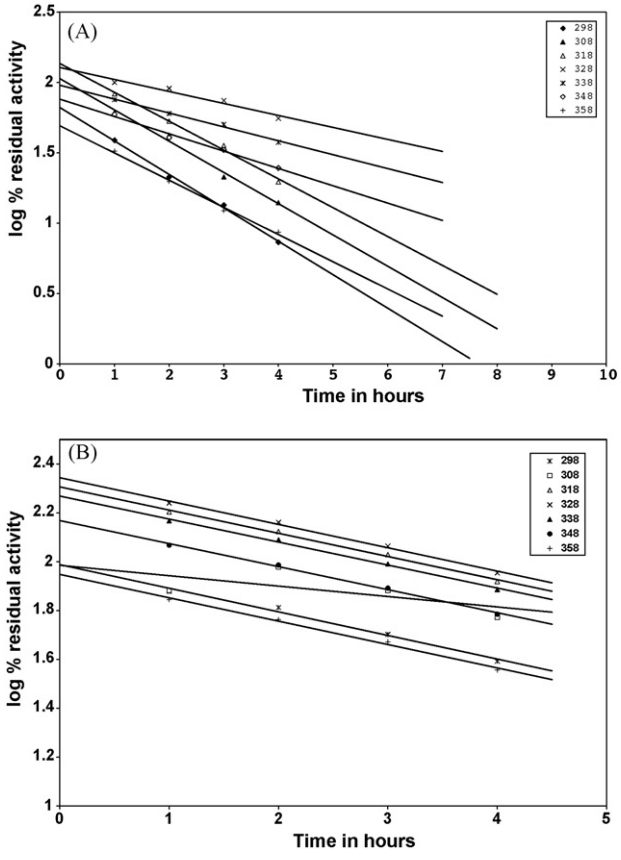

Fig. 3. First-order plots of thermal inactivation of *Aspergillus awamori* feruloyl esterase. (A) Native FAE-II and (B) activated FAE-II. Slopes yield k_i, the first-order inactivation rate constants.

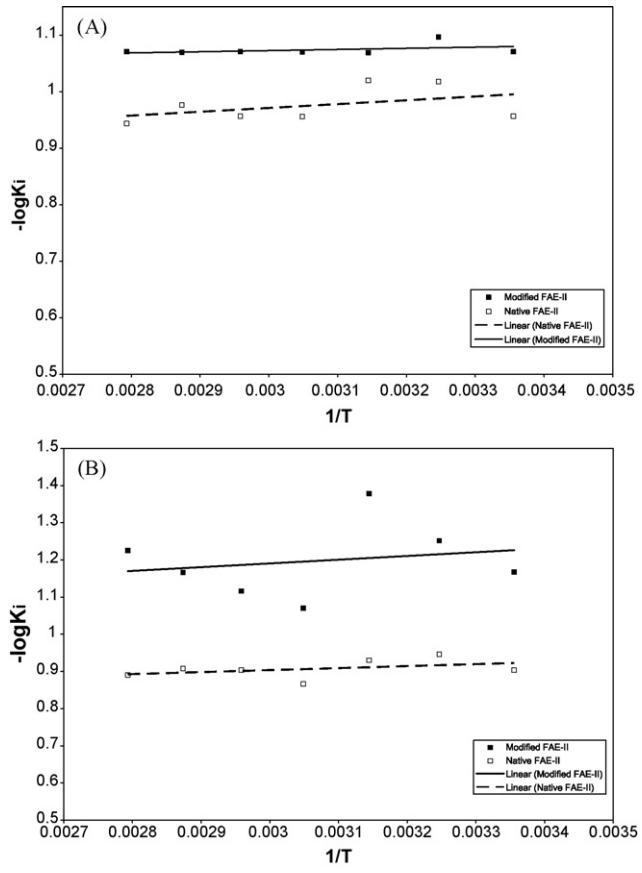

Fig. 4. Arrhenius plots for thermal inactivation for native and activated feruloyl esterase (FAE-II): (A) without GdnHCl chemical denaturant and (B) with GdnHCl chemical denaturant. Slopes of the plots yield E_{ai}, inactivation energy.

determined and listed in Table 2. Temperature-dependent stabilization factors for the activated *A. awamori* feruloyl esterase (FAE-II) were calculated and tabulated in Table 3. Far UV-CD spectra of native and activated feruloyl esterase (FAE-II) with and without GdnHCl, chemical denaturant are shown in Fig. 6. The densities and solubilities of activated *A. awamori* feruloyl esterase (FAE-II) are illustrated in Table 4, and represented in Figs. 7 and 8.

As shown in Fig. 1, one can conclude that calcium ions have a significant effect on the activity of native *A. awamori* feruloyl esterase (FAE-II). The relative activity of FAE-II enzyme in the absence of GdnHCl chemical denaturant increases with increasing calcium ion

Table 2
Thermodynamic parameters related to the sugarcane bagasse hydrolysis reaction catalyzed by native feruloyl esterase (FAE-II) and activated feruloyl esterase enzymes.

	ΔG^* (KJ/mol)	ΔH^* (KJ/mol)	ΔS^* (J/mol K^{-1})	E_a (KJ/mol)	E_{ai} (KJ/mol)	A (s^{-1})	ΔG_{E-S} (KJ/mol)	ΔG_{E-T} (KJ/mol)
Without GdnHCl[a]								
Native FAE-II	−26.94 ± 0.23	18.90 ± 0.18	139.80 ± 1.32	21.4 ± 0.15	14.67 ± 0.25	43.96 ± 0.24	−0.25 ± 0.07	−1.57 ± 0.1
Activated FAE-II	−24.73 ± 0.19	8.80 ± 0.16	102.23 ± 3.22	11.3 ± 0.12	25.62 ± 0.18	44.84 ± 0.41	−0.17 ± 0.03	−0.42 ± 0.03
With GdnHCl[a]								
Native FAE-II	−28.81 ± 0.17	23.10 ± 0.15	228.38 ± 6.13	25.6 ± 0.14	5.87 ± 0.24	43.21 ± 0.27	−0.24 ± 0.009	0.48 ± 0.01
Activated FAE-II	−20.62 ± 0.20	9.40 ± 0.26	90.43 ± 2.45	11.9 ± 0.10	23.92 ± 0.22	44.08 ± 0.29	−0.19 ± 0.03	−0.21 ± 0.07

[a] Guanidine hydrochloride (1.5 M in 50 mM phosphate buffer (pH ~ 5.5) containing 0.15 M NaCl). The data given are average values ± standard deviations (S.D.) of $n = 3$ independent determinations.

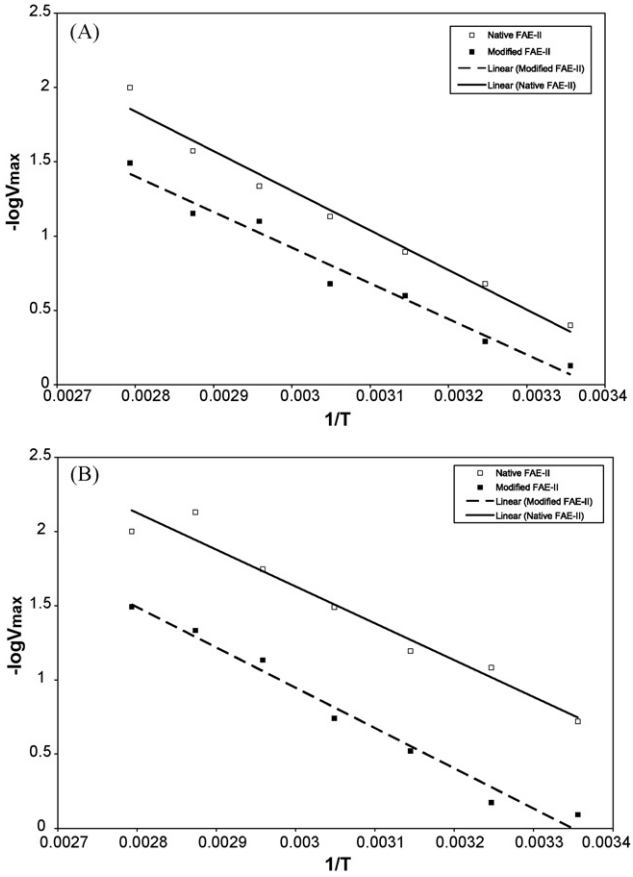

Fig. 5. Arrhenius plots for thermal activation for native and activated feruloyl esterase (FAE-II): (A) without GdnHCl chemical denaturant and (B) with GdnHCl chemical denaturant. Slopes of the plots yield E_a, activation energy.

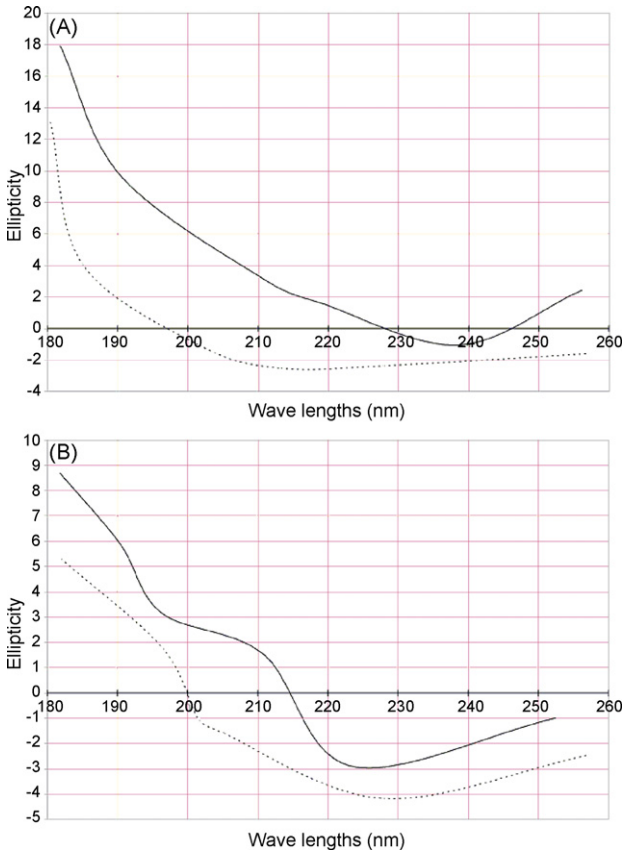

Fig. 6. Far-UV-CD spectra of native and activated feruloyl esterase (FAE-II): (A) without GdnHCl and (B) with GdnHCl. Solid line: activated FAE-II, dotted line: native FAE-II.

concentration added to the native enzyme till a maximum relative activity of 257% is obtained at $[Ca^{2+}] = 0.27\ mol\ dm^{-3}$, and then the relative activity starts to decrease with increasing calcium ion concentration. The same behavior happens in the case with the presence of GdnHCl chemical denaturant, as the relative activity increases to maximum (131%) at $[Ca^{2+}] = 0.27\ mol\ dm^{-3}$, and then starts to decrease with increasing calcium ion concentration.

Thermal inactivation results plotted in Fig. 2 indicates that calcium ions [$Ca^{2+} = 0.2\ mol\ dm^{-3}$] increased the stability of the native FAE-II enzyme. However, the addition of a higher amount of calcium ions ($\geq 0.3\ mol\ dm^{-3}$) induced a decrease in enzymatic thermostability, independent of the amount of Ca^{2+} added. We proposed that the stability arises from the calcium ion contribution to the enzyme tertiary structure. K_m (Michaels constant) is the true dissociation constant of the enzyme–substrate complex. The values of K_m calculated for ferulic acid product from the Lineweaver–Burk double reciprocal plots were found to be almost 2.50, 2.33 mM for the native FAE-II enzyme and activated FAE-II by calcium ions, respectively, in the absence of GdnHCl chemical denaturant. The changes in K_m values were found to be limited.

The calculated maximum velocity (V_{max}) shown in Table 1 indicates that the incubation of native *A. awamori* FAE-II in 0.2 mM Ca^{2+} resulted in approximately twofold increase of V_{max} (V_{max} were $(22.2 \pm 0.46$ and $54.05 \pm 0.26)\ \mu mol\ min^{-1}$ for the native FAE-II enzyme and activated FAE-II enzyme, respectively, in the absent of GdnHCl chemical denaturant; V_{max} were $(10.44 \pm 0.42$

Table 3
Temperature-dependent stabilization factors (SF) of activated feruloyl esterase enzyme.

	T (K)						
	298	308	318	328	338	348	358
Without GdnHCl[a]	2.52	2.24	2.04	3.06	1.92	1.22	1.02
With GdnHCl[a]	1.01	0.99	0.98	1.15	1.03	0.98	0.98

[a] Guanidine hydrochloride (1.5 M in 50 mM phosphate buffer (pH ~ 5.5) containing 0.15 M NaCl). SF = $t_{1/2}$ (activated FAE-II)/$t_{1/2}$ (Native FAE-II). The half-life ($t_{1/2}$) of the enzyme at different temperatures was estimated by incubation at (25, 35, 45, 55, 65, 75 and 85 °C). Aliquots were removed at definite time intervals over a period of 90 min and assayed for activity at 55 °C.

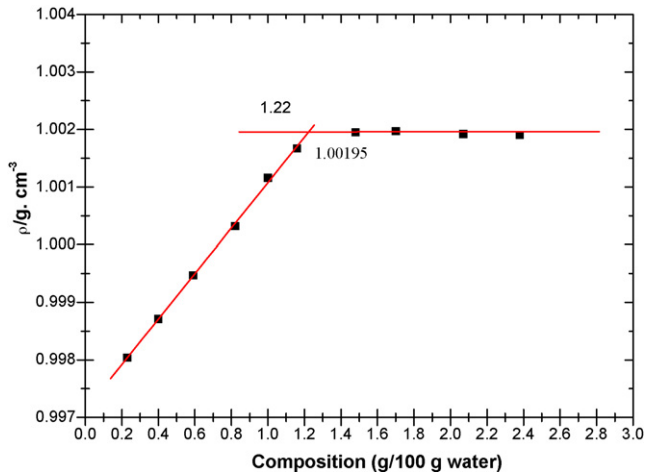

Fig. 7. Density of activated feruloyl esterase (FAE-II) in water solutions at 298.15 K and pH 4.5.

Table 4
Enzyme densities (g cm^{-3}) and solubilities of activated feruloyl esterase (FAE-II) in water solutions.

T (K)							
298.15		308.15		318.15		328.15	
Water	ρ (g cm^{-3})	Water	ρ (g cm^{-3})	Water	ρ (g cm^{-3})	Water	ρ (g cm^{-3})
pH 4.5							
0.23	0.99804	0.25	1.10782	0.27	1.14575	0.28	1.23058
0.4	0.99871	0.44	1.10857	0.48	1.14652	0.48	1.23141
0.59	0.99947	0.65	1.10941	0.7	1.14739	0.71	1.23235
0.82	1.00032	0.9	1.11036	0.98	1.14837	0.99	1.23339
1	1.00116	1.1	1.11129	1.19	1.14933	1.2	1.23443
1.16	1.00167	1.28	1.11185	1.38	1.14992	1.4	1.23506
1.48	1.00195	1.63	1.11216	1.77	1.15024	1.78	1.2354
1.7	1.00197	1.88	1.11219	2.03	1.15026	2.05	1.23543
2.07	1.00192	2.28	1.11213	2.47	1.1502	2.49	1.23537
2.38	1.0019	2.63	1.11211	2.84	1.15018	2.86	1.23534
Solubility = 1.22 g/100 g water		Solubility = 1.33 g/100 g water		Solubility = 1.39 g/100 g water		Solubility = 1.44 g/100 g water	
pH 5.5							
0.25	1.08088	0.27	1.19977	0.3	1.24085	0.25	1.08088
0.43	1.0816	0.48	1.20058	0.52	1.24168	0.43	1.0816
0.64	1.08243	0.7	1.20149	0.76	1.24263	0.64	1.08243
0.89	1.08335	0.98	1.20251	1.06	1.24368	0.89	1.08335
1.08	1.08426	1.19	1.20352	1.29	1.24473	1.08	1.08426
1.26	1.08481	1.39	1.20414	1.5	1.24536	1.26	1.08481
1.6	1.08511	1.77	1.20447	1.91	1.24571	1.6	1.08511
1.84	1.08513	2.03	1.2045	2.2	1.24573	1.84	1.08513
2.24	1.08508	2.47	1.20444	2.67	1.24567	2.24	1.08508
2.58	1.08506	2.84	1.20441	3.08	1.24565	2.58	1.08506
Solubility = 1.27 g/100 g water		Solubility = 1.38 g/100 g water		Solubility = 1.52 g/100 g water		Solubility = 1.87 g/100 g water	
pH 6.5							
0.25	1.18327	0.28	1.21528	0.3	1.25574	0.3	1.34872
0.44	1.18417	0.48	1.2161	0.52	1.25658	0.53	1.34962
0.65	1.18518	0.71	1.21702	0.77	1.25754	0.78	1.35065
0.9	1.18618	0.99	1.21806	1.07	1.25861	1.08	1.3518
1.1	1.18678	1.21	1.21908	1.31	1.25967	1.32	1.35294
1.27	1.18711	1.4	1.2197	1.52	1.26031	1.53	1.35362
1.62	1.18714	1.79	1.22004	1.94	1.26066	1.95	1.354
1.86	1.18708	2.06	1.22007	2.22	1.26069	2.24	1.35403
2.27	1.18705	2.5	1.22001	2.71	1.26062	2.73	1.35396
2.61	1.09608	2.88	1.21998	3.11	1.2606	3.14	1.35394
Solubility = 1.92 g/100 g water		Solubility = 2.18 g/100 g water		Solubility = 2.37 g/100 g water		Solubility = 2.52 g/100 g water	

and 25.25 ± 0.24) μmol min^{-1} for the native FAE-II enzyme and activated FAE-II enzyme, respectively, in the presence of GdnHCl chemical denaturant). The catalytic constant (K_{Cat}) is the first-order rate constant that refers to the properties and reactions of the enzyme–substrate, enzyme–intermediate and enzyme–product complexes. The data presented in Table 1 show that K_{Cat} for activated FAE-II enzyme is two times higher than that for the native FAE-II enzyme with or without GdnHCl chemical denaturant.

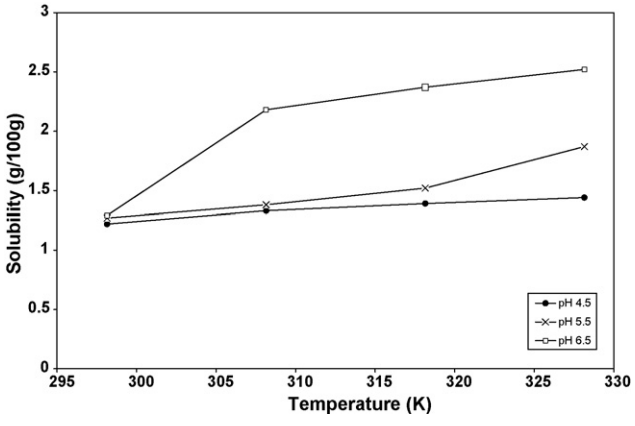

Fig. 8. Effect of temperature and pH medium on the solubility of activated feruloyl esterase (FAE-II).

The specificity constant (K_{Cat}/K_m) is an apparent second-order rate constant that refers to the property and the reactions of the free enzyme and free substrate. The data presented in Table 1 also show that K_{Cat}/K_m for activated FAE-II enzyme is two times higher than that for the native FAE-II enzyme with or without GdnHCl.

Thermodynamic and activation parameters provide a detailed mechanism for many kinds of chemical and biological reactions. It is evident that the inactivation rate is retarded by the calcium ion. In order to understand the process involving the binding of the calcium ion to the enzyme protein, we examined the effect of temperature on the inactivation process. In Table 2, the thermodynamic parameters; ΔH^* (enthalpy of activation), ΔG^* (free energy of activation), ΔS^* (entropy of activation), ΔG^*_{E-S} (free energy of substrate binding), ΔG^*_{E-T} (free energy of substrate binding), related to the sugarcane bagasse hydrolysis reaction catalyzed by native FAE-II enzyme and activated FAE-II enzyme by calcium ion were listed. The examination of the thermodynamic data in Table 2 reveals that:

(i) The free energy of activation (ΔG^*) and the corresponding enthalpy (ΔH^*) and entropy (ΔS^*) values do not change markedly in the temperature range (298–358 K) and all of this

parameters are highest at 328 K, which indicates maximum stability at this temperature.

(ii) A more adequate measure of stability is obtained from E_a, the activation energy of activation process and E_{ai}, the activation energy of inactivation. A large value of E_{ai} and a small value of E_a imply that more energy is required to inactivate the enzyme.

(iii) The positive entropic change is considered to be due to the release of some water molecules from the calcium ion and proteins on the bindings.

(iv) The increment of ΔG^*_{E-S} (free energy of substrate binding) and ΔG^*_{E-T} (free energy of substrate binding) in case of activated FAE-II enzyme than native one with or without GdnHCl chemical denaturant indicates that the activated enzyme work better than the native one.

(v) The frequency factor A, which may be regarded as the frequency of collisions with the proper orientation to produce a chemical reaction, does not changes markedly from the native FAE-II enzyme to the activated FAE-II enzyme.

In the present investigation, the stability of enzymes is defined by their half-life under given conditions. The stabilization factor is the ratio of the half-life after treatment relative to that for the untreated enzyme. Our calculations (Table 3) indicate that stabilization for the activated feruloyl esterase FAE-II was higher in the absence of GdnHCl chemical denaturant than with GdnHCl chemical denaturant.

Circular dichroism (CD) spectroscopy in the far-UV region is a tool to investigate the conformational stability of globular proteins and follow the changes in the secondary structure of biological proteins in solvent composition and in biomolecular interactions and to see whether the structure determined from X-ray crystallography is the same as the solution structure. The structural features of the investigated proteins by means of far-UV-CD spectra show that the secondary structure of both native and activated FAE-II enzyme at 25 °C was markedly changed with GdnHCl (Fig. 6A and B). This result implies that FAE-II enzyme is chemically denaturated by the presence of GdnHCl indicating that this enzyme is highly sensitive to GdnHCl. Based on this experimental findings with available structural information [20], we suggested that the weak resistance of FAE-II enzyme against GdnHCl is because GdnHCl has a positive charge delocalized over the planar structure. It is reasonable to suppose that GdnHCl, being preferentially adsorbed on the protein surface due to its preferential interaction with hydrogen bonding groups and can perturb and weaken the optimized electrostatic interactions between charged side-chains.

It well known that, protein solubility in a spiritual medium is a function of solvent environment, such as pH, temperature, ionic strength, and presence of additives and their concentrations [19–22] and relates to surface hydrophobic (protein–protein) and hydrophilic (protein–solvent) interaction. The mechanism of how a variety of additives affect the protein solubility has been poorly understood. Solubilization capability of activated FAE-II enzyme in the presence of calcium ions was determined by measuring its densities in water solution and the data listed in Table 4. From our density measurements for the activated FAE-II enzyme, we can observe how temperature and pH medium affect the solubility of the enzyme. Solubility measurements are tedious but inevitable to correctly characterize the solution properties of the system of interest, which is crucial for the development of reliable crystallization processes for bulk enzyme recovery. We showed that the minimum solubility of FAE-II was always near the *p*I, as has been observed previously [23,24]. The effect of temperature on the protein solubilities in different pH mediums (pH 4.5, 5.5 and 6.5) was given in Table 4. From this table, we see the solubility increases with temperature under different pH mediums and the results are illustrated in Figs. 6 and 7. When the temperature of the solution is raised high enough for a given time, the protein is denatured.

4. Conclusion

One of the primary objectives of enzyme engineering is to produce stable enzymes useful for various biotechnological applications including organic synthesis. Native enzymes can be structurally engineered to suppress their inactivation under different conditions. This can be achieved by mutagenesis, immobilization, or chemical modification. In our experiments we conclude that:

(1) It was found that calcium is unique among metal ions in enhancing the feruloyl esterase activity and also acts to depress the thermal inactivation and chemical denaturation.

(2) The increment of the values of the parameters like V_{max}, K_{Cat}, ΔH^* (enthalpy of activation), ΔG^* (free energy of activation), and E_{ai} of the activated FAE-II over the native one indicates that the catalytic activity of the activated enzyme also increases.

(3) As E_a (the Arrhenius activation energy) decreases and E_{ai} (the activation energy of inactivation) increases, it implies that the native FAE-II enzyme has become a better catalyst.

(4) Briefly, the thermodynamic interpretation attempts to present a clear relationship between the kinetic as well as thermodynamic parameters and calcium binding.

(5) Solubility of activated *A. awamori* feruloyl esterase (FAE-II) could be altered by temperature and pH changes, we conclude that both temperature and pH affect this functional property. Besides, it was also observed that an interaction between both temperature and pH on activated FAE-II enzyme solubility, but the interaction is rather limited near its isoelectric point (pI ~ 5.5).

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