Enzymatic Degumming of Soybean Oil by Immobilized Phospholipase A_1 on Plasma Surface Modified Chitosan Nanofibrous Membrane

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ABSTRACT: *The paper presents the enzymatic degumming of soybean oil by immobilization of phospholipase A_1 (PLA_1) on atmospheric plasma surface modified chitosan nanofibrous membrane. As evidenced by Scanning Electron Microscopy (SEM) images, chitosan nanofibers exhibited a number of properties indicating this polymer as a good enzyme carrier. Fourier Transform InfraRed (FT-IR) spectroscopy showed the reaction between the enzyme and functional groups of chitosan nanofibers. The properties of immobilized PLA_1 were compared to free PLA_1 in order to evaluate the possibility of using the immobilized enzyme for the degumming of soybean oil. The pH tolerance and thermal stability of the immobilized PLA_1 were significantly improved. In addition, the immobilized PLA_1 can be easily recovered and retained at 80% of its initial activity after 10 times of recycling. The enzymatic degumming process was carried out at 60°C and pH 6.0. The residual phosphorus content decreased to 8.6 mg/kg after 6 h, which is to meet oil safety standards and suitable for the physical refining of soybean oil.*

KEYWORDS: *Enzymatic degumming; Phospholipase A_1; Immobilization; Soybean oil; Chitosan nanofibers.*

INTRODUCTION

The degumming process is the initial step in edible oil refining that removes phospholipids and some portions of trace metals along with mucilaginous substances. The removal of the phospholipids is required for producing

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high quality refined oil [1]. Phospholipids are negative components in vegetable oils during oil deodorization and steam distillation [2]. Traditional degumming processes such as acid treatment, water degumming, super degumming, total degumming, and ultrafiltration process cannot guarantee the achievement of low phosphorus content (<10 mg/kg) required for industrial applications [3-4]. Although the traditional water degumming process is effective for hydratable phosphatides, a significant amount of Non-Hydratable Phosphatides (NHP) will remain in the oil (especially in soybean oil). The NHP can be removed simultaneously with the Free Fatty Acids (FFA) by a chemical refining process that must involve the utilization of a greater amount of acid thus resulting in high cost for wastewater treatment. Other technical and environmental demerits of the chemical refining process include low yields, nutrient loss, high-energy consumption, and a large amount of water consumption. Some physical refining processes have gained popularity as more environmentally friendly treatments with lower operating costs. Nonetheless, a small amount of NHP will remain in the degummed soybean oil even after the physical refining process [5]. It is therefore important that a new approach be developed for the complete removal of phosphatides from vegetable oils.

Enzymatic degumming is probably the best process available today for reducing the phosphorus content of vegetable oils. In this process, enzymes changed NHP into a hydratable form, which was then removed by centrifugation [4]. The result of Xiaoyang *et. al.* showed the phosphorus content of dewaxed rice bran oil after phospholipase A1-catalyzed degumming could be decreased from 332.5 mg/kg to 9.3 mg/kg while the phosphorus content of degummed rice bran oil after water and acid degumming was more than permitted limit [6]. For a degumming process to be successful, the selection of enzyme and its catalyzed mechanism is very important. Most studies on the degumming of crude vegetable oils are based on the free phospholipase A_1 [7-10]. Free phospholipase A_1 (PLA_1) is very sensitive to pH and temperature and cannot be easily reused. Therefore, the immobilization of PLA_1 is a promising technology and has a large impact on industrial scale degumming operations.

To achieve relatively high enzyme loading and catalytic efficiency for large-scale operations and applications supports with high surface-to-volume rations -an inherent feature of nanoscale materials- are often desirable. Among the various nanoscale materials, it appears that electrospun nanofibrous membranes are promising supports for enzyme immobilization due to advantages such as large specific surface, fine porous structure, easy recoverability from the reaction media, and applicability for continuous operations in comparison with nanoparticles [11].

Chitosan is an abundantly available low-cost biopolymer that can be obtained from natural resources. As compared with other commercial polymers, it has received a lot of focus due to its specific properties such as cationic, nontoxicity, high adsorption capacity, abundance, and low price. These significant chemical and biological characteristics make chitosan a desirable biomaterial for enzyme immobilization [12].

Plasma treatment is a simple process to modify the physical and chemical characteristics of biomaterials without altering their bulk properties and without changing the mutagenic and toxic effects of chemical materials like glutaraldehyde. It can be performed using different gases to modify the physical and chemical characteristics of biomaterials and to enhance their biological activity [13].

This paper presents pioneering research for enzymatic degumming by immobilized PLA_1 on plasma-modified chitosan nanofibers. The conditions for PLA_1 purification [14] and its immobilization on plasma-modified chitosan nanofibrous membrane [15] were determined in our previous studies. The effect of pH and the reusability of immobilized PLA_1 were investigated when used in the degumming of soybean oil and finally, the quality of the enzymatic degummed oil was assessed.

EXPERIMENTAL SECTION

Materials

The PLA_1 (Lecitase™ Ultra), soybean oil (with phosphorus content of 168.5 mg/kg), soy-phospholipid (SPL), chitosan (CS of low molecular weight, 91.2 % degree of deacetylation), and Polyethylene oxide (PEO, MW 900 KDa) were obtained from Novozymes A/S (Bagsvaerd, Denmark), Behshahr oil Industry (Tehran, Iran), Behpak Co. (Behshar, Iran), Easter Groups (Dong Chen Co., Ltd, China), and Acros Organics Co. (New Jersey, USA), respectively. All other chemicals of analytical grade were purchased from Merck Chemical (Darmstadt, Germany).

Preparation and surface modification of chitosan nanofibers

Chitosan nanofibers were prepared using the method described by *Mirzaei et al*. [16]. Chitosan solution and PEO solution were prepared separately in aqueous acetic acid (80 V/V%) under magnetic stirring at 37°C for 24 h. The obtained solutions were then mixed together in a weight ratio of 90:10 CS/PEO. The process was carried out using Electroris (FNM, Tehran, Iran). The voltage, distance between needle to the collector, injection rate, drum speed, and time were fixed at 20 kV, 12 cm, 3 mL/h, 200 rpm, and 1 h, respectively. Electrospun fibers were treated by homemade air-Dielectric Barrier Discharge (DBD) plasma. A power of 30 W and a frequency of 6 kHz was applied to the DBD reactor by DC-pulsed high voltage.

Characterization

To characterize the morphology of nanofibers before and after plasma treatment, a Scanning Electron Microscope (SEM) was used (Philips XL30, USA). To test changes in the chemical structure, the Attenuated Total Reflection-Fourier Transform InfraRed (ATR-FTIR; Bruker Optics, TENSOR 27, USA) spectra were measured from 4000 to 500 cm^{-1} at a resolution of 4 cm^{-1} [13,17].

Immobilization of PlA_1 on CS/PEO nanofibrous mat

The coupling reaction used to immobilize PLA_1 onto the Atmospheric Plasma Surface Modified CS/PEO Nanofibrous Membrane (APSM CS/PEO NM) was carried out under different conditions to determine the optimum conditions for immobilization. The preliminary trials investigated changes in the pH of the reaction mixture, the immobilization time, and the amount of PLA_1 at 4.5-7.5, 3-10 h, and 200-1000 U/kg, respectively. PLA_1 solution was prepared by solving the appropriate amount of enzyme in phosphate-citrate buffer (50 mM, pH 4.5-7.5). Varying amounts of PLA_1 were added to the APSM CS/PEO NM, and incubated at 4ºC [18]. Immobilization efficiency is defined as the ratio of the activity of the immobilized enzyme to the activity of the free enzyme used [19].

Enzyme assay

PLA_1 assay was performed with soy-phospholipid emulsion using the method of *Jiang et al*. [9]. Four mL of substrate emulsion (25% SPL and 4% polyvinyl alcohol solution with a volume ratio of 1:4), 5 mL of 100 mM citric acid buffer (pH 5.0), and 1 mL of enzyme solution were mixed and incubated at 37ºC for 10 min. The reaction was terminated with the addition of ethanol 95% (15 mL) after incubation, and the liberated FFAs were titrated with 50 mM NaOH. Blanks were measured with heat-inactivated PLA samples (95 ºC, 10 min). One unit of PLA is the amount of enzyme which releases 1 µmol of titratable Free Fatty Acids (FFAs) per minute under the described conditions.

Effect of pH and temperature on enzyme activity

The pH and temperature optima of the free and immobilized PLA_1 were investigated on emulsion consisting of 4% SPL in 50 mM phosphate-citrate buffer in the range of pH 2.0-10.0 (at 35°C for 3 h) and 4-90ºC (10 min), respectively [20]. The highest activity measured under the corresponding pH or temperature was designated as 100%, and the activities at all the remaining pH and temperatures were values proportional to the highest activity.

Reusability

To evaluate the reusability, the immobilized enzyme was placed into soybean oil at pH 6.0 and 60°C. The hydrolysis of the phospholipids for 7 h was monitored over 10 cycles. After each cycle, the immobilized enzyme was washed with 50 mM phosphate buffer at pH 6.0 and then reused [3,21]. The residual enzyme activity after each cycle was defined as the value proportional to the original activity (100%).

Batch degumming process of soybean oil

Batch degumming was performed with free and immobilized enzymes using the optimum conditions determined in the previous sections. 300 g of soybean oil and 0.36 mL citric acid (45%) were mixed in a 500 mL Erlenmeyer flask, heated to 80°C, and stirred at 500 rpm for 20 min. The oil was cooled to 60°C and then adjusted to 5.5–6.0 pH with 1 M NaOH while stirring at 500 rpm. Six (6) mL of water was added to the oil and stirred at 30 rpm for 20 min. The free or immobilized enzyme solution was added to the oil at an appropriate dose rate of PLA_1 (U/kg oil mass). The mixture was incubated at 60°C and stirred at 30 rpm. The samples for phosphorus analysis were taken at 1 h intervals [3,21]. Then the reaction mixture was centrifuged at 6,000 *g* to remove

the mucilaginous gums. The residual phosphorus content in the oil phase was assayed according to the AOCS method Ca12–55 [22].

Determination of the oil quality indices

Free Fatty Acids (FFA) and Peroxide Value (PV) were determined according to the AOCS official methods F 9a-44 and Cd 8-53, respectively [22].

Statistical analysis

All experiments were performed in triplicate. The data were reported as means and standard deviations. One-way ANOVA was performed using SPSS 16 Statistical software (SPSS Inc., Chicago, IL.). Differences were considered to be significant at $p \leq 0.05$ according to Duncan's Multiple Range Test.

RESULTS AND DISCUSSION

Surface morphologies of unmodified and APSM CS/PEO nanofibrous membrane

The original CS/PEO nanofibrous membrane presented a homogeneous, continuous, and uniform network with a diameter range of 70–240 nm (Fig. 1a). A series of atmospheric plasma with different initial times ranging from 2 to 10 minutes was applied to investigate the effect on surface functionalization and enzyme immobilization. One practical concern for solid supporting materials is the stability of nanofibrous structures during functionalization and biocatalytic applications. Surface-modification by atmospheric plasma for 6 min caused remarkable changes in the stability of CS/PEO nanofibers (Fig. 1b') in comparison with non-treated CS/PEO NM (Fig. 1a') after immersion in Distilled Deionized Water (DDW) and remained intact after plasma treatment (Fig. 1b).

The chemical structure changes were characterized *via* ATR-FT-IR. The results demonstrated that the quantities of chitosan functional groups could be increased after plasma treatment from 2 to 6 min but reduced after 10 min, in which the 6 min APSM CS/PEO NM exhibit the most hydroxyl, carboxylic acid, –NH, and – OH groups on the surface in comparison to the untreated CS/PEO NM. Therefore, the 6 min atmospheric plasma treatment was chosen as the suitable plasma reaction time for CS/PEO NM (Fig. 2). The unmodified CS/PEO NM exhibited a stretching of C – O bond of the hydroxyl groups at approximately 1030 and 1085 cm^{-1} and then an amino

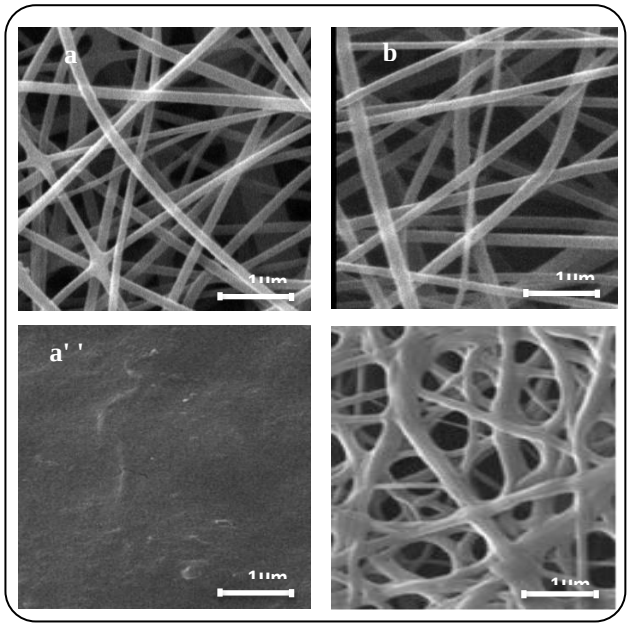

Fig. 1: SEM images of (a) untreated, and (b) 6 min plasma treated CS/PEO NM before immerging in double distilled water, and (a') untreated, and (b) 6 min plasma treated CS/PEO NM after immerging in double distilled water

stretching bond at approximately 1600 cm^{-1}. The C = O carbonyl stretch of a carboxylic acid bond appears at approximately 1650 cm^{-1}. The absorption band at approximately 3000-3500 cm^{-1} was contributed by the stretching vibrations of –NH and – OH groups [23,24]. Furthermore, the ATR-FT-IR spectra for APSM CS/PEO NM are similar to those of the unmodified CS/PEO NM; however, the APSM CS/PEO NM exhibited more significant hydroxyl, carboxylic acid, –NH and – OH groups absorption bonds. This means that the atmospheric plasma could involve functional groups. *Ni et al.* found that surface modified chitosan using open-air plasma could introduce chitosan with bioactive components more efficiently [25].

Immobilization of PLA_1

During the immobilization procedure, the effects of time and pH on the activity of the immobilized PLA_1 were determined and the results shown in Fig. 3. Data show that the process at 5.5 pH and 5 h reaction time can obtain 75 and 80% immobilization efficiencies. When the pH value was higher or lower than 5.5, the immobilization efficiency was lower. The change in pH value can influence the electrical charges on the surface of the nanofibers and limit the interactions between functional groups on the surface

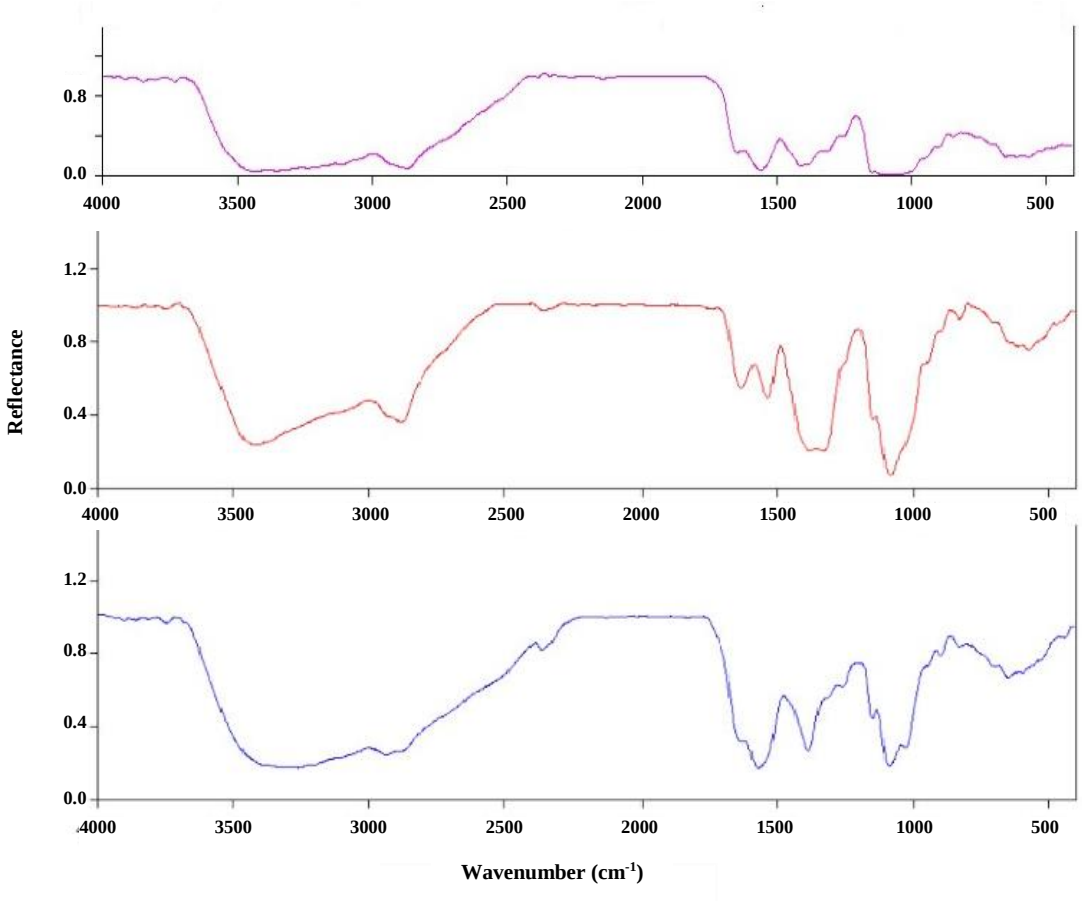

Fig. 2: ATR-FTIR spectra of (a) untreated, (b) 6 min plasma-treated CS/PEO NM, and (c) 6 min plasma-treated CS/PEO NM after PLA_1 immobilizing in optimum condition (pH 5.5, 5 h, 600 PLA1 dosage U/kg)

of APMS CS/PEO NM and those of PLA_1. Protein denaturation can occur under conditions of excess acid or alkali [26-27]. The immobilization efficiency significantly increased with treatment time until it reached a maximum at 5 h and then steadily declined over the next 5 h. The longer immobilization times could result in a greater load of PLA_1 molecules on the surface of the support and limit substrate diffusion due to steric inhibition [28]. The initial concentration of an enzyme can also greatly affect the activity of the immobilized enzyme. In order to find the optimal concentration of PLA_1, the immobilization of various concentration ranges of PLA_1 from 200 to 1000 U/ kg on 1 cm^2 APSM CS/PEO NM was investigated and the results are shown in Fig. 3C. The highest immobilization efficiency was 96.5% at the lowest PLA_1 concentration of 200 U/ kg. Further increases in PLA_1 concentration caused a decrease in PLA_1 activity that followed a corresponding decrease in immobilization. The highest activity (2362 U/g) was observed at 600 U/kg concentration of PLA_1. Moreover, the saturation and multi-layering of the enzyme on the surface could cause intermolecular steric hindrance to substrate diffusion at the higher enzyme/support ratio [29]. The enzymatic aggregation on the surface of APSM CS/PEO NM could block the active sites of the enzyme.

In this system, plasma activation is an important factor that affects the performance of the immobilized PLA_1. As shown in Fig. 2c, in optimum conditions (5 h, pH 5.5, 600 U/kg PLA_1 concentration and 82.3% immobilization efficiency), the enzyme loading significantly increases with an increase in plasma effect which is attributed to the increased surface density of functional groups such as $–NH^{3+}$ on the membrane surfaces. The results obtained for PLA_1 immobilization are directly associated and almost simultaneously, reflecting the observation made by other researchers concerning the effect of pH, time, and different enzyme concentrations [19,21].

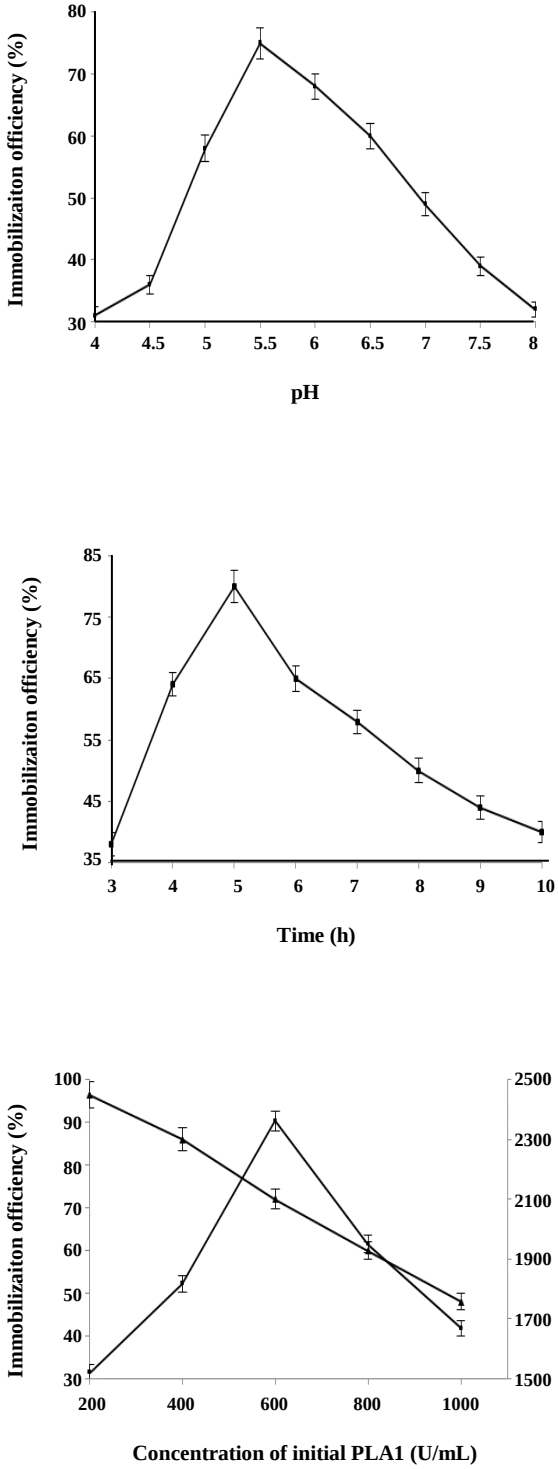

Fig. 3: Effect of (a) pH and (b) time on the immobilization efficiency of immobilized PLA1; (c) Effect of initial concentration of PLA on the immobilization efficiency (▲) and activity (■) of immobilized PLA1.

Effect of pH and temperature on the activities of free and immobilized enzymes

Figure 4a shows the pH-activity profiles of the free and immobilized enzymes from pH 2.0 to 10.0 (35°C, 3h). When the pH was within the 5.5–6.5 range, the immobilized PLA_1 had greater relative activity. The optimum pH for immobilized PLA_1 was 6.0. The free PLA_1 activity intensely decreased at pH values greater than 5.0. Compared to free PLA_1, the pH-activity profile of the immobilized enzyme showed an upward shift in pH with considerable broadening due to greater pH stability. This was attributed to the partitioning effects of the supports. Since the reaction medium and the insoluble support are two different phases, depletion in one of the phases is possible. When this occurs, the immediate microenvironment of the enzyme on the surface of the support material will be different from that of the bulk solution. Similar results have been reported by other researchers [7,18]. The broadened pH/activity profile of immobilized PLA_1 may have important benefits when developing industrial applications.

The optimum temperature for the free PLA_1 was 50ºC, while the maximum activity of the immobilized PLA_1 was at 60ºC (Fig. 4b). The shift in optimal temperature toward higher values could be due to the immobilization of the enzyme which increased instability and resulted in the formation of the enzyme-substrate complex which hindered the access of substrates to the active sites and covalent bond between the enzyme and support medium [13,18]. Enzyme immobilization using plasma surface modification is an effective tool for its thermostabilization due to the restriction of linkage and free movement at higher temperatures. Therefore, the amide linkages (present in enzyme) are protected from disruption thus leading to the stability of the enzyme at higher temperatures [13]. There are many advantages of running bioprocesses at elevated temperatures such as higher diffusion rates, lower substrate viscosities, increased reactant solubility, and reduced risk of microbial contamination [27].

Reusability of immobilized enzyme

The reusability of immobilized enzymes is an important aspect of the application, especially in industrial applications. The variation of the activity of immobilized PLA_1 after multiple-reuse is shown in Fig.5 where it could be observed that the residual activity remained about 80% of

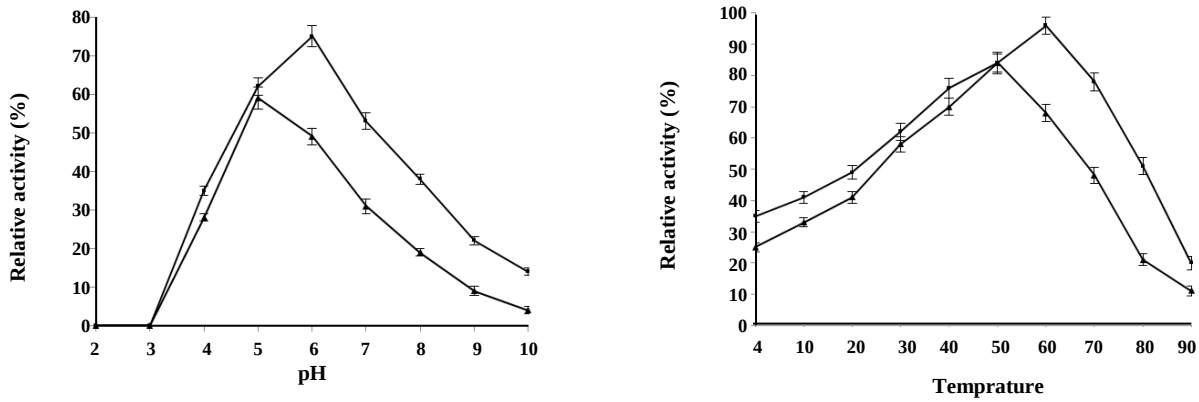

Fig. 4: The effect of (a) pH and (b) temperature on the activity of free (▲) and immobilized (■) PLA1

the initial activity after the 10th recycle. The decrease inactivity was considered as the denaturation and leakage of protein from the supports during use [18]. Therefore, the current results indicate that prepared APMS CS/PEO NM has good stability and reusability for PLA_1.

Batch enzymatic degumming

The free and Immobilized PLA_1 enzymes were used to reduce the residual phospholipid contents below 10 mg/kg in soybean oil. The initial phosphorus content in water-degumming soybean oil was 168.5 mg/kg. The temperature and pH values were maintained at 50 and 60°C, and pH 5.0 and 6.0, respectively for free and Immobilized PLA_1. The hydrolysis time and residual phosphorus levels are shown in Table 1. Free PLA exhibited a sharp decline in phosphorus content and reached less than 10 mg/kg after 4 h. The final residual phosphorus content reached almost 7.6 mg/kg after 6 h. The final residual phosphorus content for immobilized PLA_1 was 8.6 mg/kg after 6 h that would be suitable for physical refining in the industry. The results indicate that the free enzyme was more mobile and more accessible to the phospholipid substrates but could only be used for one cycle.

For the water degumming, the residual phosphorus content of degummed rice bran oil could not be reduced to less than 50 mg/kg. The citric acid dosage of 0.10%, high shearing rate of 23000 rpm, chelation time of 60 min, NaOH dosage of 1.5 mole equivalent to the amount of citric acid, reaction temperature of 50°C, and total water dosage of 2.5%, while the phosphorus content of degummed rice bran oil after water and acid degumming was 120.5 mg/kg and 66.4 mg/kg, respectively. The phosphorus content of dewaxed rice bran oil after phospholipase A1-catalyzed degumming could be decreased from 332.5 mg/kg to 9.3 mg·kg [6].

The result of *Yang et al.* showed the highest activity of the 15 units of recombinant free PLA1 from *Serratia marcescens* (18.9 U/mL) could decrease the phosphorus content in 150 g of crude rapeseed oil decreased from 22.6 mg/kg to 9.3 mg/kg [2]. *Sheelu et al.* used immobilized PLA_1 on gelatin cross-linked with glutaraldehyde to degum rice bran oil without loss of enzyme activity even after six recycles [7]. The calcium alginate-chitosan was used for immobilization of PLA_1 by *Yu et al*. with a high fixation level but the immobilized enzyme could only be used for four cycles [21]. Immobilization of PLA_1 on magnetic nanoparticles showed better stability and in a batch, the final residual phosphorus content was reduced to 9.6 mg/kg after 7 h [3]. Oil degumming process, under the condition of pH 5.5 and T = 50 °C, the phosphorus content was reduced to less than 10 ppm after 4-5 h for free PLA_1 and after 7 h for immobilization PLA_1 on bentonite [1]. In comparison with previous researches, immobilized PLA_1 on APSM CS/PEO NM has a broad range of temperature and pH, compared to the free PLA_1 with 10 cycles reusability. Also, the final residual phosphorus content was reduced to less than 10 mg/kg in less time. Enzymatic degumming is a relatively recent adaptation of the standard water-degumming process that offers the benefit of increasing the efficiency, reducing losses, during the degumming step. The enzymatic process variation uses phospholipases to separate polar and nonpolar moieties of phospholipids, thereby improving the removal of the polar

Table 1: The residual phosphorus contents (mg/kg) after degumming by free and immobilized PLA_1 in batch process.

Time (h)	Phosphorous content (mg/kg)	
	Free PLA_1	Immobilized PLA_1
0	168.5 ± 2.8 [a]	168.5 ± 2.8 [a]
1	69.9 ± 1.8 [b]	96.4 ± 2.5 [a]
2	30.2 ± 1.1 [b]	52.9 ± 1.9 [a]
3	15.7 ± 0.5 [b]	28.3 ± 0.9 [a]
4	9.8 ± 0.3 [b]	18.1 ± 0.6 [a]
5	8.7 ± 0.3 [b]	10.5 ± 0.4 [a]
6	*7.6 ± 0.2 [b]*	*8.6 ± 0.2 [a]*

Values in the same raw followed by different letters are significantly different ($p<0.05$)

Table 2: Overview of the FFA and oxidative stability of degummed soybean oil after different kinds of treatment.

Parameters	Crude oil	Enzymatic degumming with free PLA_1	Enzymatic degumming with immobilized PLA_1
FFA (g/100g) *	2.10 ± 0.05[a]	2.22 ± 0.11[b]	2.23 ± 0.06[b]
PV (mmol/kg)	3.55 ± 0.07[a]	3.64 ± 0.13[b]	3.62 ± 0.09[b]

Values in the same raw followed by different letters are significantly different ($p<0.05$)

**. Expressed as the oleic acid*

components and reducing the amount of oil that is entrained in the gum phase [30]. Although some studies show a combination of new techniques to increase the efficiency and productivity of the degumming process were used [31]. The combination of different types of phospholipases [32], accelerates enzymatic processes with ultrasound used to intensify mass transfer rates and, in consequence, to increase the rates of biochemical reactions [33].

The effect of enzymatic degumming on the quality of degummed oil

It is well known that PLA_1 hydrolyzes the acyl-ester bond of phospholipids to release FFA thus, treatment of crude oil with PLA_1 could result in an increase in free fatty acid in the oils. In our study, there existed a significant difference between crude and enzymatic degummed oils ($P \leq 0.05$). *Sampaio et al*. found that with increasing enzymatic degumming time to achieve good degumming efficiency, FFA also increases (Table 2) [10]. The PV gives a measure of the extent to which an oil sample has undergone primary oxidation. Enzymatic degummed soybean oils showed a significantly higher value of PV compared with crude oil. However, a significant difference was not observed between soybean oils degummed by free (at 4 h) and immobilized PLA_1 (at 6 h). This increase means that the crude oil was partially oxidized during the enzymatic degumming process (Table 2). It might be explained that phospholipids were a kind of natural antioxidant and could delay the rate of oil oxidation [34].

CONCLUSIONS

Oil degumming is a crucial and costly process in oil refining. A maximum of 10 mg/kg is the final acceptable phospholipids content for vegetable oils and so achieving this value was the aim of this study. The results show the immobilization of PLA_1 on APSM CS/PEO NM not only provides a simple pathway to reach high enzyme loading, effective catalytic activity, easy recovery, and reusability but also remarkably improves the enzyme stability under common pH and thermal conditions compared to free PLA_1. The immobilized PLA_1 can be reused 10 times without a significant loss in the enzyme activity. In addition, the results demonstrate that immobilized PLA_1 can be used for industrial-scale degumming and appears to be robust enough for repeated use in batch reactors and other biotechnological processes.

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