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ORIGINAL ARTICLE

Enhancing Enzyme Stability and Functionality: Covalent Immobilization of Trypsin on Magnetic Gum Arabic Modified Fe_3O_4 Nanoparticles

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Abstract

This study aimed to fabricate gum Arabic (GA)–coated Fe_3O_4 nanoparticles bearing numerous active aldehyde groups on their surface, followed by an assessment of their capability as a magnetic support for the covalent immobilization of the trypsin enzyme for the first time. FT-IR, XRD, TGA, and SEM results demonstrated the successful synthesis of GA-coated Fe_3O_4 nanoparticles, along with the covalent immobilization of the enzyme onto the support. Immobilization enhanced the relative enzymatic activity across a range of aqueous solution pH levels (ranging from 4 to 11) and temperatures (ranging from 20 to 80 °C) without altering the optimum pH and temperature for trypsin activity. Kinetic studies using Michaelis-Menten plots revealed changes in kinetic parameters, including a lower V_{max} and higher K_m for immobilized trypsin compared to the free enzyme. The immobilization onto magnetic gum Arabic nanoparticles resulted in an improved stability of trypsin in the presence of various solvents, maintaining a stability order comparable to that of the free enzyme due to the stabilizing effect of the support. The reusability results showed that the immobilized enzyme can retain over 93% of its activity for up to 15 cycles.

Keywords Trypsin · Gum Arabic · Covalent immobilization · Magnetic nanoparticles · Enzymatic stability · Enzymatic activity

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Introduction

Enzymes are catalysts with high effectiveness and versatility in numerous processes. They find widespread use in catalyzing a variety of reactions in organic synthesis and industry. The primary advantages of enzymes, in comparison to chemical catalysts, are their adaptable activity, enantio-specificity, ability to function in mild conditions, and high catalytic efficiency [1–4]. Additionally, enzyme-based catalysts offer economic and environmental benefits over chemocatalysts. Enzymes are completely biodegradable due to their production from renewable resources [5]. However, the use of enzymes in their native form is often constrained by several limitations, such as high cost, high sensitivity to the changes in environmental conditions such as temperature and pH [1, 4]. Moreover, enzymes become inactive in organic solvents, while the use of organic solvent systems instead of aqueous media for many enzymatic reactions offers numerous advantages, such as providing the feasibility of using variety of reactants and substrates that are not soluble or dispersible in aqueous solutions. Despite these challenges, the remarkable catalytic capabilities offered by enzymes for numerous applications have spurred extensive research aimed at addressing these issues [6–9]. One successful and straightforward strategy to overcome these limitations is the immobilization of enzymes onto a support [8, 10–15]. The advantages of immobilized enzymes over their free counterparts include the preservation of activity and stability at various temperatures, pH levels, and even in the presence of organic solvents [16–26]. Additionally, in the case of proteases, the rate of the autolysis process can be substantially reduced through immobilization [27–29].

The literature widely reported the immobilization of enzymes on magnetic nanoparticles [30] due to ease of separation of enzymes from media and reusability. Various polysaccharides, including chitosan, alginate, carrageenan, dextran, and starch, have been utilized to modify magnetic to enhance enzyme stability in the media and reduce agglomeration of the nanoparticles [30–34]. However, surprisingly, the use of gum Arabic-modified magnetic Fe_3O_4 nanoparticles as a magnetically separable support for stabilizing enzymes is very rare in the literature [35, 36]. While, gum Arabic is a branched polysaccharide characterized by its negative charge and remarkable emulsifying properties, has demonstrated exceptional stabilizing effects on nanocarriers in various media, finding widespread applications in the food and pharmaceutical industries [37–39]. Thus, this work aims to delve deeper into the potential of gum Arabic-modified magnetic Fe_3O_4 nanoparticles as an efficient platform for stabilizing and immobilizing enzymes with comprehensive investigations into its activity and stability across diverse media and environmental conditions. By doing so, we seek to broaden the scope of biotechnological applications, particularly those requiring robust, reusable, and efficient enzyme systems.

In this study, the synthesized Fe_3O_4 nanoparticles are first surface modified with GA. Subsequently, the numerous -OH groups on the GA-coated Fe_3O_4 nanoparticles are activated using glutaraldehyde (Fig. 1). These active aldehyde groups on the GA-coated surface readily react with the amino groups of enzymes, enabling their immobilization through strong covalent bonds, as depicted schematically in Fig. 1.

Trypsin is a serine protease in the digestive system of both humans and animals. The proteolytic function of trypsin has found widespread applications in protein chemistry, proteomics, and nutrition research [40]. To the best of our knowledge, this is the first work reporting on the covalent immobilization of trypsin onto magnetic GA nanoparticles, along with an investigation into its activity and stability across diverse media and environmental conditions.

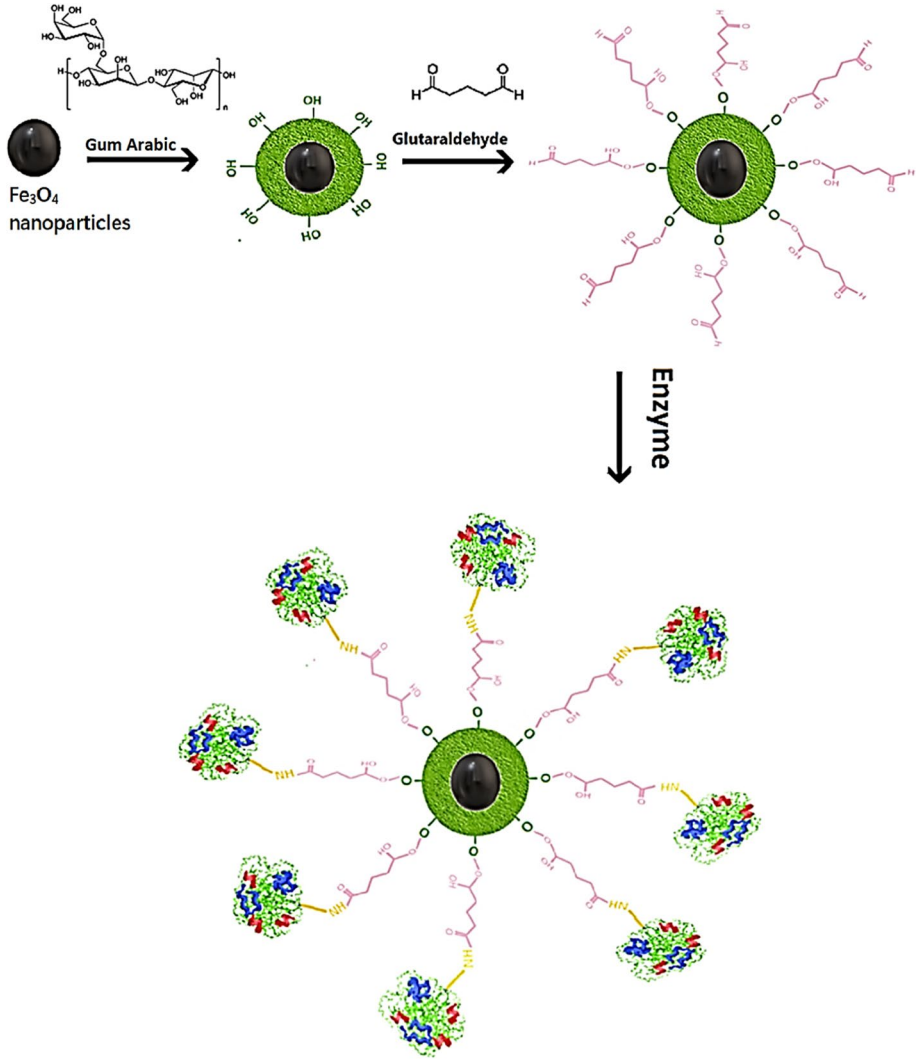

Fig. 1 Schematic representation of the surface modification of Fe_3O_4 nanoparticles with gum Arabic, surface activation with glutaraldehyde, and the trypsin enzyme immobilization on the magnetic support

We expect that the covalent immobilization of the trypsin onto the biopolymer- coated Fe_3O_4 nanoparticles bearing numerous –OH groups (and aldehyde groups after modification) will enhance the enzyme's stability and activity at varying temperatures, pH levels, and even in the presence of organic solvents. The structure and morphology of the gum coated Fe_3O_4 nanoparticle will be characterized using FT-IR, XRD, TGA, and SEM techniques. The enzymatic activity of both the free and immobilized trypsin was evaluated across different aqueous solution pH levels and temperatures. Subsequently, the enzymes' kinetic parameters were studied using casein as a substrate. Lastly, the thermal stability of both free and immobilized trypsin was assessed in the presence of various solvents.

Experimental

Materials

$FeSO_4.6H_2O$ (=99%), $FeCl_2.4H_2O$ (≥99%), ethanol (≥99.9), methanol (≥99.9), dimethylformamide (DMF, ≥99.8), dimethyl sulfoxide (DMSO, ≥99.8), trichloro acetic acid (TCA, ≥99.9), gum Arabic (GA) with a microbiological purity (Total aerobic microbial count (TAMC) ≤1E+03 CFU/g, monosodium phosphate (≥99%), N-propanol (≥99%), ammonium hydroxide (25% aqueous solution), glutaraldehyde (GA, 25% aqueous solution), Coomassie brilliant blue G-250 (≥85%), trypsin (from porcine pancreas) lyophilized, pur. rating: 60% of 100, casein (≥95%) were purchased from Merck (Darmstadt, Germany). Sodium phosphate buffer (50 Mm, Merck) with a pH of 7.5 was used for preparing enzyme solutions. All aqueous solutions were prepared with deionized water and all chemicals were of analytical grade and used without further purification.

Preparation of Magnetic Iron Oxide Nanoparticles

Fe_3O_4 magnetics nanoparticles were prepared using the simple chemical co-precipitation method [41–43]. First, a mixture of $FeCl_2.4H_2O$ (1.0 g, 5.0 mmol) and $FeCl_3.6H_2O$ (2.6 g, 9.6 mmol) was prepared in 100 mL distilled water at 80° C under a N_2 gas atmosphere [44]. After thorough mixing for 60 min, the solution was added dropwise to a 10 mL ammonia solution. When the pH remained constant at about 10, the addition of ammonium hydroxide was stopped, and the resultant black solution was further stirred for 30 min at 80 °C. The black precipitate was collected by magnetic decantation and washed with deionized water repeatedly to remove unreacted impurities. Separately, GA was dissolved in deionized water (5 g/l) at a temperature of 50° C to achieve a homogenous solution. The resultant GA solution was added dropwise into the vigorously stirring magnetic nanoparticles suspension in the presence of GA solution (25%). After stirring for 24 h at room temperature, the resultant GA-modified magnetic nanoparticles were separated from the solution using a magnet, washed repeatedly with distilled water, and dried in a vacuum oven at 50 °C for 24 h.

Immobilization of Trypsin

0.02 g of the dried support was added into 0.45 ml sodium phosphate buffer (50 mM, pH 7.5) and dispersed using an ultrasonic bath for 30 min. The dissolved nanoparticles were then added into 5 ml trypsin solution (3 mg/ml) and incubated at 4 °C for 24 h while shaking. The immobilized enzyme on the support was separated using a magnet and washed with sodium phosphate buffer.

Determination of Protease Activity the Enzymes and Protein Assay

The activity of free trypsin and the immobilized enzyme were evaluated in 50 mM sodium phosphate buffer (pH 7.5) containing 1% (w/v) casein as substrate. Enzymatic reaction was performed with 50 µL of the enzyme solution (1mg/mL). The final volume of reaction was 500 µL. After 20 min incubation, the enzymatic activity stopped by adding 500 µL of 10% (w/v) of TCA. The reaction mixture was centrifuged in 14,000 × g

for 5 min. Finally, the absorbance of the supernatant was determined by a UV–vis spectrophotometer at 280 nm. One unit of protease activity was defined as the amount of the trypsin required to release 1 µmol of tyrosine in 1 min at 25 ºC. Protein concentration was measured by Bradford method using bovine serum albumin as the standard [45]. to determine the amount of free and immobilized enzymes.

Characterization of Support

UV–vis spectra were collected using a UV–vis spectrophotometer (Jinan Hanon Instruments Co., Ltd, China). The particle size and morphology of the samples were investigated by field emission-scanning electron microscope (FE-SEM, MIRA3 TESCAN-XMU, Czech Republic). Fourier-transformation infrared spectrometer (FT-IR, Vector 22, Bruker, USA) was used to investigate the structure of materials. X-ray patterns of samples were recorded by an X-ray diffractometer (XRD, Bruker D8 advance diffractometer, Germany). Thermogravimetric analysis (TGA) of samples was carried out with a thermogravimetric analyzer (Perkin–Elmer Cetus Instruments, Norwalk, CT) at a heating rate of 10 °C/min under N_2 atmosphere.

Investigating the Effect of pH on the Activity of Trypsin

To investigate the effect of pH on the enzyme activity, the activity of free and immobilized enzymes at pH values ranging from 4 to 11 was assessed in 100 mM solutions of sodium acetate buffer for pH 4–6, sodium phosphate buffer for pH 7–8, and glycine buffer for pH 9–11 media containing casein (1% w/v) for 20 min at 25 ºC by using the procedure mentioned above.

Investigating the Effect of Temperature on the Activity and Stability of Trypsin

To study the effect of temperature on the activity of free and immobilized trypsin, an enzyme reaction mixture containing sodium phosphate buffer (50 mM, pH 7.5) and casein (1%, w/v) was incubated at temperature ranges of 20–80 °C, the enzymes activity was then determined as described above.

Thermal stabilities of both free and immobilized trypsin were determined by measuring the residual activity of enzyme exposed to 40 °C in sodium phosphate buffer (pH 7.5) for different incubation times (0–60 min), followed by placing samples on ice bath for 30 min to ensure re-folding of all protein structures. The residual activity was also measured at 25 °C as described above.

The activity of the sample that was placed on the ice bath from the beginning was considered as a control sample and its activity rate was considered to be 100%. The residual activity of the other samples was calculated as a percentage of total activity.

Kinetic Parameters

To determine the kinetic parameters of the free and immobilized enzymes, the trypsin enzyme activity was measured in different concentrations of casein (0.25, 0.40, 0.80, 1.00, 2.50, 5.00, and 10.00 g/L) as described above. The V_{max} and K_m of the enzyme were then calculated by using Michaelis-Menten curve and Lineweaver–Burk equation.

Assessing the Thermostability of Trypsin in Organic Solvents

To study the effect of the presence of organic solvents (DMSO, DMF, ethanol, methanol, and n-propanol) on the thermostability of the free and immobilized trypsin, the enzyme's solutions containing 10%(v/v) of different organic solvents was first prepared. The resulting solution was then incubated at 40 °C for different times (0–60 min) followed by determining residual activity of each sample after cooling in ice bath for 30 min (the control sample was placed in an ice bath from the beginning).

Calculation of Half-Life and k_{in} of the Free and Immobilized Trypsin

To determine the half-life and rate constant of enzyme inactivation (k_{in}), the time course of the decrease in logarithmic values of the enzyme's residual activity was evaluated and demonstrated a linear decrease in residual activity over the course of enzyme incubation (results not shown). This observation suggests a similarity between the enzyme inactivation process and first-order chemical reactions. As a result, Eqs. 1 and 2 were employed to calculate the rate constant of enzyme inactivation (k_{in}) and the corresponding half-lives, respectively.

$$\mathrm{Ln(Activity)} = \mathrm{ln(Activity)_0} - \mathrm{k_{in}t} \tag{1}$$

$$\mathrm{Half-life} = \frac{0.693}{\mathrm{k_{in}}} \tag{2}$$

Study of the Reusability of the Immobilized Trypsin

To assess the reusability of immobilized trypsin, 100 µL of the solution (50 mM sodium phosphate buffer, pH 7.5) containing the immobilized enzyme added to the 400 µL of the solution containing 50 mM sodium phosphate buffer (pH 7.5) and 1% (w/v) casein. After incubation of solution at room temperature (25 ºC) for 10 min, the immobilized enzyme was separated from the solution using external magnet and then 500 µL of TCA (10% w/v) added to the solution. The solution was centrifuged in 15,000 × g for 5 min and the supernatant absorbance measured at 280 nm to determine the enzymes activity. The separated immobilized enzyme washed by sodium phosphate buffer (50 mM, pH 7.5) for three times, and used in the next cycles. The activity of immobilized trypsin in the first cycle of the reaction was considered 100%.

Results and Discussion

FT-IR Spectroscopy

FTIR spectra of the synthesized Fe_3O_4 nanoparticles, neat GA, GA modified Fe_3O_4 nanoparticles, and trypsin immobilized on GA modified Fe_3O_4 nanoparticles are compared in Fig. 2. The strong absorbance peak in Fig. 2a appeared at 567 cm^{-1} was assigned to

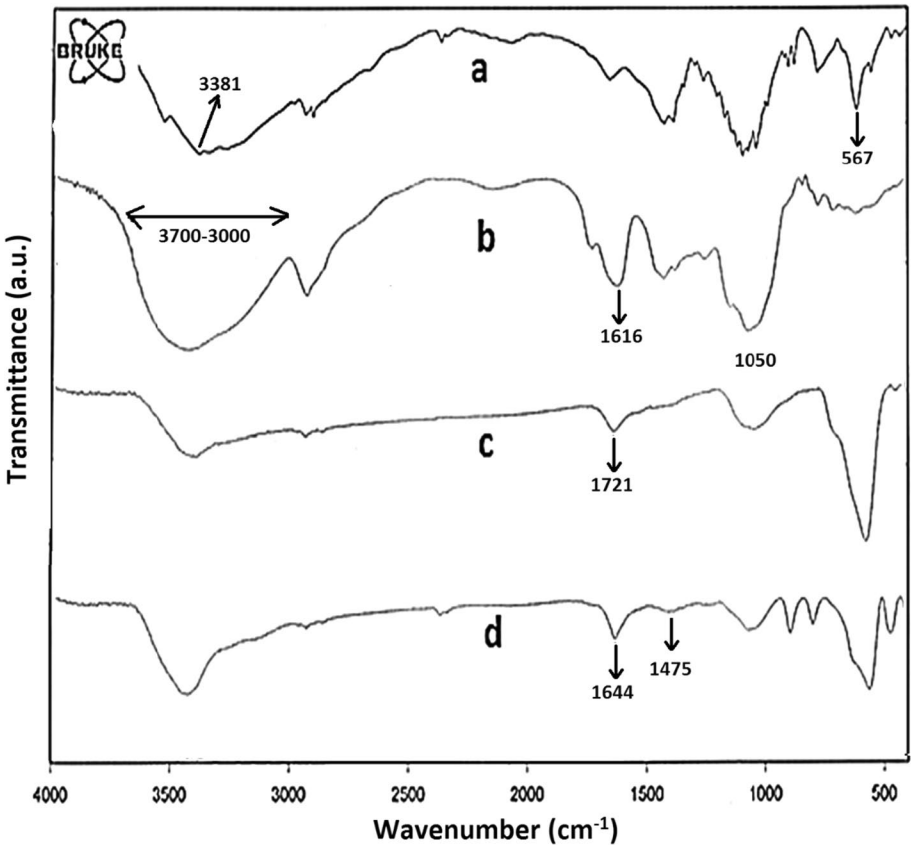

Fig. 2 FTIR spectra of (**a**) Fe_3O_4 nanoparticles, **b** neat GA, **c** GA modified Fe_3O_4 nanoparticles, and (**d**) trypsin immobilized on GA modified Fe_3O_4 nanoparticles

the stretching vibration of Fe–O bonds in Fe_3O_4 [39]. In addition, stretching vibration of hydroxyl groups was located at 3381 cm^{-1}. Figure 2b shows the FTIR spectrum of neat GA. The absorption peak at around 1050 cm^{-1} 1 belongs to the stretching vibration of C-O bonds. The relatively strong peak appeared at 1616 cm^{-1} corresponds to the carbonyl (C = O) of carboxylic acid groups on the structure of the biopolymer [39]. The strong and broad band in the range of 3000 to 3700 cm^{-1} can be attributed to the stretching vibrations of the hydroxyl groups presented in the structure of GA.

Figure 2c indicates the FTIR spectrum of the magnetic nanoparticles coated with glutaraldehyde crosslinked GA. This spectrum shows the characteristic peaks of both neat Fe_3O_4 nanoparticles and GA indicating successful surface modification of the magnetic nanoparticles with GA. Above all, a new peak was appeared at the wavelength of 1721 cm^{-1} in Fig. 2c, which can be attributed to the presence of free aldehyde groups of glutaraldehyde. Figure 2d shows the FTIR spectrum of trypsin immobilized on GA modified Fe_3O_4 nanoparticles. The presence of trypsin enzyme on the matrix is confirmed by the appearance of the peaks in the wavenumbers of 1457 and 1644 cm^{-1}, which are correlated to the stretching vibration of C = O of amide I and the bending vibration of N–H of amide II in the trypsin molecular structure, respectively [46]. Besides, the lack of the

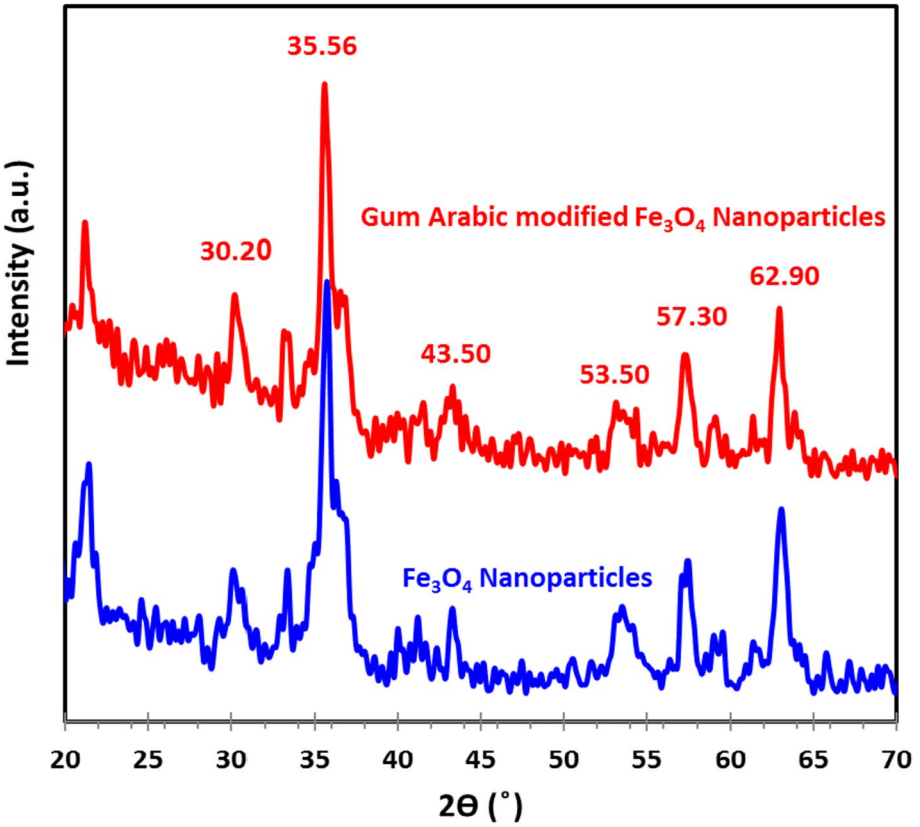

Fig. 3 XRD patterns of Fe_3O_4 nanoparticles and GA modified Fe_3O_4 nanoparticles

peak originated from the free aldehyde groups in the enzyme-immobilized substrate can be attributed to the covalent bond formation between these groups and the amino (or hydroxyl) groups of the enzyme [47–49].

Microstructure Analyses

The XRD pattern of Fe_3O_4 nanoparticles is shown in Fig. 3. The peaks appeared at 2θ values of 30.20, 35.56, 43.50, 53.50, 57.30, and 62.50◦ are the characteristic peaks of the Fe_3O_4, and confirm that magnetic nanoparticles with a highly crystalline cubic spinel structure were successfully formed [50].

After surface modification of magnetic nanoparticles with GA, almost the same characteristic peaks were observed, which indicates that their crystalline structure was preserved during this process. Due to amorphous structure of GA, it has no specific peak in the XRD spectrum [39].

SEM micrographs of Fe_3O_4 nanoparticles and Fe_3O_4 nanoparticles modified with GA are shown in Fig. 4a and b, respectively. Figure 4a shows that Fe_3O_4 nanoparticles have irregular shapes with a size of less than 100 nm. The surface modified magnetic

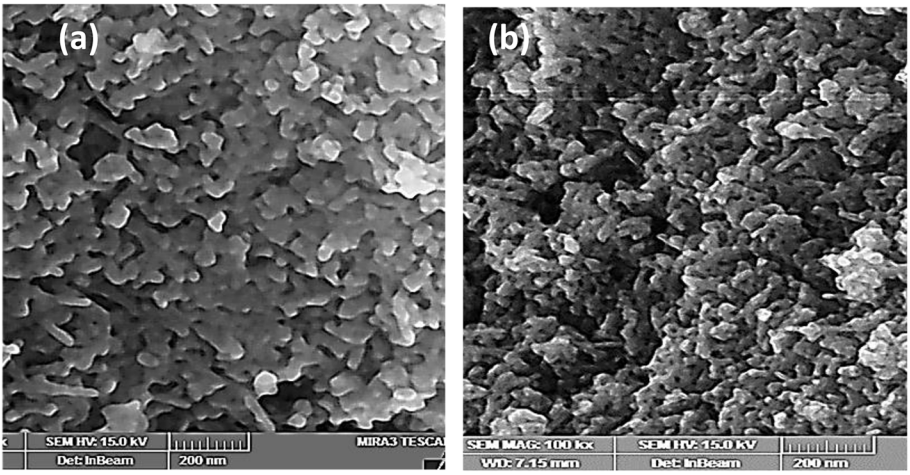

Fig. 4 SEM micrographs of (**a**) Fe_3O_4 nanoparticles and (**b**) Fe_3O_4 nanoparticles modified with GA

nanoparticles exhibit relatively smaller particle sizes (Fig. 4b), which could be attributed to the emulsifying and stabilizing effect of the GA.

Thermogravimetric Analysis Results

Figure S1 shows the thermograms of unmodified and GA modified Fe_3O_4 nanoparticles. The neat Fe_3O_4 nanoparticles showed a slight weight loss up to a temperature of about 200 °C and their weight reached 96.5%, which is most likely related to the evaporation of moisture presented in the sample. Nevertheless, after this temperature, the weight of the sample remained almost constant. Gum Arabic modified Fe_3O_4 nanoparticles

Table 1 Effect of different organic solvents on stability parameters of trypsin

Enzyme form	Solvent	[a]$k_{in}(min^{-1})\times10^3$	Half-Life (min)	LogP
Free	–	13 ± 0.3	53.3 ± 1.2	–
	DMSO	16 ± 0.4	43.3 ± 1.0	−1.34
	DMF	17 ± 0.4	40.7 ± 0.9	−1.01
	Methanol	22.0 ± 0.5	31.5 ± 0.7	−0.74
	Ethanol	23.3 ± 0.6	26.6 ± 0.6	−0.32
	n-propanol	26.0 ± 0.7	23.3 ± 0.6	0.34
Immobilized	–	4.9 ± 0.2	141.0 ± 5.6	–
	DMSO	8.9 ± 0.2	77.8 ± 2.4	−1.34
	DMF	11.0 ± 0.3	63.0 ± 2.0	−1.01
	Methanol	12.0 ± 0.4	57.7 ± 1.9	−0.74
	Ethanol	14.0 ± 0.4	49.5 ± 1.4	−0.32
	n-propanol	15.0 ± 0.4	46.2 ± 1.3	0.34

[a] Rate constant of enzyme inactivation

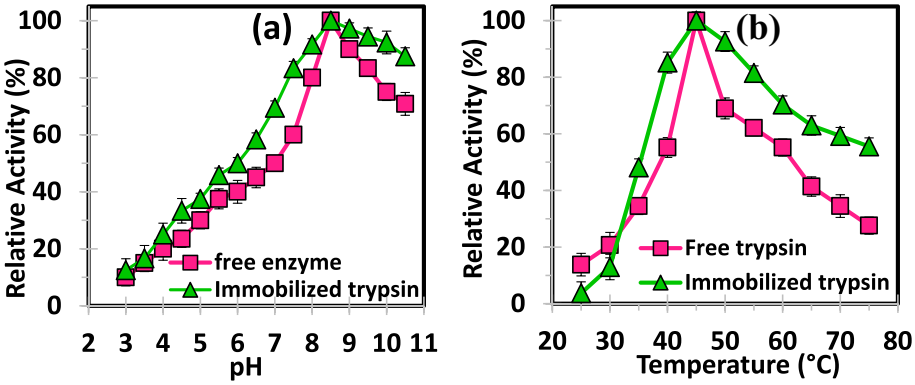

Fig. 5 Effects of (**a**) pH and (**b**) temperature on the activity of free and immobilized trypsin at 40 °C

showed a similar trend up to nearly 200 °C. However, with further raising the temperature, the weight of sample was reached to 80.4% at 600 °C. The weight loss in this temperature range is related to the destruction and thermal degradation of organic part of the sample, i.e., gum Arabic. Based on the difference in the weight percentage of these two samples at 600° C, the amount of gum Arabic on the surface of Fe_3O_4 nanoparticles was obtained to be 16%.

The Effects of pH and Temperature on the Activity of Free and Immobilized Trypsin

Variations in the pH of the reaction medium can significantly impact enzyme activity. To investigate the effect of different pHs on the enzyme activity, the activity of both free and immobilized trypsin was measured at different pHs. According to Fig. 5a, both the free- and immobilized-trypsin exhibit maximal activity at pH 8.5. Figure 5a also shows that the enzyme immobilization slightly increased the activity of trypsin in different pHs. This finding suggests that the employed matrix has not altered the chemical environment of the immobilized enzyme, likely due to the presence of hydroxyl groups on the gum Arabic matrix [51].

In addition, the activity of free and immobilized enzymes in the temperature range of 25 to 80 °C were studied. As shown in Fig. 5b, the highest activity is seen at 45 °C for both the free and immobilized enzymes. At temperatures of 35 to 70 °C, the immobilized enzyme shows higher relative activity than the free enzyme, revealing that the activity of enzyme has been increased at different temperatures by its immobilization on the gum Arabic magnetic nanoparticles without changing the optimum temperature of the enzyme activity. This indicates that magnetic gum Arabic nanoparticles are suitable support for the enzyme immobilization without making any changes in the optimum temperature.

Furthermore, the activity of both free and immobilized enzymes was examined within the temperature range of 25 to 80 °C. As depicted in Fig. 5b, the maximum activity is observed at 45 °C for both the free and immobilized enzymes. Within the temperature range of 35 to 70 °C, the immobilized enzyme exhibits a higher relative activity than the free enzyme. This outcome indicates that enzyme immobilization on the gum Arabic-coated magnetic nanoparticles has heightened the enzyme's activity at various temperatures, while still maintaining the enzyme's optimum temperature for activity. This underscores that magnetic gum Arabic

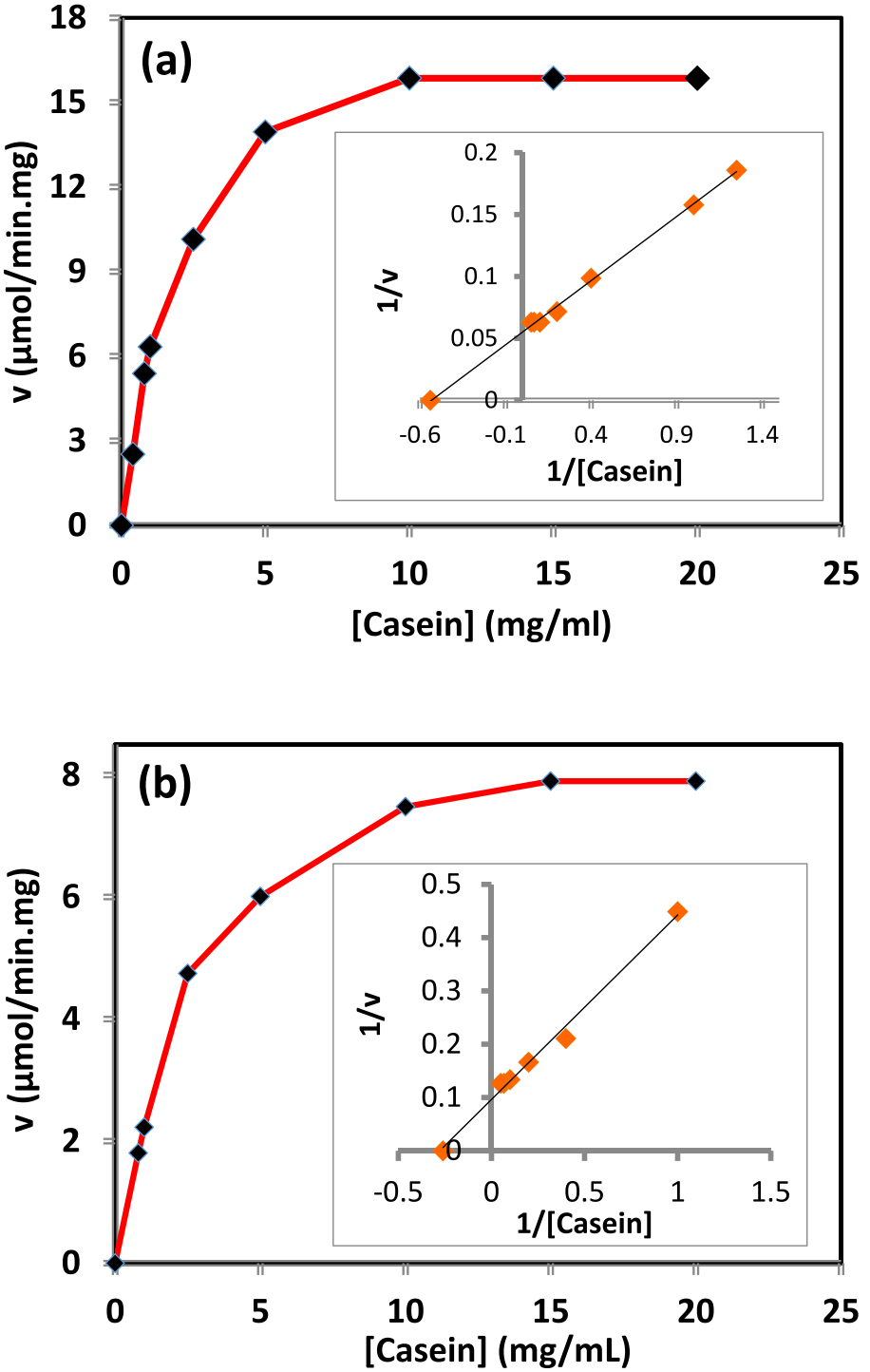

Fig. 6 Michaelis–Menten and Linweaver-Burk (inlets) curves of (**a**) free trypsin and (**b**) immobilized trypsin

nanoparticles serve as a suitable support for enzyme immobilization without altering the enzyme's optimum temperature.

Kinetic Study of Free- and Immobilized-Trypsin

The kinetic parameters of maximum reaction rate (V_{max}) and the Michaelis–Menten constant (K_m) for both free and immobilized enzymes were determined using the Lineweaver–Burk plot in the presence of casein as substrate and the results were shown Fig. 6a and b and Table S1. The immobilized trypsin exhibited a lower V_{max} (10.50 µmol/mg.min for the immobilized trypsin compared to 20.00 µmol/mg.min for the free trypsin) and a higher K_m than free enzyme (2.00 g/L for the free enzyme to 3.65 g/L compared to the immobilized enzyme). Enzyme immobilization rigidifies the enzyme structure and reduces its flexibility, a factor crucial for enzyme activity [52–56]. Thus, the decrease in V_{max} and the increase in K_m for immobilized trypsin could be correlated with the enhanced rigidity of the enzyme and the chemical characteristics of gum Arabic (including the hydrophilic property of the support and the slight hydrophobicity of the substrate).

Thermostability of Free and Immobilized Trypsin in the Absence and Presence of Organic Solvents

The time-course thermal stability of both free and immobilized trypsin was assessed at 40°C. After exposure to 40°C for 60 min, the relative activity of the free enzyme reached 45% of its initial value, while the immobilized enzyme retained 75% of its initial activity, demonstrating a noticeable increase in thermal stability due to immobilization (Fig. 7a). The chemical structure of the supports plays a crucial role in enhancing enzyme stability [52, 57–59]. In this work, the significant increase in the stability of the enzyme can be attributed to the formation of covalent bonds as well as hydrogen bonding between the hydroxyl groups on gum Arabic and trypsin.

The effect of organic solvents on the activity and stability of free and immobilized trypsin were studied at 40 °C over varying time intervals. As shown in Fig. 7b, the stability order of the free enzyme in the absence/presence of 10% solvent is as follows: without solvent > DMSO > DMF > methanol > n-propanol > ethanol. Figure 7b and Table 1 exhibit that the immobilization of the enzyme onto the magnetic gum Arabic nanoparticles enhances the enzyme stability (or enzyme half-life) in both absence and presence of the solvents. and the stability order of the immobilized enzyme is as follows: without solvent > DMSO > DMF > methanol > n-propanol > ethanol (Fig. 7c). Thus, the immobilization of trypsin on gum Arabic enhances enzyme stability following a similar order. The chemical properties of supports or cross-linkers influence the stability of immobilized enzymes when exposed to organic solvents [60, 61].

The immobilization of trypsin onto gum Arabic did not alter the stability order of trypsin in the presence of solvents. Gum Arabic, being a polysaccharide rich in hydroxyl groups, maintains a hydrophilic microenvironment around the immobilized trypsin similar to water. Consequently, this leads to comparable behavior (or stability order) of the immobilized enzyme and the free enzyme in the presence of solvents with varying degrees of hydrophobicity (or log P) [61].

Among the solvents listed in Table 1, DMSO and DMF are highly polar like water, while methanol, ethanol, and n-propanol are moderately polar in comparison. Therefore, the rate constant of enzyme inactivation (K_{in}) in the solvents with moderate polarity

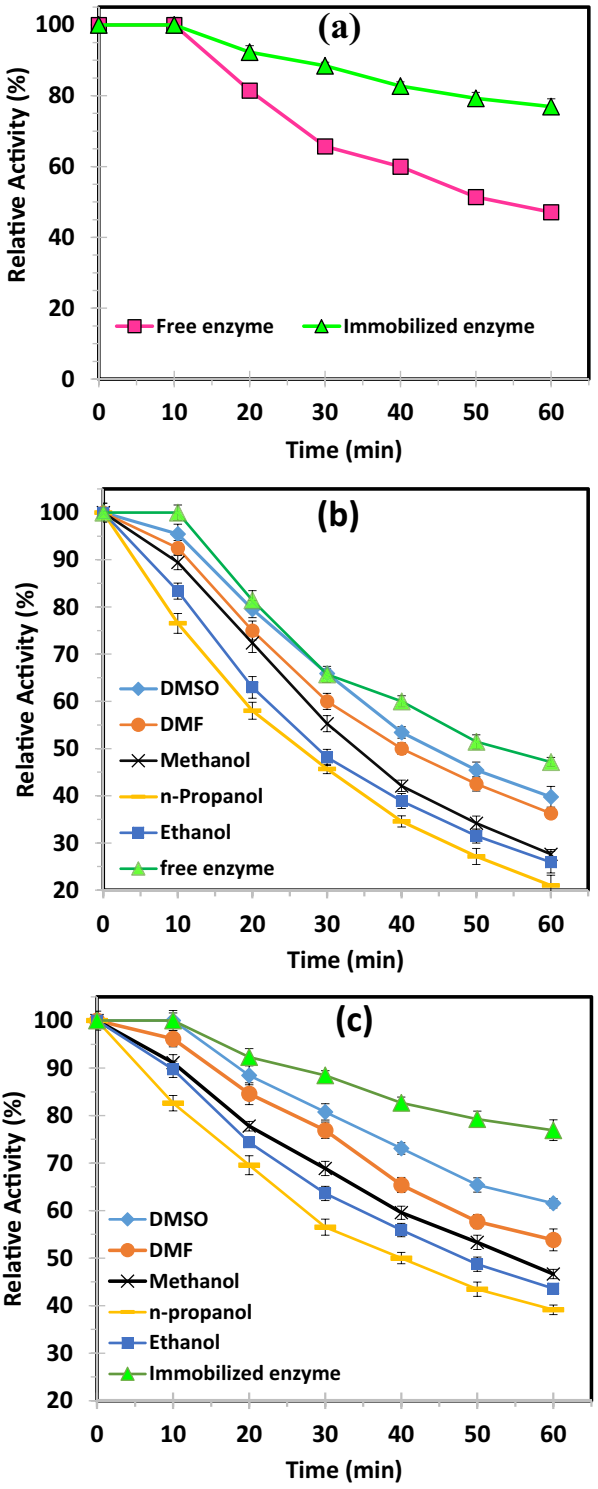

Fig. 7 **a** Comparison of the thermal stability of free and immobilized trypsin in the absence of organic solvent at 40 °C. Effect of organic solvents on the thermal stability of (**b**) free and (**c**) immobilized trypsin as a function of time at 40 °C

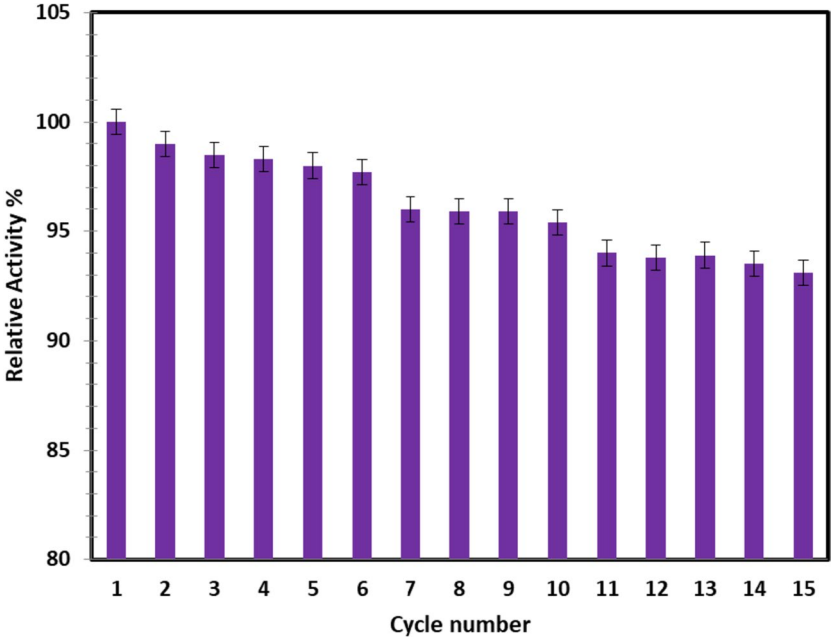

Fig. 8 Reusability of immobilized trypsin on GA-coated Fe_3O_4 nanoparticles

(methanol, ethanol, and propanol) is higher than that of the solvents with high polarity. While the K_{in} of propanol is close to that of methanol and ethanol for both free and immobilized enzymes. These alcohol solvents exhibit similar capabilities in dissolving polar and moderately polar substances, with small differences arising from their carbon chain lengths, resulting in slight variations in their polar nature.

Reusability of Immobilized Trypsin

The immobilization of an enzyme on magnetic nanoparticles presents a notable advantage in terms of facilitating the reusability of the immobilized enzyme. In this study, the reusability of immobilized trypsin was investigated to assess the effect of GA-coated Fe_3O_4 nanoparticles as a platform on the reusability of the immobilized enzyme. Figure 8 shows that the immobilized enzyme retained over 93% of its activity for up to 15 cycles.

Recently, our group showed that the immobilized trypsin onto Fe_3O_4@SiO_2–NH_2 magnetic nanoparticles maintained 85% of its activity after 6 cycles [53]. Sun et al. immobilized trypsin on carboxymethyl chitosan functionalized magnetic nanoparticles cross-linked with carbodiimide and glutaraldehyde, which retained 71 and 88.5% of their initial activities after 6 cycles, respectively [33]. Moreover, in an another study, magnetite/lignin/trypsin and magnetite/chitin/trypsin systems preserved around 60% and 80%, respectively, of their initial activity after 10 consecutive biocatalytic cycles [34]. Mahmood et al. immobilized surfactant-coated lipase on gum Arabic coated magnetic Fe_3O_4 nanoparticles using glutaraldehyde as a coupling agent, which retained around 65% of its original activity after reusing for seven times [35]. The higher reusability of

the trypsin immobilized on gum Arabic coated Fe_3O_4 nanoparticles prepared in the current work as compared to the lipase immobilized on gum Arabic coated magnetic Fe_3O_4 nanoparticles [35] (retaining approximately 93% activity of trypsin after 15 cycles *vs.* maintaining 85% of activity of lipase after 6 cycles) can be attributed to the extended exposure of the gum Arabic coated magnetic Fe_3O_4 nanoparticles in glutaraldehyde solution in this work (24 h *vs.* 2 h). During this period of time, the gum Arabic coating on magnetic nanoparticles is crosslinked and stabilized by glutaraldehyde as well as numerous -OH groups of the gum Arabic are converted to active aldehyde groups to readily react with the amino groups of enzymes during enzyme immobilization process. As a result, the enzyme molecules will exhibit higher reusability on the magnetic nanoparticles with higher crosslink density and increased number of aldehyde groups on the gum Arabic coating. This denser and more reactive surface can effectively reduce enzyme auto-digestion and minimize enzyme leakage [33].

Conclusions

In this study, gum Arabic modified Fe_3O_4 nanoparticles were successfully fabricated as confirmed by FTIR, TGA, and XRD results. SEM micrographs revealed the effective reduction in particle size due to the surface coating of nanoparticles with GA. These nanoparticles served as a magnetic platform for the covalent immobilization of the trypsin enzyme for the first time.

The immobilized trypsin maintained its catalytic efficiency across a wide range of pH values and temperatures, indicating the robustness of the immobilization process. This approach retained the enzyme's inherent activity while enhancing its stability, making it a promising candidate for biotechnological applications. Moreover, the kinetic study highlighted the altered kinetic parameters of the immobilized enzyme, underscoring the impact of immobilization on enzyme rigidity and function. The observed increase in the thermal stability of immobilized trypsin further substantiated the advantages of the proposed immobilization strategy. The evaluation of the enzyme's behavior in the presence of organic solvents affirmed the stabilizing effect of the magnetic gum Arabic nanoparticles, effectively maintaining enzyme integrity. The order of stability observed for the immobilized enzyme mirrored that of the free enzyme, indicating the efficacy of the immobilization approach in retaining enzyme stability even in challenging solvent environments.

Briefly, the successful immobilization of trypsin on magnetic gum Arabic nanoparticles demonstrates the potential of this technique to enhance enzyme stability and functionality. The results pave the way for broader applications in various fields that require immobilized enzymes with improved performance and resilience.

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Declarations

Ethics Approval This study does not contain any experiments involving human or animal subjects.

Consent to Participate Not applicable.

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