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Stabilization of lactoperoxidase by tragacanth-chitosan nano biopolymer

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Abstract

Lactoperoxidase (LPO) is a glycoprotein enzyme with a wide antimicrobial activity. Its stabilization is a key instrument for use LPO in industry. Gum tragacanth is a biopolymer, which is used for encapsulation and chitosan is used as a matrix for protein immobilization. This paper attempts to immobilize LPO by tragacanth-chitosan nano-biopolymers. Start with enzyme activity evaluating, LPO loading was done after analyzing tragacanth-chitosan nanocomposites by zeta sizer, FT-IR and SEM. Determining the nanocomposites proper size, the stability of immobilized and free LPO during storage was evaluated after 14 days of storage in 4 °C and 25 °C. Thermal stability also estimated by storing the immobilized and free LPO in different temperatures from 0 to 80 °C. It was found that, using nanocomposites with the size of 356.8 nm would result to preserving more stability during storage. The immobilized enzyme would have also more thermal stability. The results may be the consequences of encapsulation of LPO in tragacanth-chitosan nano-biopolymers. These findings suggest the application of tragacanth-chitosan nano-biopolymers in LPO immobilizing.

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Introduction

Lactoperoxidase (LPO; EC 1.11.1.7) is a glycoprotein consisted of 595 amino acids, which belongs to mammalian peroxidase superfamily. LPO plays an important role in strengthening the non-immune host defense system by oxidizing halide and pseudo halide ions to generate products with a wide antimicrobial activity (Kussendrager and Van Hooijdonk, 2000; Singh *et al*., 2008). Given the role of LPO in food industry (as natural bio preservative), cosmetics and tumor therapy (Boscolo *et al.,* 2007), it is not surprising that particular consideration has been dedicated to stabilizing this enzyme (Booth *et al*., 1989; Jafary *et al*., 2012).

Recently, researchers have shown an increased interest in nanotechnology, especially using nano-cages (with a diameter of 2–6 times the diameter of the native protein) for enzyme immobilizing (Garcia-Galan *et al*., 2011; Wei *et al*., 2000; Zhou and Dill, 2001). Application of chitosan in enzyme immobilizing is investigated by a numerical study (Luan *et al*., 2014; Monier *et al*., 2010; Piñuel *et al*., 2011) and gum tragacanth is one of the most widely used polysaccharides for encapsulation of active agents in food industry (Nedovic *et al*., 2011).

Gum tragacanth is a highly branched polysaccharide, which has been defined as the dried gummy exudation flowing from the trunk and branches of different species of Astragalus (fam. Leguminosae) by the food chemical codex. The remarkable property of gum tragacanth is the capacity to modify the rheology of aqueous media and form a mucilaginous gel even at fairly low concentration. Tragacanth gum polysaccharide is widely used as stabilizer, laxative, and emulsifier in pharmaceutics, cosmetics, and food products (Balaghi *et al*., 2010; Moghbel *et al*., 2005; Singh and Sharma, 2014). Chitosan is a polymer of glucosamine, which is derives of chitin. Chitosan properties have been defined as improved mechanical strength, resistance to chemical degradation and high molecular weight. In addition, it can be a suitable polymeric material for mucosal delivery due to its positive charge. Chitosan forms a gel-like layer in an aqueous environment, which makes it favorable for interacting with mucosal glycoproteins. Many characteristics like bioavailability, nontoxicity, biocompatibility, low cost, and avoiding the disturbance of metal ions to enzyme, make chitosan an ideal support material for enzyme immobilization (Dai *et al*., 2011; Islam *et al*., 2011; Jiang *et al*., 2005) To the best of author's knowledge, no report has been found so far using tragacanth-chitosan nanocomposite for Lactoperoxidase immobilization. Accordingly, the primary goal of this study was to investigate the stabilization of LPO by encapsulation of enzyme in tragacanth-chitosan nanocomposite. The paper also explores the thermal stability of the complex and the optimum particle size of nanoparticles.

Materials and methods

LPO activity assay

LPO (Sigma Chem. Co) activity was measured spectrophotometrically (using Shimadzu spectrophotometer uv260- model TCC-240A, Tokyo, Japan; equipped with a constant temperature cell holder working at 22 ◦C). Before activity determination, the lyophilized LPO was diluted 10-fold with 0.1 M potassium phosphate buffer (Merck co.) at pH 6. The reaction mixture consisted of 1860 µlit of 1 mM ABTS (Sigma Chem. Co), prepared in 0.1 M potassium phosphate buffer at pH 6, 1030 µlit of 0.3 mM H_2O_2 (Sigma Chem. Co) solution, and 0.1 mL of enzyme solution. The absorbance was monitored continuously at 412 nm for 2 min. The enzyme activity was calculated with the following equation (Barrett *et al*., 1999). The average of the three measurements was used to calculate enzyme activity. The activities of immobilized enzymes were determined in the same way.

$$K = \frac{\Delta A}{\Delta t} \times \frac{V}{\varepsilon dv} \times \text{dilution factor} \qquad \mu mol \times min^{-1} \times ml^{-1} (u/ml)$$

Synthesis of tragacanth-chitosan nanocomposite

In the first place, a solution of tragacanth (local market of Isfahan, Iran) 0.01% was prepared in the distilled deionized water. The 0.005% of this solution was made with 0.1 M potassium phosphate buffer.

The 0.005% of chitosan (Yuhuan Marine Biochemistry, China) was prepared in the same way. Various ratio of chitosan solution was poured bit by bit into tragacanth solution, which was under vigorous vortex. Then, nanoparticles size and charge was determined by zeta sizer (Marvelon Nano ZS3600, UK). The nanoparticles with proper size and charge were selected. Their interactions were evaluated by FT-IR (Jasco- 6300, Japan) and their shape and morphology was evaluated by scanning electron microscope (SEM) (Leo 1400, England). Making sure of suitable condition for enzyme loading, the LPO loading was done (Gazori *et al*., 2009).

Synthesis of tragacanth-chitosan-LPO nanocomposite

Firstly, 11.36 µlit of LPO was added into 125 µlit of 0.005% chitosan slowly in room temperature. After 10 minutes, this solution was poured bit by bit into 1 mlit 0.005% tragacanth and then went under vigorous vortex. Afterwards, zeta sizer was used for size and charge determination and SEM was used for nanocomposite-LPO morphology evaluation. Ultimately, the activity was measured by mentioned assay. All experiments were performed in triplicate.

Evaluation of enzyme stability

Evaluation of immobilized enzyme stability during storage

To determine the storage stability of immobilized enzyme, immobilized and free enzyme were stored at 4 °c and at room temperature (25 °c). Then for 14 days at regular intervals of time, both were tested to assay the enzyme activity according to the method described previously.

Evaluation of thermal stability of immobilized enzyme

To determine the optimum temperature and thermal stability for immobilized and free LPO, their activities were assayed at different temperature ranging from 0 to 80 °c by heating it in a water bath for 1 min. After appropriate time, the mixture was immediately transferred to an ice bath and then tested to measure the remaining activity.

Evaluation of the effect of tragacanth-chitosan nanocomposite size on stability of immobilized LPO

For this purpose, two different sizes (under 500 nm and above 500 nm) of enzyme loaded tragacanth-chitosan nanocomposite were selected, and the activities were assayed within 14 days (samples were stored at 4 °c).

Statistical analysis

Independent t-test was used to compare the mean activity of immobilized and free enzyme. The differences were considered statistically significant if $P< 0.05$.

Results

Preparation and characterization of tragacanth-chitosan nanoparticles and LPO loaded nanoparticles

The results obtained from the preliminary analysis of particle size, zeta potential and polydispersity index (PDI) of tragacanth-chitosan nanoparticles without LPO (measured by zeta sizer), are shown in Table 1. Concentrations are presented as gr/dl and 0.01% concentration of tragacanth-chitosan, which was prepared with water, was diluted in potassium phosphate buffer to prepare 0.005% concentration. There is a clear trend of increase in nanoparticle's size and zeta potential with the increase of chitosan portion in tragacanth-chitosan nanoparticles.

Effect of tragacanth-chitosan nanocomposite size on stability of immobilized LPO

Immobilization was done using two different sizes of nanoparticles (formula code 5, under 500 nm, and formula code 8, above 500 nm) and catalytic activities were assayed within 14 days at 4 °C. Table 2 presents the results from the analysis of particle size, zeta potential and PDI of LPO loaded nanoparticles, using formula code 5. As Fig. 1 shows, using formula code 5 (with the size of 356.8 nm) for immobilization process, the immobilized LPO preserved 48.8% of its activity within 14 days after immobilization. Meanwhile, using formula code 8 (with the size of 651.1 nm) resulted in 19.5% of activity preservation. Formula code 5, therefore was selected as the proper

formula for LPO loading.

Fig. 2 exhibits the FT-IR analysis of nanoparticle code 5. Morphology studying of tragacanth-chitosan nanoparticles revealed spherical and cube particles in SEM images (Fig. 3.a), so LPO loading was done. Cube particles are probably salts crystals in the buffer. As shown in Fig. 3.b, enzyme loaded nanoparticles had also spherical morphology.

Table 1. Particle size, zeta potential and PDI of tragacanth-chitosan nanoparticles without the enzyme, prepared in water (1-4) and potassium phosphate buffer (5-8).

	Formula code	Concentration of tragacanth-chitosan	Tragacanth:chitosan portion	Nanoparticle size (nm)	Nanoparticle zeta potential (mV)	PDI[1]
Diluted in water	1	0.01%	1:8	432.2	-9.6	0.125
	2	0.01%	1:4	488.3	-5.8	0.212
	3	0.01%	1:2	582.8	-5.19	0.427
	4	0.01%	1:1	596.7	-2.53	0.157
Diluted in potassium phosphate buffer	5	0.005%	1:8	356.8	-8.42	0.174
	6	0.005%	1:4	495.1	-4.25	0.241
	7	0.005%	1:2	504.5	-3.2	0.325
	8	0.005%	1:1	651.1	-2.1	0.314

[1]Polydispersity Index.

Enzyme stability during storage

Fig. 3 shows the remaining activity of immobilized and free enzyme after 14 days of incubation at 4 °C or 25 °C. Based on the results, after 14 days, immobilized LPO preserve 48.8% of its activity, whereas only 12.1% of free enzyme activity remained at 4 °C (Fig. 3.a). Storing the immobilized and free enzyme in 25 °C for 14 days resulted in the preservation of 19.9% and 1.01% of activity respectively (Fig. 3.b). In both temperatures, the mean activity of immobilized LPO during 14 days was significantly higher compared to the free enzyme (P-value< 0.001). The data was average of triplicate experiments and are shown as the percentage of the remaining activities.

Table 2. Particle size, zeta potential and PDI of LPO loaded nanoparticles.

Formula code	Enzyme concentration (unit/ml)	Enzyme quantity (µl)	Nanoparticle size (nm)	Nanoparticle zeta potential	PDI[1]
5	80	11.36	521.2	-9.41	0.396

[1]Polydispersity Index.

Thermal stability

The activity of the immobilized and free LPO was assayed in a wide range of temperatures from 0 to 80 °C (Fig. 4). The optimum temperature was approximately 30 °C for both forms. In spite of the tendency of the catalytic activities to fall after 40 °C, it seemed that the immobilized LPO preserved its activity significantly more than the free enzyme (P-value< 0.001). In 70 °C, less than 30% of the free LPO activity remained, but at the same condition, the immobilization resulted in preservation of more than 60% of LPO activity. At higher temperatures, namely 80 °C, the free LPO lost more than 90% of its activity, meanwhile the immobilized enzyme could preserve more than 30% of its activity. The data was average of triplicate experiments and are shown as the percentage of the remaining activities.

Discussion

The present study was accomplished to stabilize LPO by introducing it into nano sized tragacanth-chitosan cages. Gum tragacanth is one of the most widely used

materials as a matrix for encapsulation of active agents (Nedovic *et al.*, 2011), and chitosan had been successfully used for peroxidase stabilization earlier (Luan *et al.*, 2014; Monier *et al.*, 2010).

FT-IR analysis

In the FT-IR spectrum of gum tragacanth (Fig .2a), bands related to methyl and non-ionized carboxyl group could be observed at 1628.59 cm^{-1}. The amine band at 1522.52 cm^{-1} was observed in the IR spectrum of chitosan (Fig. 2b). The spectrum of tragacanth-chitosan (Fig. 3c) reveals the main changes in 1439.71 cm^{-1}, which relates to carboxylic salt and could not be observed in the spectrum of tragacanth or chitosan. Increasing intensity of the ionized carboxyl group at 1617.98 cm^{-1} and shifting to a lower frequency at 1743.33 cm^{-1}, indicates the formation of ionic crosslinks between chitosan and tragacanth.

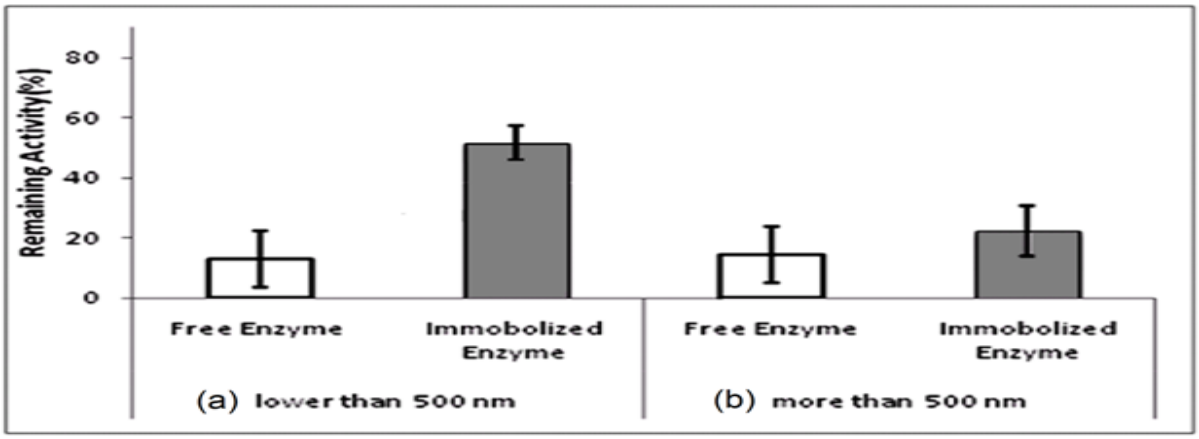

Fig. 1. The remaining activity of immobilized enzyme, after 14 days of incubation in 4 ºC. Using formula code 5 (356.8 nm) for immobilization resulted in 48.8% activity preservation (a), while using formula code 8 (651.1 nm) resulted in 19.5% activity preservation (b). The data was average of triplicate experiments and are shown as the percentage of the remaining activities.

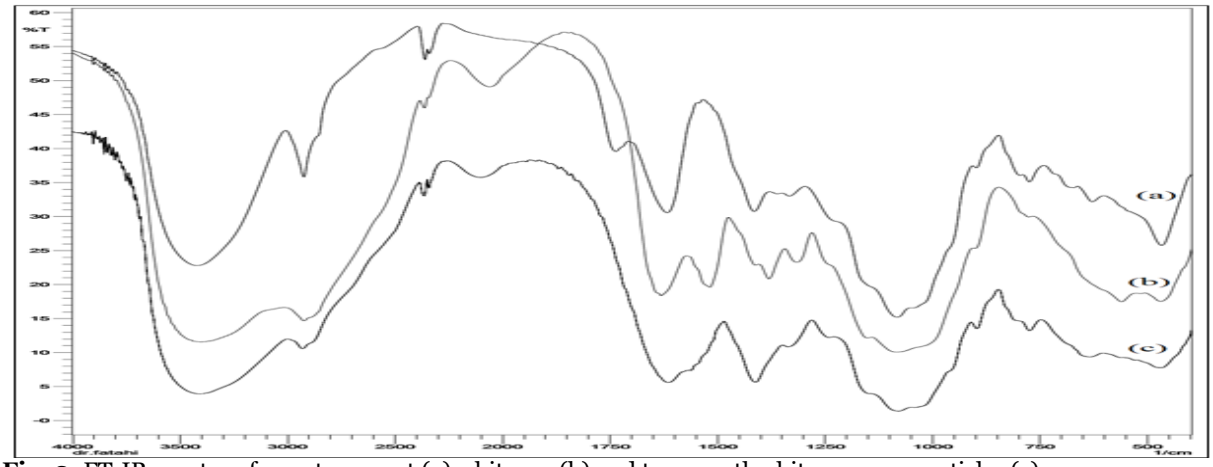

Fig. 2. FT-IR spectra of gum tragacant (a), chitosan (b) and tragacanth-chitosan nanoparticles (c).

Immobilization of LPO on tragacanth-chitosan nanocomposites

The existence of negatively charged carboxyl and hydroxyl groups on tragacanth (Singh and Sharma, 2014) and positively charged amino groups on chitosan (Islam *et al.*, 2011) facilitated the interaction between tragacanth and chitosan. Considering the cationic properties of chitosan, LPO will be encapsulated in chitosan molecule and the coo^- groups on tragacanth and NH_3^+ on chitosan will electrostatically binding together afterwards, to produce the tragacanth-chitosan-LPO complexes.

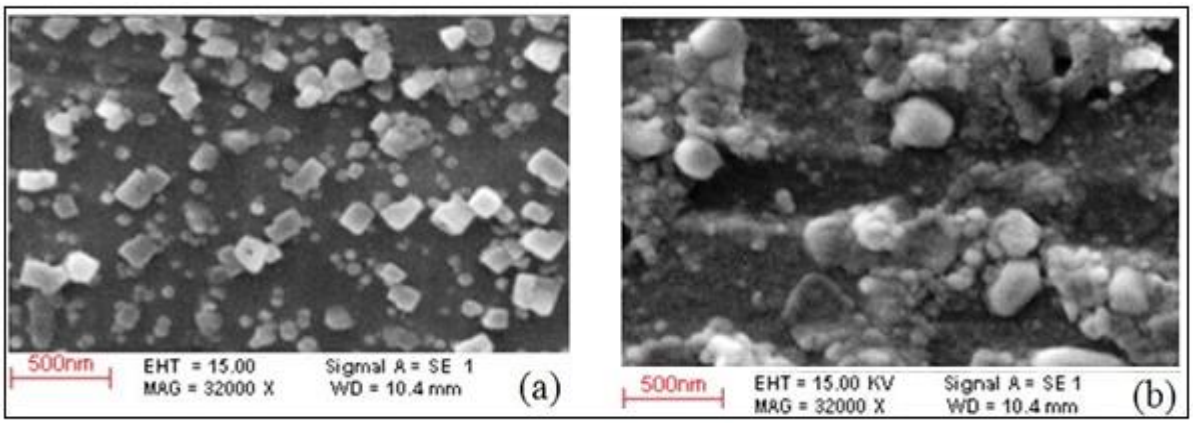

Fig. 3. SEM imaging of the tragacanth-chitosan nanoparticles (a) and tragacanth-chitosan-LPO nanoparticles (b). Cube particles are probably salts crystals in the buffer.

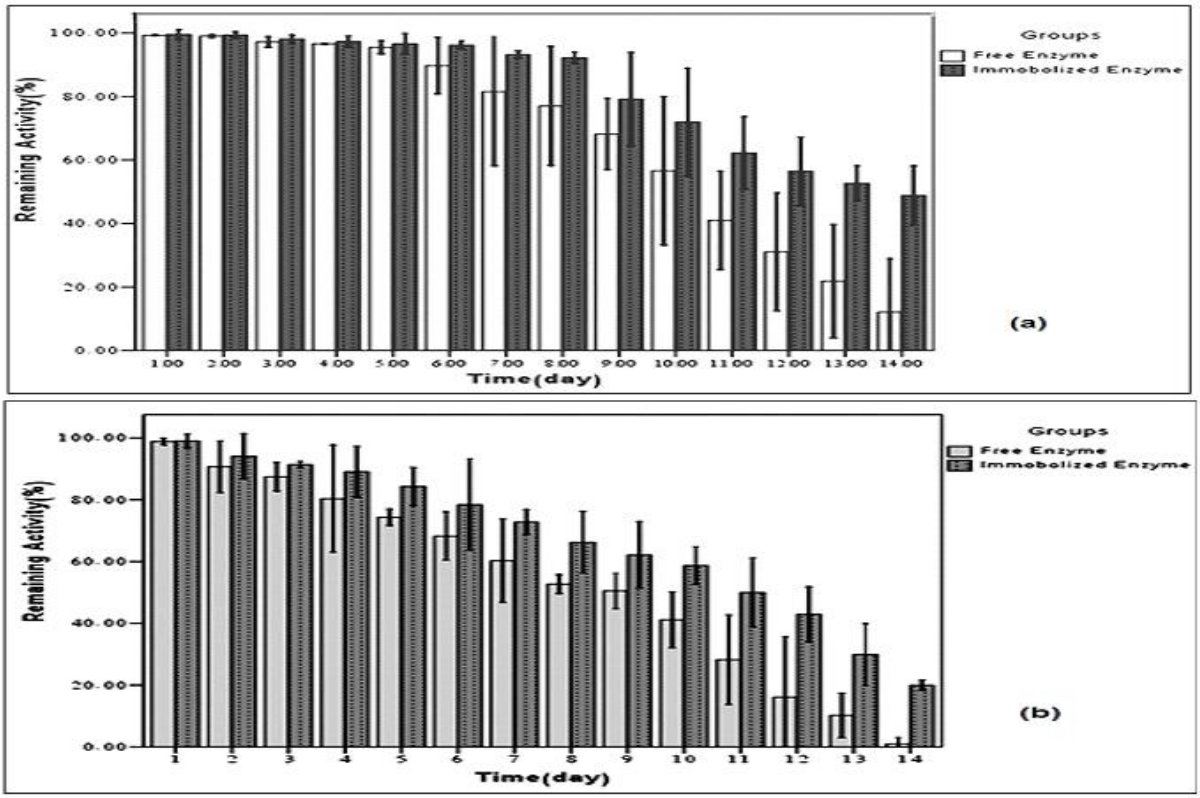

Fig. 4. Stability of free and immobolized LPO during14 days incubation at 4 °C (a) and 25 °C (b). After 14 days, the remaining enzyme activity of free and immobilized LPO at 4 °C was 48.8% and 12.1% respectively, and at 25 °C was 19.9% and 1.01% respectively. The data was average of triplicate experiments and are shown as the percentage of the remaining activities.

Effect of nanocages size on enzyme stabilization

The results suggest that, using nanocomposites smaller than 500 nm, could preserve the LPO activity more than the larger one (Fig. 1). These findings further support the idea of Zhou and Dill (2001), which suggests that by using relatively small cages; stabilization of proteins against unfolding can be achieved. Based on the study done by wei *et al* (2000), this effect is a consequence of thermodynamic laws. Theoretically, in such small cages, the unfolded configurations of the chain are not thermodynamically favored. Also in spherical cages with a diameter of 2–6 times the diameter of the native protein, proteins can achieve maximum stabilization. Considering the norm diameter of a hydrated protein, which is within the range of 10 nm,

it is concluded that the use of meso to nanoporous materials will accommodate the maximum stabilization for the proteins. Therefore, formula code 5 (Table 1) is probably the best formula for LPO stabilization with tragacanth-chitosan nanocomposites.

Enzyme stability during storage

During 14 days of storage in 4 °C and 25 °C, the immobilized LPO showed a significantly (P-value< 0.001) higher activity compared to the free enzyme. The findings suggest that, tragacanth-chitosan nanocomposites can significantly preserve the catalytic activity of LPO in vitro in room temperature and also in low temperatures.

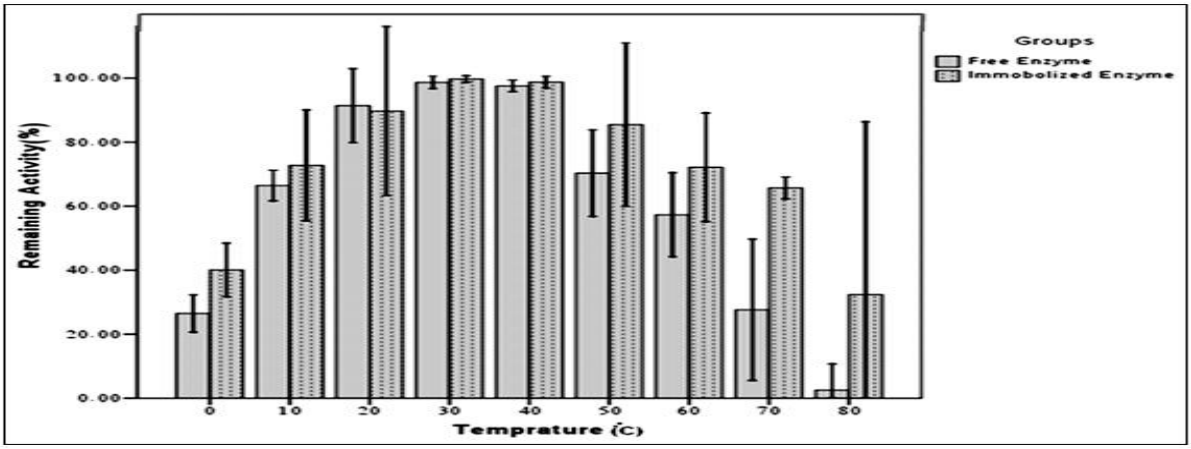

Fig. 5. Thermal stability of immobilized and free LPO. The data was average of triplicate experiments and are shown as the percentage of the remaining activities.

Thermal stability

According to Boscolo *et al* (2007), 70 °C is an apparent midpoint transition temperature for irreversible thermal denaturation of LPO. The finding of the current study is in agreement with Miroliaei *et al* (2007) findings, which showed that immobilization of LPO on Con A-Sepharose 4B would considerably increases thermal stability of the enzyme. The findings of this study suggest that, immobilizing LPO with tragacanth-chitosan nanocomposites would make the enzyme more stable in high temperatures. Encapsulation the LPO in tragacanth-chitosan nanocomposites may explain the observed stabilization.

Conclusion

In conclusion, immobilizing LPO in tragacanth-chitosan nanocomposites preserves the enzyme catalytic activity during storage and enhances its thermal stability. These results show the possibility of application of tragacanth-chitosan nanocomposites as a novel optional matrix for immobilizing LPO.

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