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Kinetic and thermodynamic features of nanomagnetic cross-linked enzyme aggregates of naringinase nanobiocatalyst in naringin hydrolysis

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ABSTRACT

In this work, the structural thermostabilization of the characterized nanomagnetic cross-linked enzyme aggregates of naringinase have been considered. Comparisons have been made between free and immobilized enzyme by the determination of temperature-dependent half-lives ($t_{1/2}$), energy barriers of thermal inactivation ($E_{a(in)}$) process, and thermodynamic parameters (ΔH^*, ΔG^*, and ΔS^*) in a storage thermostability approach. Samples of NM-NGase-CLEAs were treated at different temperatures in the range of 40–80 °C for 90 min. The K_m values of immobilized enzyme was reduced about 10.7 folds compared to the free one. The catalytic efficiency (k_{cat}/K_m) was raised about 10.5 folds after immobilization. Enzyme half-life ($t_{1/2}$) of NM-NGase-CLEAs increased from 18.7 to 52.9 min (about 3 folds) at 80 °C. The thermodynamics study indicated that $E_{a(in)}$ of the free enzyme increased from 38.51 to 49.14 ($KJ \cdot mol^{-1}$) and ΔH^* increased from 35.57 to 46.20 ($KJ \cdot mol^{-1}$) after immobilization, which indicates an increase in the thermostability of this multimeric enzyme after nanomagnetic CLEAs fabrication. The NM-CLEAs of naringinase preserved 73% of its original activity after 10 cycles, which implies strong operational stability. Thus, the developed method for nanomagnetic CLEAs preparation has provided an efficient and simple approach for the productive and reusable nanobiocatalyst together with ease in enzyme handling.

1. Introduction

Naringinase is an intricate enzyme that involves α-L-rhamnosidase (EC 3.2.1.40) and β-D-glucosidase (EC 3.2.1.21). It has a significant role in reducing the bitterness of citrus juices by naringin hydrolysis via two step reactions into prunin and conversion of prunin to naringenin (4, 5,7-trihydroxyflavonone), which is bitterless [1]. A general scheme for the stepwise hydrolysis of naringin by the action of naringinase is outlined in Fig. 1.

Some details of naringinase molecular structure are specified by researchers. In 1984, Gabor and Pittner isolated naringinase from *Penicillium* sp. and reported that this enzyme is a glycoprotein which is electrophoretically homogenous, possesses both α-rhamnosidase (EC 3.2.1.40) and β-D-glucosidase (EC 3.2.1.21) activities based on electrofocussing and SDS-PAGE studies [2]. Schalkhammer and Pittner (1986), determined the holoenzyme and subunit structure for this enzyme by using of gel filtration, electrophoresis and immobilization methods. Based on the resulted kinetic parameters (K_m, V_{max}, and k_i) for glucose and rhamnose, they ascertained the existence of two catalytic sites for naringinase. By chemical modification of both the active site regions, they revealed the existence of an essential tyrosine site for both enzyme active sites. Modification of lysine group at one of the sites increased glucosidase activity by up to 5-fold. [3]. The existence of an enzyme with 2 active sites for naringinase from *Aspergillus niger* has been approved by Roitner et al. [4]. They reported that, The enzyme complex can be separated into various oligomers by gel filtration. The naringinase of *A. niger* appears to be a single enzyme with two active sites, one for the α-L-rhamnosidase activity and the other for β-D-glucosidase activity. The ratio of these activities varies with the protein concentration and the pH [5]. The catalytic mechanism of naringinase follows from glycoside hydrolase (GH) family 78 enzymes. Acidic residues generally function as general acid and base catalysts in glycoside hydrolases. Several negatively charged residues, such as Asp567, Glu572, Asp579, and Glu841, conserved in GH family 78 enzymes, interact with rhamnose, and mutant of these residues have drastically reduced enzyme activity, indicating that the residues are crucial for enzyme catalysis [6]. A naringinase from *Aspergillus aculeatus* JMUdb058 was purified, identified, and characterized by Chen et al., 2013. This naringinase had a molecular mass of 348 kDa and contained four subunits with MWs of 100, 95, 84, and 69 kDa. Mass spectrometric analysis revealed that the three larger subunits were β-d-glucosidases and that the smallest subunit was an α-l-rhamnosidase. The naringinase and its α-l-rhamnosidase and β-d-glucosidase subunits all had optimal activities at approximately pH 4 and 50 °C, and they were stable between pH 3 and 6 and below 50 °C. This naringinase was able to hydrolyze

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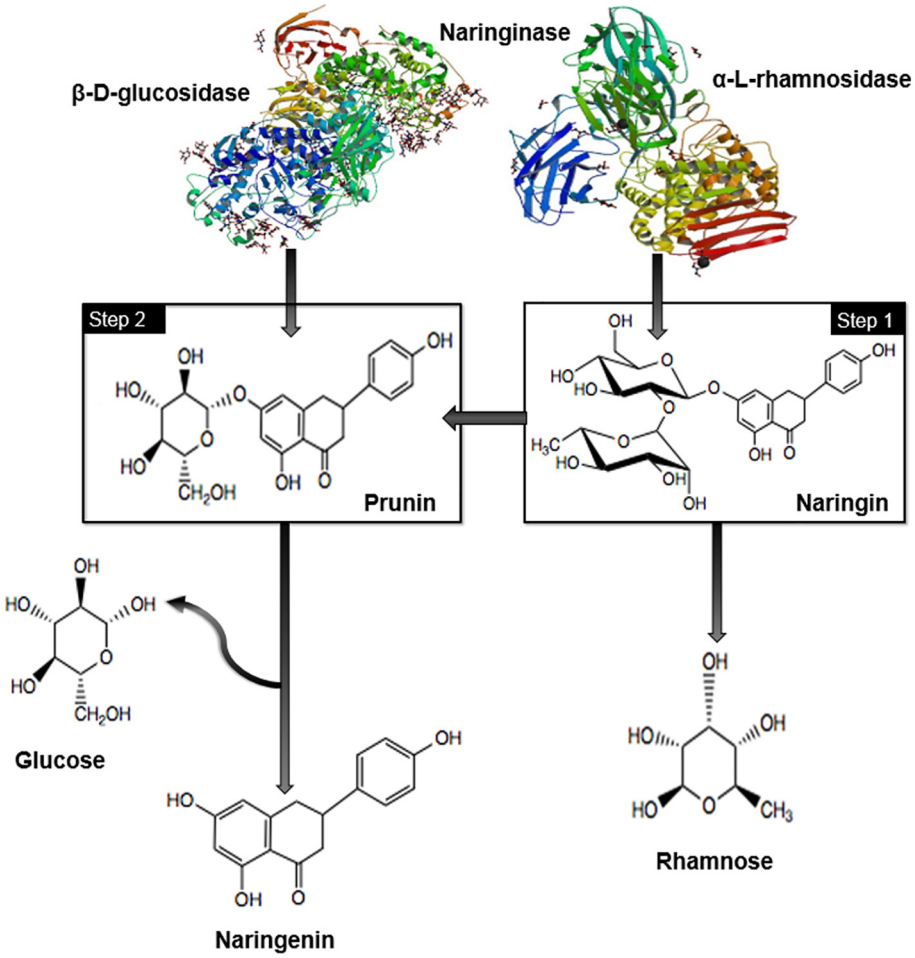

Fig. 1. Schematic representation of stepwise hydrolysis of naringin by the action of naringinase.

naringin, aesculin, and some other glycosides. The enzyme complex had a K_m value of 0.11 mM and a k_{cat}/K_m ratio of 14,034 s^{-1} mM^{-1} for total naringinase [7]. According to mentioned previous studies, naringinase is a multimeric enzyme and dissociation of subunits leads to inactivation of the enzyme. It seems that, multipoint noncovalent assembly between monomers could stabilize the three dimensional structure of each individual monomer correctly assembled in the multimer [8]. Hence, preventing the dissociation of subunits has been recognized as an important strategy to stabilize multimeric enzymes [9]. Use of naringinase in naringin hydrolysis, has become a conventional process in citrus juice industries since, this kind of process conserve the valuable nutrients in the end products. The main reasons for enzyme immobilization are the need to improve the stability, reusability, and easier separation of the biocatalyst compared to the free enzyme [10,11]. On an industrial scale applying of recyclable and thermostable 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 of final product [12,13]. Moreover, in the case of multimeric enzymes such as naringinase, immobilization stabilize the quaternary structure and prevents dissociation-related inactivation of subunits [11,14]. For this reason, enzyme immobilization by using of various methods have been proposed. Among these methods, cross-linked enzyme aggregates (CLEAs), is presented as an efficient, carrier free, and simple way for enzyme immobilization and purification. Easily preparation from crude enzyme extracts, elimination of the expensive carriers, improvement of storage and operational stability against denaturation by heat, organic solvents, and autoproteolysis, high catalyst productivities and possibility to co-immobilize of two or more enzymes to provide CLEAs as well as improved stability, selectivity and specificity compared to soluble enzymes are considered to be the major benefits of this procedure [15]. This process involves cross-linking of physically aggregated enzymes and formation of insoluble, stable and catalytically active CLEAs through bonding between accessible NH_2 groups located on the surface of the enzyme, and aldehyde groups of cross-linking agents such as glutaraldehyde. [16–19]. This reaction leads to establishment of inter- and intramolecular aldol condensations or Schiff's base formation and subsequently, reduction of the Schiff's base by a reducing agents such as cyanoborohydride to form irreversible amine linkages [15]. Most proteins contain several lysine residues that are located at protein surface and are exposed to the aqueous media owing to the polarity of the amine groups. Moreover, lysine residues are generally not involved in the catalytic site hence, during moderate crosslinking, protein conformation and thus biological activity of the enzyme are conserved. Glutaraldehyde exists in multiple forms (monomer, oligomer, and polymer) in aqueous solution, and all of these forms might be reactive toward lysine residues (ε-amino group) of the enzymes [20,21]. In glutaraldehyde-mediated aqueous reaction systems, a reversible equilibrium has been observed between the polymeric, cyclic and open chain forms [22]. Immobilization by CLEAs technique is more effective mainly for complex enzymes than multisubunit covalent attachment [23,24] in preexisting supports owing to stabilizing the quaternary structure of multimeric enzymes [25,26]. Concurrently, this is an excellent method for stabilizing the quaternary structures of multimeric enzymes. [17]. It should be noted that some problems are encountered during industrial application of CLEAs. Particle size that is generally in the region of 5–50 mm which noticeably has a direct effect on mass transfer limitations and

filterability [10]. The increased size of CLEAs clusters causes internal mass-transfer limitations about special accessibility problems for macromolecular substrates, and how to avoid the dramatic modification of some essential ε-amino groups by glutaraldehyde (which results in CLEAs with a significant loss of biological activity) [27]. Due to having quite small pore size, the softness of the CLEAs structure leading to the dispersing of enzyme mass in the solution, and formation of clumps during separation process (e.g. centrifugation and filtration), as well as, difficulties in CLEAs separation from the reaction media [27]. Because most CLEAs are prepared in solution, the particle size and cross-linking efficiency may not be uniform. Large particles may result in diffusion constraints and low catalytic efficiency, and tiny clusters have poor recoverability. CLEAs that consist of small particles cannot be filtered off or recycled. Control of the CLEA particle size and a uniform size distribution are therefore essential to overcome these drawbacks [16]. Among the factors determining the CLEA size, the amount of enzyme and the concentration of the cross-linker play a major role. Both parameters can modify the final result of the biocatalyst [28]. All of these conditions can lead to decrement of catalytic efficiency of the enzyme [25,26]. The low content of lysine in the target enzyme will lead to the failure of CLEAs preparation because it plays an important role in the crosslinking step [29]. Some efforts have been carried out to improve the structure of the CLEAs by various strategies such as use of lysine-rich proteins like bovine serum albumin (BSA), and soy protein isolate (SPI) that have been used as feeder proteins to reduce diffusion problems [30,31] as well as, lysine-rich compounds such as poly-lysine, and/or polyamines [16,32]. Use of a feeder protein such as bovine serum albumin (BSA) that is rich in lysine residues (with 118 accessible surface lysine residues) facilitates CLEAs preparation when the enzyme concentration is low and/or high concentration of glutaraldehyde is used for CLEAs preparation [16]. It can contribute to the formation of CLEAs with high activity, excellent operational stability, and low mass transfer limitation and diffusion problems [27]. The cross-linking efficiency depends on the number of surface lysine residues of the enzyme. An acidic or neutral enzyme with a low content of lysine residues cannot be cross-linked efficiently purely by using a cross-linker. To overcome this problem, coaggregation of enzymes and ionic polymers such as aminated polymer (polyethylenimine, PEI) is performed. Recently metal-ion-chelated PEI is used for immobilization of a multimeric oxidoreductase by CLEAs method [16]. To improving CLEAs stability for repeated usage in continuous processes and ease of CLEAs separation from the reaction media, CLEAs modification by adding magnetic nanoparticles and amino-functionalized magnetic nanoparticles could be performed [33]. Among these methods, preparation of magnetic CLEAs is a favorable techniques for CLEAs preparation owing to ease of separation by an external magnetic field and increasing stability and reusability of the immobilized enzymes. In this case, Martins et al. produced magnetic cross link enzyme aggregates (mCLEAs) of rhamnopyranosidase (Rhmnase) by chemical cross-linking with functionalized magnetite nanoparticles for glycompounds biosynthesis in microbioreactors [34]. Highly stable and easily recyclable hybrid magnetic cross-linked lipase aggregates (HM-CSL-CLEAs) were prepared by Cui et al. via coaggregation of lipase aggregates with nonfunctionalized magnetic nanoparticles and subsequent chemical cross-linking with glutaraldehyde [35]. Accordingly, there is growing interest in developing and improving strategies of CLEAs technology by some efficient approaches [27]. Some of the smart magnetic CLEAs were fabricated by conducting the cross-linking in the presence of functionalized magnetic nanoparticles. These mCLEAs can be used in a magnetically stabilized fluidized bed reactor, bringing novel combinations of bioconversions and downstream processing [10]. Generally, application of CLEAs method on macro/micro scale involves some technical difficulties such as alteration of protein structure, steric hindrance, diffusion rate limitation, and low mechanical stability, and difficulties in handling the gelatinous CLEAs [10]. For overcoming of these drawbacks, enzyme immobilization in nanoscale (1–100 nm) has been several advantages due to large surface area to volume ratios of nanomaterials that mainly increases mass transfer as well as substrate affinity [36]. The necessity of kinetic parameters assessment of the enzymes are to specify the interactions between the enzyme and substrate, the affinity of the enzyme for binding to the substrate molecules, effect of modification or immobilization on the functional properties of the enzyme, to design favorable conditions for achieving an optimal enzymatic process and the explanation of the effect of operational conditions on the structural and functional stability of the enzymes [37]. In the present study kinetic and thermodynamic characteristics of a lysine functionalized nanomagnetic CLEAs of naringinase with a narrow particle size that was reduced by ascorbic acid for Schiff-base formed reduction as a new nanobiocatalyst for naringin hydrolysis were assessed. The effect of this immobilization on kinetic and thermodynamic characteristics of NM-CLEAs of naringinase compared with free one was specified.

2. Experimental

2.1. Materials

Viscozyme L produced from a selected strain of *Aspergillus aculeatus* was supplied by Novozymes (Bagsvaerd, Denmark). Naringin, glutaraldehyde (25% v/v in water), sodium potassium tartrate, 3,5 dinitrosalicylic acid, $FeCl_2$, $FeCl_3$, D(+) glucose, lysine and ascorbic acid were purchased from Merck. All other chemicals were supplied by Merck (Darmstadt, Germany). Perkin Elmer Lambda 25UV/VIS spectrophotometer (USA) was employed for absorbance intensity in cells with 1 cm path length against the blank. All values were expressed as mean ± standard deviation of the three replicate experiments.

2.2. Methods

2.2.1. Enzyme activity assay

Naringinase was estimated for its activity by measuring the amount of glucose released from the two-step hydrolysis of naringin to prunin and rhamnose and then, prunin to naringenin and glucose. The glucose concentration as a reducing sugar was determined using the dinitrosalicylic acid (DNS) microassay at 575 nm (using a Perkin Elmer, Lambda 25 UV/VIS spectrophotometer in cells with 1 cm path length). The absorbance was estimated via the standard calibration curve of glucose. All experiments were repeated at least 3 times to ensure reproducibility. One unit of naringinase activity corresponds to the amount of liberated glucose as a reducing sugar per minute under standard assay conditions [38–40].

2.2.2. Synthesis of magnetic nanoparticles

Nanomagnetite was fabricated by co-precipitation of iron salts in NaOH environment [41]. The co-precipitation method is the simplest chemical process and the most economical, efficient approach to acquire Fe_3O_4 nanoparticles. The MNPs were supplied by mixing $FeCl_3$ and $FeCl_2$ in 2:1 M ratio. The aqueous solutions of Fe^{2+} and Fe^{3+} were made in distilled water. The solution of NaOH was added dropwise to the mixture while keeping the pH at the range of 9.0 to 9.5. Black colored particles of iron oxide were precipitated in the solution at room temperature by strongly stirring with a mechanical stirrer in alkaline medium. Moreover, an N2 gas stream was constantly bubbled through all solutions during the process. The particles were isolated by high-speed centrifugalization at 11357 × *g* for 20 min, washed with distilled water for 3 times. Lastly, the attained particles were dispersed in the distilled water by an ultrasonic bath [42,43]. The FE-SEM analysis was accomplished by EDX evaluation of the supplied MNPs.

2.2.3. Functionalization of magnetic nanoparticles by lysine addition

MNPs have a large surface area/volume ratio, which leads to particle aggregation; consequently, the surface energy decreases owing to strong magnetic attractions between particles, inhibiting particle

dispersion in aqueous solutions. Some effective protection techniques such as coating were employed to overcome this limitation. Various materials have been involved in this process, such as polylysine, BSA, 3-amine propyltrimethoxysilane (APTMS), 3-aminopropyl triethoxysilane (APTES) a toxic compound with health hazards [44]. In this research, lysine with reactive ε-NH2 groups were applied for coating the MNPs [45,46]. The appropriate amount of lysine was dissolved in 50 ml 100 mM sodium acetate buffers (pH 5.0); then, the water-based Fe_3O_4 MNP equal to the amount of lysine was injected to the lysine solution. After that, fast mechanical stirring was carried out for lysine binding directly onto Fe_3O_4 nanoparticles at room temperature. Then 24 h later, the Fe_3O_4@lysine nanoparticles acquired were separated centrifugally at 11357 × *g* and washed with distilled water to eliminate the unreacted lysine as much as possible. Interaction between Fe_3O_4 and lysine should be carried out in reactive forms in aqueous solution. It is specified that, the zeta potential of Fe_3O_4 at pH of approximately 7.5–7.7 is around zero (isoelectric point) and the particles are aggregated [47,48]. Below this isoelectric point, the surface of MNPs is positively ($\equiv Fe\text{-}OH_2^+$) and above isoelectric point is negatively ($\equiv Fe\text{—}O^-$) charged respectively. In L-lysine structure there is an α-carboxyl group ($—COO^-$), an α -amino group (NH_3^+), and a side-chain amino group (NH_3^+). Depends on pH of the solution, the charged state of these groups is altered. The dissociation constants (pK_a) of the acidic carboxyl group and the basic amino groups are 2.2, 9.1 and 10.8, respectively. Based on data reported by Viota et al. and Antal et al., the maximum adsorption of L-lysine onto magnetic nanoparticles are achieved when the pH of the solution is between 3 and 5 [47,48]. Since, our research is operated in 100 mM sodium acetate buffer pH 5.0, it is expected that, bonding between lysine and Fe_3O_4 is formed through negatively charged α-carboxyl group ($—COO^-$) of lysine and positively charged ($\equiv Fe\text{—}OH_2^+$) of nanomagnetite. In this way, the presence of free NH_3^+ groups on the surface of MNPs are conserved for conjugation with naringinase CLEAs by reaction between glutaraldehyde (cross-linker) and accessible surface lysine amino groups of the enzyme.

2.2.4. Preparation of nano-magnetic CLEAs of naringinase

Fabrication of NM-Ngase-CLEAs was performed by aggregation of naringinase-lysine (0.0133 mg ml^{-1} enzyme along with 0.133 mg lysine monohydrate) accompanied by simultaneous addition of lysine functionalized Fe_3O_4 in tert-butanol (enzyme/tert-butanol ratio was 1:9), 10 mM purified glutaraldehyde (distillation at 70 °C, 236 mmHg with a A235/A280 ratio of 0.117), use of ultrasonic bath (200 Hz) for 10 min at room temperature and holding cross-linked nanostructures at 3–4 °C for 3 h. Then prepared NM-NGase-CLEAs was separated from the supernatant by centrifuging at 11357 × *g* and washing with 1000 μl phosphate buffer (pH 7) three times.

2.2.5. Characteristics of free and NM-CLEAS of naringinase

These specifications were determined in previous our reports [49] as follow:

The optimal conditions for the immobilization process required 10 mM purified glutaraldehyde, 1 to 10 ratio of enzyme to lysine, and 3 h crosslinking at 3–4 °C. The activity of free naringinase was 107.76 U/mg that was decreased after immobilization to 93.23 U/mg (about 13.5%). The pH optimum of free and immobilized enzymes for naringin hydrolysis was found to be 5.5. The optimum temperature of immobilized enzyme was shifted upward by 10 °C since the NM-NGase-CLEAs reached their maximum activity at 60 °C, while the maximum activity of free naringinase was obtained at 50 °C. The morphology of the NM-NGase-CLEAs implied a non-uniform, semi-pyramid and semi-cubic rods. The dynamic light scattering (DLS) results showed that the nanomagnetite particle size was around 81.9–96.5 nm, with a polydispersity index (PDI) of 0.238. After NM-NGase-CLEAS formation, the particle size was reduced to around 13.2–15.3 nm, with PDI of 0.177, respectively. Moreover, the Ζ-potential of −28 mV also confirms the improvement of CLEAs stability [49].

2.2.6. Thermostability and enzyme half-life

The thermostability designation of the free and immobilized naringinase was carried out in the absence of naringin as the enzyme substrate with residual activity assessment at five representative temperatures: 40, 50, 60, 70, and 80 °C respectively. For this reason, both the free and immobilized enzymes were incubated in 100 mM sodium acetate buffer at pH 5.5 in the absence of substrate at similar conditions. Samples were withdrawn at proper periodic stages up to 90 min from the incubated enzymes. Afterwards, hydrolysis reaction was performed by adding 100 μl (0.0133 mg) of incubated enzyme to 900 μl of naringin (1% w/v) in 9000 μl of 0.1 M sodium acetate buffer at pH 5.5 and enzyme activity assay was carried out by DNS method at 575 nm. The relative activity was defined as the ratio of the residual activity to the initial activity. Following, based on the data captured by thermostability study, the inactivation rate constants (k_d) were determined and the apparent half-lives ($t_{1/2}$) were estimated by using Eq. (1).

$$t_{1/2} = Ln2/k_d \tag{1}$$

The half-life of free and immobilized enzyme is the time in minute, needed for 50% initial activity loss [50].

2.2.7. Kinetic parameters determination

The Lineweaver-Burk plot (double reciprocal) procedure was employed to acquire the Michaelis-Menten kinetic models for the description of the hydrolysis of naringin by the free and the immobilized naringinase. The apparent values of K_m (substrate concentration at which V_o equals 1/2 Vmax) and V_{max} (maximum velocity) were measured by plotting 1/[S] against 1/V, respectively.

$$[S]/V_0 = 1/V_{max}.[S] + K_m/V_{max} \tag{2}$$

where [S] is the substrate concentration (naringin), V_0 is the initial enzyme velocity, V_{max} is the maximum enzyme velocity, and K_m is the Michaelis constant and is defined only in experimental terms and equals the value of [S] at which V_0 equals 1/2 V_{max}. The assay mixture comprised 0.0133 $mg.ml^{-1}$ free and immobilized enzyme NM-NGase-CLEAs and numerous naringin concentrations (0.25–5 mg mL^{-1}) in sodium acetate buffer (0.1 M, pH 5.5) at 50 °C for 60 min [38,51,52]. The turnover number (k_{cat}) of the free and immobilized enzyme was determined based on the calculated values of V_{max} and $[E]_0$ (the concentration of enzyme active sites) of free and immobilized nariginase was estimated by using the Eq. (3)

$$k_{cat} = V_{max}/[E]_0 \tag{3}$$

where $[E]_0$ is the concentration of enzyme active sites.

$$[E]_{0\ (\text{Free enzyme})} = 0.668 \times 10^{-6}\ \mu\text{mol}$$

$$[E]_{0\ (\text{NM-NGase-CLEAs})} = 0.633 \times 10^{-6}\ \mu\text{mol}$$

$[E]_0$ of free and immobilized naringinase was estimated based on the final enzyme protein in reaction over the molecular mass of naringinase (1.99×10^5 g/mol).

2.2.8. Thermodynamic parameters determination

The thermodynamics of the thermo-inactivation of free and immobilized naringinase was determined by calculating the first-order rate constants for deactivation (k_d) at different temperatures (40–80 °C). Then, using the Arrhenius plot obtained by plotting the natural logarithm of k_d versus the reverse of absolute temperature afterwards, the Eyring's absolute rate equation was employed, derived from the transition state theory [53,54].

$$k_d = (k_B\ T/h)\ e^{(-\Delta H*/RT)}.e^{(\Delta S*/R)} \tag{4}$$

where, h represents the Planck's constant (6.63×10^{-34} Js), k_B is the Boltzman's constant (1.38×10^{-23} JK^{-1}), R is the gas constant (8.314 JK^{-1} mol^{-1}), N is the Avogadro's number (6.02×10^{23} 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. (5)–(7).

$$\Delta H^* = E_{a(in)} - RT \quad (5)$$

$$\Delta G^* = -RT \ \ln (k_d \cdot h)/(k_B \cdot T) \quad (6)$$

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T \quad (7)$$

The energy barriers of thermoinactivation, $E_{a(in)}$, relevant to the free and immobilized naringinase were estimated by applying the Arrhenius equation (Eq. (8)) and the plot.

$$\ln k_d = -E_{a(in)}/RT \quad (8)$$

2.2.9. Reusability of NM-CLEAs

Enzyme immobilization supplies an interesting opportunity for multiple use of the same biocatalyst. The main purpose for the synthesis of NM-CLEAs is to finally design suitable reusable immobilized enzymes that can be easily eliminated from the reaction medium during downstream processing, with maximum recovery of enzymatic activity for reducing production costs of the industrial processes. To evaluate the reusability, The hydrolysis reactions were conducted at 60 °C with naringin solution (1% w/v) as the substrate under the optimal pH of 5.5. and NM-CLEAs was separated from the reaction media by magnetic field and washed three times with 0.1 M phosphate buffer (pH 7). The same procedure was repeated up to 13 times.

3. Results and discussion

3.1. Kinetics study of NM-NGase-CLEAS

The kinetic constants of free and immobilized naringinase were estimated using the double reciprocal plot method (Lineweaver-Burk plots) and the resulted kinetic parameters are illustrated in Table 1.

The calculated K_m for the free enzyme was 1.45×10^{-3} (M) after immobilization, the apparent K_m decreased to 1.36×10^{-4} (M), (10.66 folds decrease), exhibiting that only a small amount of substrate is needed to saturate the enzyme, indicating a high affinity for substrate as well. The decrease in K_m value on immobilization displayed that interaction between enzyme and substrate may have been strengthened by a suitable orientation of the enzyme active site toward the substrate and the matrix structure caused lesser steric limitations thus, the substrate was free to interact with NM-NGase-CLEAs. It can be due to the increased flexibility of enzyme molecules after being cross-linked and bonded with glutaraldehyde. It means that the substrate has not undergone diffusional limitation with respect to CLEAs due to insolubilisation [17].

Beside, reduction in mass transfer limitation due to nano size CLEAs preparation, and the presence of Fe_3O_4 nanoparticles increases hydrophobicity of immobilized enzyme and improve the binding efficiency of substrate [55].

A 10.7 folds decrease in the K_m value after immobilization by CLEAs method can confirm the claim that, this kind of enzyme immobilization should stabilize the quaternary structure of multimeric enzyme (e.g., naringinase) perhaps due to appropriate subunit assembly of the enzyme [8].

Immobilization is in many instances associated with a decrease in enzyme activity because of structural alteration to some degree, due to unspecific enzyme support interactions or the interactions that cause the immobilization. Immobilization in most cases, will produce slight distortions in the enzymes structure, and this may alter the final properties of the enzyme [56].

The immobilized enzyme was characterized by lower V_{max} in comparison to the free enzyme. It decreased from 2.52 μmol min^{-1} for the free enzyme to 2.35 μmol min^{-1} for the immobilized one. Presumably, the main reason for the reduction of Km value (10 fold) can be attributed to the conformational changes due to immobilization during enzyme aggregation, binding to functionalized nanomagnetic particles, and cross-linking by glutaraldehyde. It seems that, owing to these conformational alterations two active centers of this multimeric enzyme have changed in such a way that, their affinity for binding to the substrate is randomly increased [56]. Due to the presence of a suitable substrate concentration as well as, lack of inhibitors (e.g., sodium acetate above 0.3 M, at pH 5, D-glucose >500 mM, and L-rhamnose >20 mM above pH 7.8) in the reaction media, the effect of substrate or product partition also, inhibitory effect of mentioned compounds does not seem to have considerable influence on the enzyme activity.

The lower K_m value of the immobilized naringinase for naringin makes it a better candidate for naringin hydrolysis. However, it was observed that the K_m value increased from free to immobilized naringinase in some studies. Awad et al. mentioned about 7-folds increasing in K_m value after covalent immobilization of naringinase from *Aspergillus niger* on grafted alginate beads, so the accessibility of the enzyme to the substrate was decreased [57]. Ono et al. reported 1.2-folds decrease in K_m after immobilization of naringinase in tannin-aminohexyl cellulose [58]. Puri and Banerjee mentioned that when naringinase from *Penicillium* sp. entrapped in cellulose triacetate fibers shows higher K_m values than in its soluble form [5]. Şekeroğlu et al. mentioned 1.77-fold increase in K_m after immobilization of naringinase on celite by simple adsorption [59]. Unlike these researches, in our study, the K_m constant considerably decreased compared to the free one by immobilization in nano scale of CLEAs. Partly decrease in turnover number (k_{cat}) of the NM-CLEAs that took a value of 6.19 $\times 10^4$ s^{-1} compared with free enzyme (6.29×10^4 s^{-1}) presumably, could be due to the conformational modification of the enzyme during aggregation and CLEAs formation especially in naringinase that is a complex enzyme with two different activities and active sites (rhamnosidase and glucosidase activities) and lead to make changes in the maximum number of conversions of substrate molecules per second by enzyme active sites. It seems that, after immobilization affinity of the enzyme for binding to the substrate (ES complex) is increased thus, products also binded strongly to the enzyme (EP Complex) and their release rate are slower.

The positive effects of immobilization on K_m increased the catalytic efficiency (k_{cat}/K_m) as well as V_{max}/K_m of immobilized naringinase about 10.5 folds compared to the free enzyme. As catalytic effectiveness of the enzymes improve, their rate constants approach limit between 10^8 and 10^9 M^{-1} s^{-1}. Therefore, owing to the closer k_{cat}/K_m of the NM-Ngase-CLEAs to mentioned values (2.80×10^9 M^{-1} s^{-1}) compared

Table 1
Kinetic parameters of free and immobilized naringinase.

	V_{max} (μmol·min^{-1})	K_m (M)	k_{cat} (s^{-1})	V_{max}/K_m	k_{cat}/K_m (M^{-1} s^{-1})
Free enzyme	2.52	1.45×10^{-3}	6.29×10^4	1.74×10^{-3}	4.34×10^7
NM-NGase-CLEAs	2.35	1.36×10^{-4}	6.19×10^4	1.73×10^{-4}	4.55×10^8

to free enzyme (2.68×10^8 M^{-1} s^{-1}) it can be claimed that, catalytic effectiveness and substrate specificity of the immobilized enzyme is improved [46,47]. This can be explained by the increase in the affinity of the immobilized naringinase to the naringin molecules.

3.2. Enzyme half-life of NM-CLEAs

Thermo-inactivation of the free and immobilized naringinase at 40–80 °C up to 90 min was analyzed to determine the inactivation rate constants or k_d. The half-lives corresponding to each sample were calculated using Eq. (1) and were plotted versus temperature. The results are indicated in Fig. 2 and imply an improved thermostability of the immobilized enzyme compared to the free one.

Table 2
Enzyme half-life of free and immobilized Naringinase at various temperatures.

$t_{1/2}$ (min)					
	40 °C	50 °C	60 °C	70 °C	80 °C
Free enzyme	239	385	462	51	19
NM-NGase-CLEAs	277	433	495	80	53

As indicated in Table 2, $t_{1/2}$ values were decreased by increasing the incubation temperature [37,56]. However, $t_{1/2}$ values of NM-NGase-CLEAs were increased 1.6 and 2.8 folds at 70 °C and 80 °C that was significantly higher than those of the free enzyme at the selected

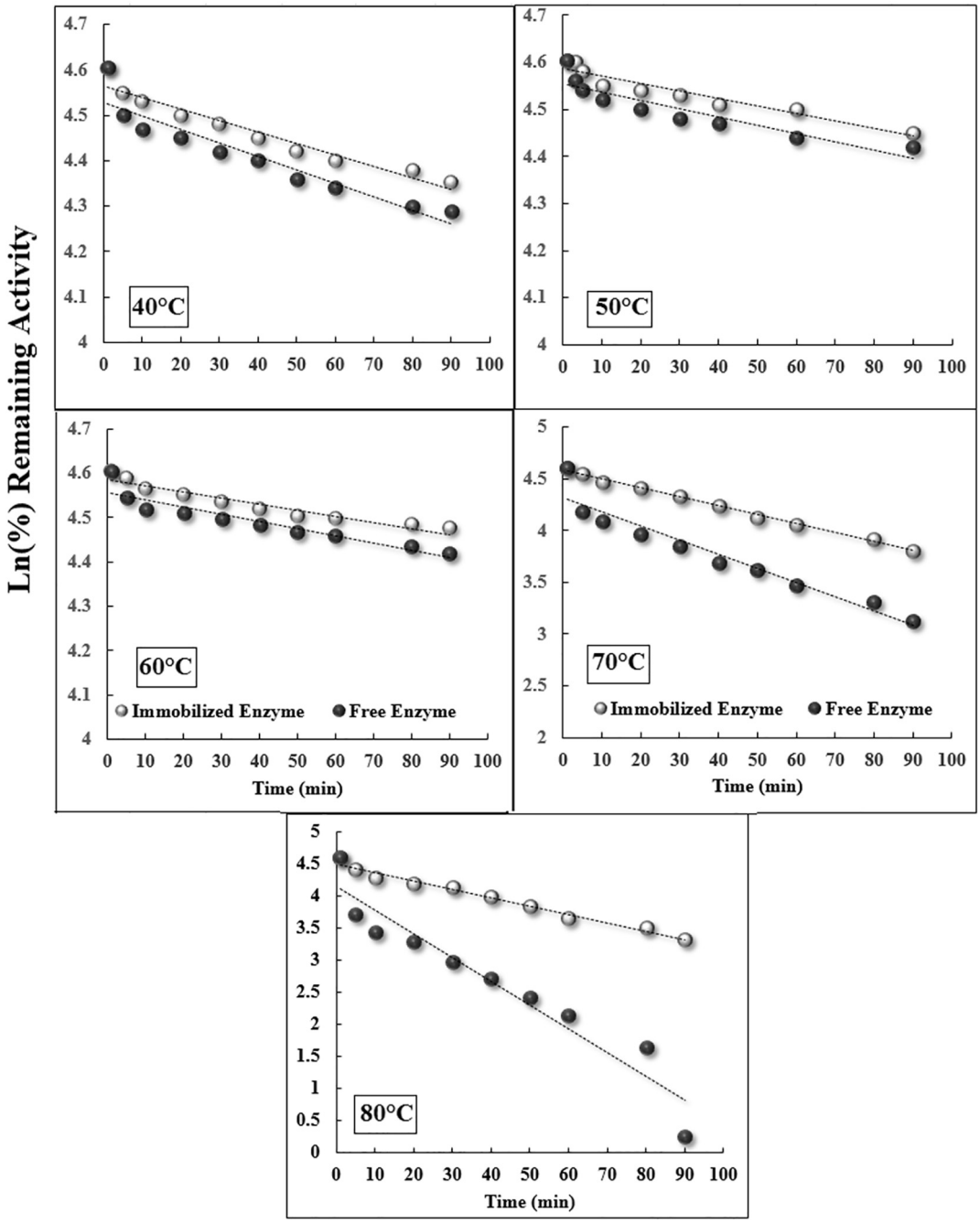

Fig. 2. The natural logarithm of residual activities of the free (●) and immobilized naringinase (○) as a function of the incubation time. All the data presented in this study are average values ± SD of three experiments.

incubation temperatures. As a result, the immobilized naringinase was more stable than the native enzyme (Table 2).

3.3. Thermodynamic study of naringinase NM-CLEAs

The energy barriers of the thermal inactivation ($E_{a(in)}$) are an important indicator of the thermostability of an enzyme. This parameter was obtained by applying an Arrhenius plot to the free and immobilized samples (Fig. 3).

Resulted $E_{a(in)}$ pertaining to the NM-CLEAs was 49.14 ($KJ \cdot mol^{-1}$) that is higher than that of the free enzyme (38.51 $KJ \cdot mol^{-1}$), which approves the stabilization of naringinase through immobilization, especially in the case of applying the cross-linking strategy. The $E_{a(in)}$ was thereafter used to calculate the thermodynamic parameters (ΔH^*, ΔG^* and ΔS^*) of free and immobilized naringinase inactivation at 80 °C using Eqs. (5)–(7). The results are presented in Table 3.

These results indicated that immobilization brings about increases in Ea(in) (27.6%), ΔH^* (29.9%), and decreases in ΔS^*values (28.6%) compared to the free one. No significant changes in ΔG^* were observed after immobilization.

The $E_{a(in)}$ as an important indicator of thermostability and ΔH^* as the amount of heat required for enzyme inactivation were increased through the cross-linking process of the enzyme (Table 3). 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, smaller changes in the free Gibbs energy of naringinase inactivation (ΔG^*) occurred that were not significant [52,53]. There is an assumption that this type of immobilization strategy increases the enzyme's 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, which resulted from improvement of the binding affinity between enzyme and substrate. The reason for further decreases in ΔS^* (28.6%) of immobilized enzyme 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 immobilized enzyme. The neutralization of excessive negative or positive charges on the surface of enzymes by CLEAs immobilization has been shown to increase the thermostability of enzymes. Another reason for the increasing thermostability of naringinase after immobilization could be the formation of new bonds by the Schiff base formation between lysine residue and glutaraldehyde (—C═N), and then the reduction of these bounded by ascorbic acid to strong covalent bonds, which imparts rigidity to the enzyme molecule, thus lowering the ΔS^* value [52,53].

Table 3
Thermodynamic parameters of free and immobilized naringinase.

	$Ea_{(in)}$ ($KJ \cdot mol^{-1}$)	ΔH^* ($KJ \cdot mol^{-1}$)	ΔG^* ($KJ \cdot mol^{-1}$)	ΔS^* ($KJ \cdot mol^{-1} \cdot K^{-1}$)
Free naringinase	38.51	35.57	50.06	−0.042
NM-NGase-CLEAs	49.14	46.20	50.49	−0.012

The amounts of ΔH^*, ΔG^*, and ΔS^* are calculated at 80 °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.

3.4. Reusability

The efficiency of reusing naringinase NM-CLEAs was evaluated up to 13 cycles (Fig. 4). The NM-CLEAs of naringinase preserved 73% of its original activity after 10 cycles, which implies strong operational stability.

4. Conclusions

Nanomagnetic CLEAs of naringinase was prepared as a new efficient nanobiocatalyst for naringin hydrolysis with a narrow particle size. Kinetic studies implied that, NM-NGase-CLEAs has a greater half-life (about 3 folds) compared with free one at 80 °C besides, about 11 folds reduction in K_m value which represents that, affinity of the enzyme for binding to its substrate is increased may be due to reasonable subunit assembly of this multimeric enzyme. The catalytic efficiency and substrate specificity of the immobilized enzyme significantly increased which can be attributed to fabrication of CLEAs in nanoscale. Thermodynamic studies revealed that, the energy barriers of the thermal inactivation also enthalpy of the inactivation were raised about 1.3 folds which represents an increase in thermostability of the enzyme after immobilization as well as, decrement in enthropy of the inactivation is accompanied with the thermostabilization of the NM-CLEAs. The operational stability of NM-CLEAs of naringinase was strongly increased since, 73% of its original activity was conserved after 10 cycles of reuses. Hence, the developed method for nanomagnetic CLEAs preparation has provided an efficient and simple approach for the productive and reusable nanobiocatalyst besides ease in enzyme handling.

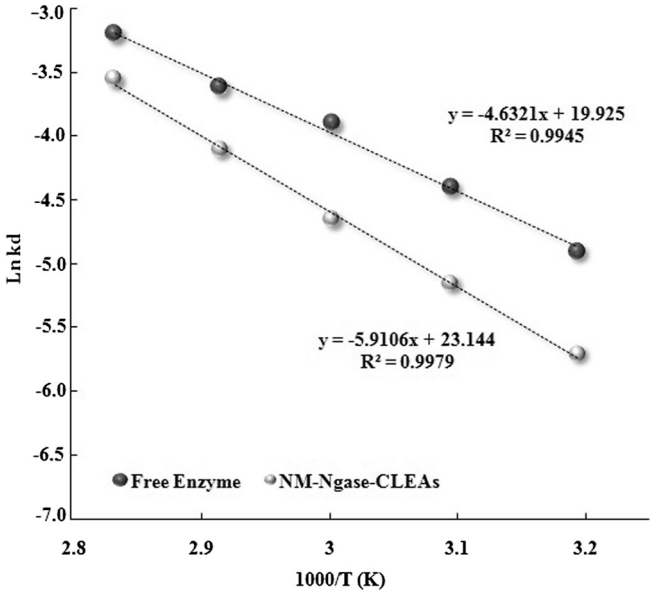

Fig. 3. Arrhenius analysis of the free and immobilized naringinase inactivation rate constant (k_d) as a function of absolute temperature (in Kelvin). (●) free enzyme, (○) immobilized naringinase. Data are average values of three repeats ($n = 3$).

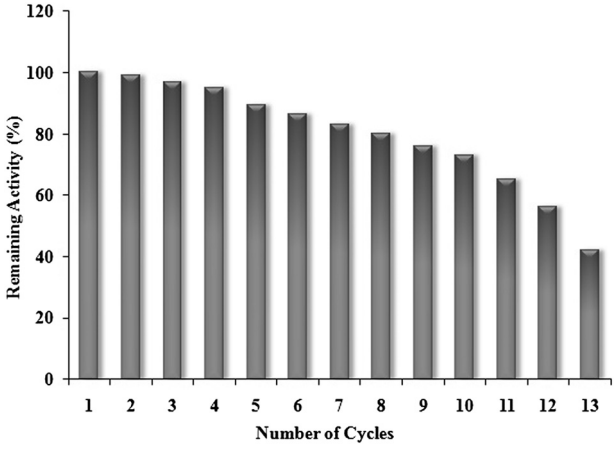

Fig. 4. Effect of the enzyme reuses on the activity of NM-NGase-CLEAs during naringin hydrolysis at pH 5.5 and 60 °C for 90 min. Each treatment was performed in triplicate.

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