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Effective Soybean Oil Degumming by Immobilized Phospholipases A_2 from *Walterinnesia aegyptia* Venom

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Cite This: *ACS Omega* 2024, 9, 21322−21332

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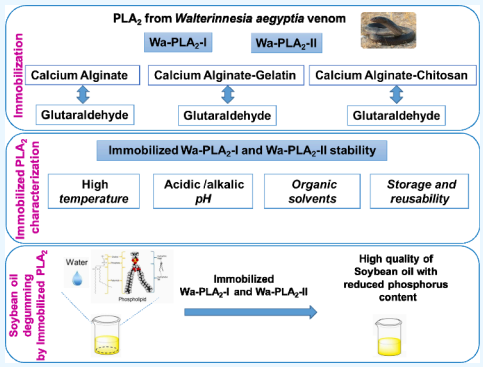
ABSTRACT: Enzymatic degumming utilizing phospholipase enzymes could be used in ecologically friendly procedures with enhanced oil recovery yields. In this study, two phospholipases A_2 of group I and II, WaPLA$_2$-I and WaPLA$_2$-II, from the snake venom of Saudi *Walterinnesia aegyptia* were evaluated for soybean oil degumming after being immobilized on three different support materials (calcium alginate (CA), CA-gelatin (CAG), and CA-chitosan (CAC), and cross-linked with glutaraldehyde). Higher yields of CAC-immobilized PLA$_2$-I (85 ± 3%) and PLA$_2$-II (87 ± 3.6%) compared to CAG (77.3 ± 2.1 and 79 ± 2.6%, respectively) and CA beads (55.7 ± 2.5% and 57.3 ± 3.1%, respectively) were observed. In addition, the optimal temperature of immobilized WaPLA$_2$-I and WaPLA$_2$-II increased from 45 to 55 °C and from 55 to 65 °C, respectively. Their stability at high temperatures was also significantly enhanced covering a larger range (70−80 °C). Likewise, the pH/activity profile of WaPLA$_2$ was greatly expanded upon immobilization with the pH-optima being shifted by 0.5 to 1 pH unit to the basic side. Similarly, the stability of WaPLA$_2$s in the presence of organic solvents was also significantly improved, while the affinity for calcium and bile salt was the same for both free and immobilized enzymes. Interestingly, the remaining activity of immobilized WaPLA$_2$ onto different supports was more than 50 or 60% after eight recycles or 120 days of storage at 4 °C, respectively. CAC−WaPLA$_2$-II was the best immobilized enzyme complex for the oil degumming process by reducing its final residual phosphorus content from 168 mg/kg to less than 10 mg/kg in only 4 h. Overall, CAC−WaPLA$_2$-II showed the most attractive profiles of temperature, pH, and reaction duration as well as significant storage stability and reusability.



1. INTRODUCTION

Phospholipases make up a class of lypolytic enzymes that hydrolyze the ester bonds of specific phospholipids. The main phospholipase families are A_1(PLA$_1$), A_2(PLA$_2$), B, (PLB), C (PLC), and D (PLD), with a target site varying according to their hydrolysis specificity.[1] Free fatty acids and 2-acyl lysophospholipid or 1-acyl lysophospholipid are produced by PLA$_1$ and PLA$_2$, respectively. PLB hydrolyzes the ester bond linking a fatty acid to the lysophospholipid. PLC is known to cleave the glycerophosphate bond in the common phospholipids. The phosphodiester bond is targeted by PLD to release the base group of the phospholipid substrate.[2]

PLA$_2$ enzymes are abundant in several biological tissues and liquids; among them is the viperidea venom.[3] According to their molecular weight, primary sequences, number and position of disulfide bonds, and their requirement of calcium for catalytic activity, five PLA$_2$ classes are defined: cytosolic calcium-dependent (cPLA$_2$), calcium-independent (iPLA$_2$), platelet-activating factor (PAF) acetylhydrolase, lysosomal PLA$_2$, and secreted PLA$_2$ (sPLA$_2$).[2] The sPLA$_2$ of snake venom is characterized by a molecular weight ranging from 14 to 18 kDa and high content on disulfide (S−S) bonds (5−8).[2] Their homology in the primary sequences and disulfide bonding pattern allow to class them onto two groups I and II. sPLA$_2$ group I is found in viperidea venom or Elapidae, and Hydrophidae snake venoms are rich with sPLA$_2$ group II.[3]

A broad range of biological processes involving snake venom sPLA$_2$ such as anticoagulant, antibacterial, antitumoral, and antiangiogenic activities makes these enzymes a potential pharmacological target. Moreover, snake venom sPLA$_2$ have been used in several industrial applications as biocatalysts especially biodiesel production, organic synthesis, and processing of vegetable oils.[4]

Due to the steadily increased demand for oils as a food and for fuel production over the past few years, a need for cost-effective approaches for discarding contaminating glycolipids and phospholipids (PLs), naturally occurring in seeds, during the refining process has been generated.[5,6] In fact, failure to remove

Received: February 18, 2024
Revised: April 18, 2024
Accepted: April 19, 2024
Published: May 1, 2024

these unwanted substances results in oil discoloration, off-taste formation, and troubles with subsequent oil processing. Chemical and physical degumming processes are considered as the first and most important step of oil refinement. These processes separate the majority of phospholipids and gums in order to produce high-quality oils with a phosphorus content of less than 10 mg/kg, indicating a high phospholipid removal efficiency. However, these conventional degumming techniques including usually water and acid degumming for phospholipids removal cannot guarantee phosphorus content industry requirements (<10 mg/kg).[1]

More recently, numerous studies have focused on enzymatic degumming because of its multiple advantages over physical and chemical techniques including better yields of refined oil, milder reaction conditions, and lesser chemical waste.[5,7] Various enzymes have been reported to be efficient for oil degumming, such as cholesterol/glycerophospholipid acyltransferases and phospholipasesA (PLA) and (PLC). Among PLA, the most commonly used are PLA_1 and PLA_2 converting nonhydratable PLs into their hydratable forms (lysoPLs), thus facilitating washout and definitely enhancing vegetable oil refining.[1,8,9] However, the industrial applications of freePLA_1 are limited by its thermal/organic solvents instability, pH requirements, and commercial unavailability, whereas several PLA_2s are lately being the most often-employed biocatalysts for the PL hydrolysis.[10]

Although the enzymes' industrial application is nowadays well established, there is still considerable research to be carried out on the development of novel applications as well as the improvement of current processes trough using promising technologies such as immobilization.[11] Compared to their free form, immobilized enzymes are preferred since they offer several advantageous especially higher stability, economic convenience, and ability of separation from reaction mixtures after hydrolysis and reuse of the insoluble enzyme several times, resulting in lower operating costs.[12]

Enzyme immobilization is a technique that involves confining enzymes to a solid phase (matrix/support) distinct from that of the substrates and products.[13] Enzyme immobilization by using different carriers and methods significantly improves the stability of the enzyme through enhancement and maintain of structural enzyme stability, which is crucial for industrial applications.[14,15] The microenvironment of the enzyme could be modified to enhance its activity as well as its specificity or selectivity could be adjusted.[16] Indeed, it has been reported that improvement of thermal stability of enzymes results from the molecular rigidity introduced by attachment to a rigid support immobilization and then creation of a protected microenvironment. Moreover, pH stability enhancement can be due to the change of the state of the immobilized enzyme ionization.[17]

Numerous methods have been described for enzyme immobilization, including cross-linking with glutaraldehyde, covalent attachment on gelatin and chitosan, entrapment in calcium alginate, or a combination of these techniques.[9,18,19]

In general, immobilization through adsorption or entrapment contributes to maintaining the catalytic activity of enzymes. However, cross-linking and covalent bonding techniques are effective in preserving stability. A combination of these methods can be a potent strategy for generating immobilized enzymes with both high catalytic activity and stability.[20] Gelatin, chitosan, and calcium-alginate are actually among the most broadly utilized polymers for immobilization.[21,22] Gelatin is a hydrocolloid material with a high content of amino acids. Its association with calcium alginate constitutes a good immobilization support for enzymes with a high yield.[14] It has been reported that alginate−gelatin−calcium hybrid and cross-linking of alginate with glutaraldehyde provided efficient encapsulation with prevention of enzyme leakage and increased mechanical stability.[14] Chitosan is a biopolymer derivative of chitin and known to be pliable and compatible for supporting attachments of enzymes.[13] Although pure chitosan beads offer many advantages, they lack the necessary robustness for widespread industrial applications. Under high temperature and pH, these beads tend to soften and become gel-like in aqueous environments. Therefore, significant research have been focused on strengthening the chitosan structure, resulting in the creation of numerous binary and ternary blended composites.[13] Chitosan has been employed in combination with alginate as an immobilization material that offers more physical and ionic interactions between the enzyme and support. Compared to pure chitosan beads, its combinatioon with other supports showed higher adsorption capacities and more resistant feature to acidic environments.[13] Glutaraldehyde has been used as a cross-linking agent with aminated immobilization support in order to promote the cationic, hydrophobic, and covalent interactions with enzymes.[23] Moreover, addition of glutaraldehyde as a cross-linker allows to cross-link the entrapped enzymes resulting to generation of aggregates and reduce their leakage.[24]

Several works on degumming of crude vegetable oils are based on the free forms of PLA_1 and PLA_2 from microbial and pancreatic origins.[1,4,25] However, few studies reported the use of immobilized PLA_2 in this industrial process.[25,26] This study aimed to immobilize two PLA_2 from *Walterinnesia aegyptia* (*W. aegyptia*) snake venom onto three different biocompatible and quite cheap mineral supports: calcium-alginate (CA), calcium alginate-gelatin (CAG), or calcium alginate-chitosan (CAC), using the cross-linking reagent, glutaraldehyde. The properties of the free and immobilized PLA_2 enzymes were studied as well as their effective use in soybean oil degumming in order to pave the way for their industrial application.

2. RESULTS AND DISCUSSION

2.1. WaPLA_2 Immobilization. Thanks to the broad application range of enzymes, their immobilization on insoluble and inert support materials is nowadays a very active field of research. In fact, this technique offers numerous advantages, such as improved storage stability, 3D structure stabilization, and an easy separation, recovery, and reuse of the enzyme. Different supports were evaluated for their efficiency to immobilize WaPLA_2-I and WaPLA_2-II enzymes (Table 1). Higher yields of CAC-immobilized PLA_2-I (85 ± 3%) and PLA_2-II (87 ± 3.6%) compared to CAG (77.3 ± 2.1% and 79 ± 2.6%, respectively) and CA beads (55.7 ± 2.5% and 57.3 ± 3.1%, respectively) were recorded. However, calcium carbonate ($CaCO_3$) support as well as the silica gel and Celite 545 carriers adsorbed remarkably much lower phospholipase enzymes (44−47.7, 22.7−25, and 31.4−32.7%, respectively) (Table 1).

Several animal and plant cells, microorganisms, enzymes, and cell organelles were efficiently immobilized on sodium alginate.[27] The high porosity of alginate beads, however, allows the entrapped enzymes with molecular weights less than 300 kDa to leak from the polymer matrix. Indeed, the pores created with 2% alginate had diameters ranging from 5 to 200 nm, allowing small-sized enzyme molecules (4−8 nm) to easily leak out.[28] This would explain the 35 kDa Wa-PLA_2-I and 14 kDa

https://doi.org/10.1021/acsomega.4c01558
ACS Omega 2024, 9, 21322−21332

Table 1. Immobilization of WaPLA$_2$-I and WaPLA$_2$-II Enzymes on Various Supports[a]

	yield of immobilized WaPLA$_2$ activity (%)	
support	WaPLA$_2$-I	WaPLA$_2$-II
CA	55.7 ± 2.5	57.3 ± 3.1
CAC	85 ± 3	87 ± 3.6
CAG	77.3 ± 2.1	79 ± 2.6
$CaCO_3$	44 ± 2	47.7 ± 2.5
Celite 545	32.7 ± 1.5	31.4 ± 1.5
silica gel	22.7 ± 1.2	25 ± 1

[a]The results are presented as the mean ± SD of three independent experiments. CA: calcium alginate, CAG: CA-gelatin, CAC: CA-chitosan, $CaCO_3$: calcium carbonate

Wa-PLA$_2$-II leakage from the gel's structure of CA.[18,29] To address this issue, both phospholipases were initially entrapped in CA followed by using glutaraldehyde to create a cross-linked matrix. The aim of incorporating the cross-linker glutaraldehyde was to facilitate the cross-linking of the entrapped enzymes, promoting the formation of aggregates and thus minimizing their leakage.[24]

Chitosan, an inert, inexpensive and hydrophilic support material, is found to be attractive for enzyme immobilization as it is nontoxic, biodegradable, and biocompatible.[30] In addition, the covalent binding of the enzyme to the support is greatly facilitated by the amino groups present on chitosan.[31] It has been documented that the immobilization could be through either the covalent binding of the enzyme to the chitosan transparent films or entrapment in the chitosan beads. These characteristics provide the system with a surface area and permeability appropriate for a selected biotransformation making chitosan one of the major immobilization techniques.[32] The use of gelatin together with glutaraldehyde as a cross-linking agent for enzyme and cell immobilization is more applicable in biochemical engineering because of its significant mechanical strength and biocompatibility. Current results were in line with several studies that supported the idea of the reactivity between amino acid residues in the immobilization support and glutaraldehyde moieties ensure the cationic or anionic charactere of support.[23] Since immobilization occurred in an acidic environment, the potential interaction between glutaraldehyde and gelatin or chitosan may result from the formation of Schiff bases between the glutaraldehyde molecules and the primary amine groups. At acidic pH levels, glutaraldehyde can produce both monomeric and polymeric cyclic hemiacetal forms, which offer stable covalent connections.[33] Compared to Wa-PLA$_2$−CA beads, the residual activities of CAG−Wa-PLA$_2$-I/CAG−Wa-PLA$_2$-II and CAC−Wa-PLA$_2$-I/CAC−Wa-PLA$_2$-II were remarkably increased by about 22 and 30%, respectively. Actually, the incorporation of gelatin into sodium alginate has been reported to highly improve the retention of both PLA$_1$ and PLA$_2$ activities.[18,19] Similarly, more than 80% retention of PLA$_1$ activity was noticed in hydrogel incorporating gelatin (43.5%) cross-linked with glutaraldehyde.[34] Therefore, one could conclude that using CA alone as the substrate for the immobilization of both Wa-PLA$_2$-I and Wa-PLA$_2$-II does not result in a perfect product. However, a much better immobilized

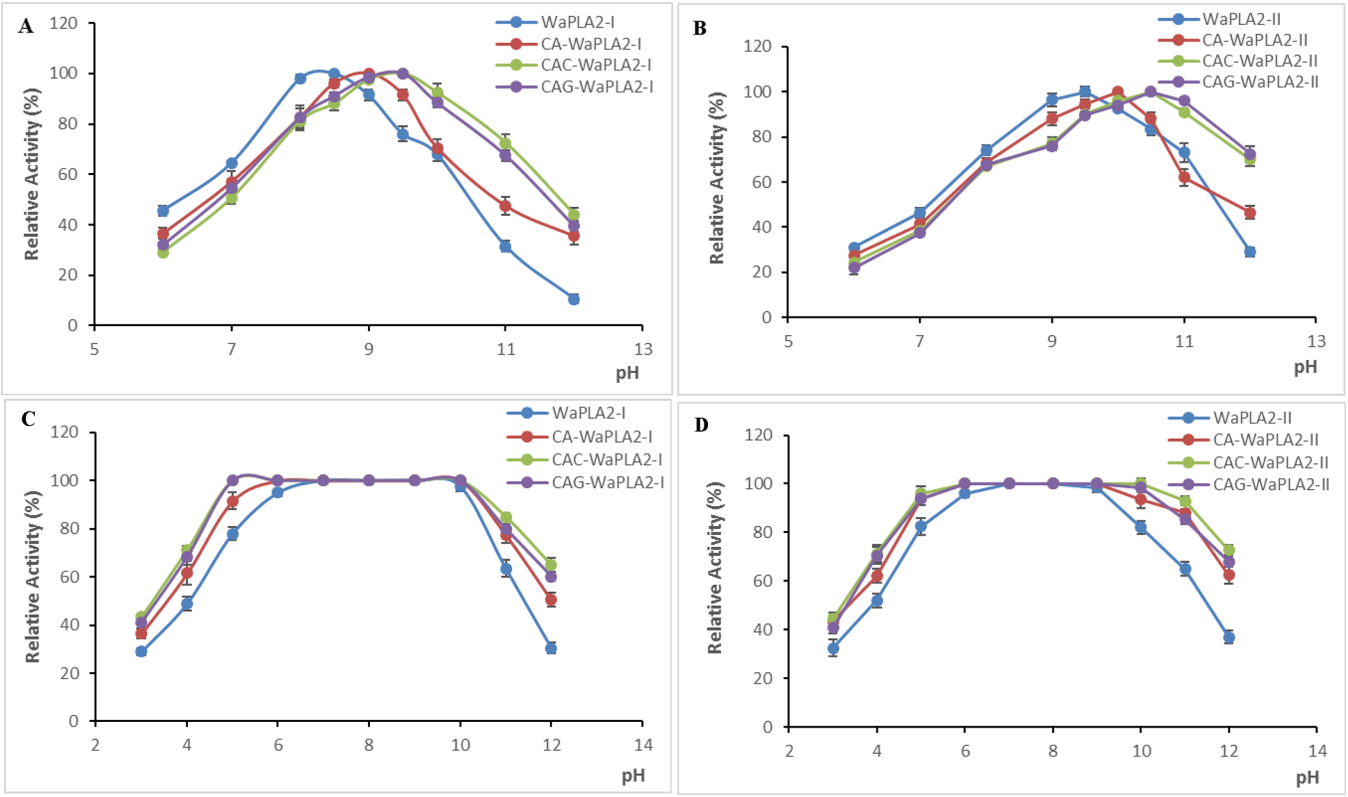


Figure 1. Effect of pH on the activity and the stability of free and immobilized WaPLA$_2$-I (A, C) and WaPLA$_2$-II (B, D).The pH stability was investigated by incubating each enzyme at ambient temperature for 1 h in different buffers at pH ranging from 3 to 12. PLA$_2$ activity was measured titrimetrically with phosphatidylcholine (PC) as the substrate at several pH values. The results are presented as means ± SD of three independent experiments.

https://doi.org/10.1021/acsomega.4c01558
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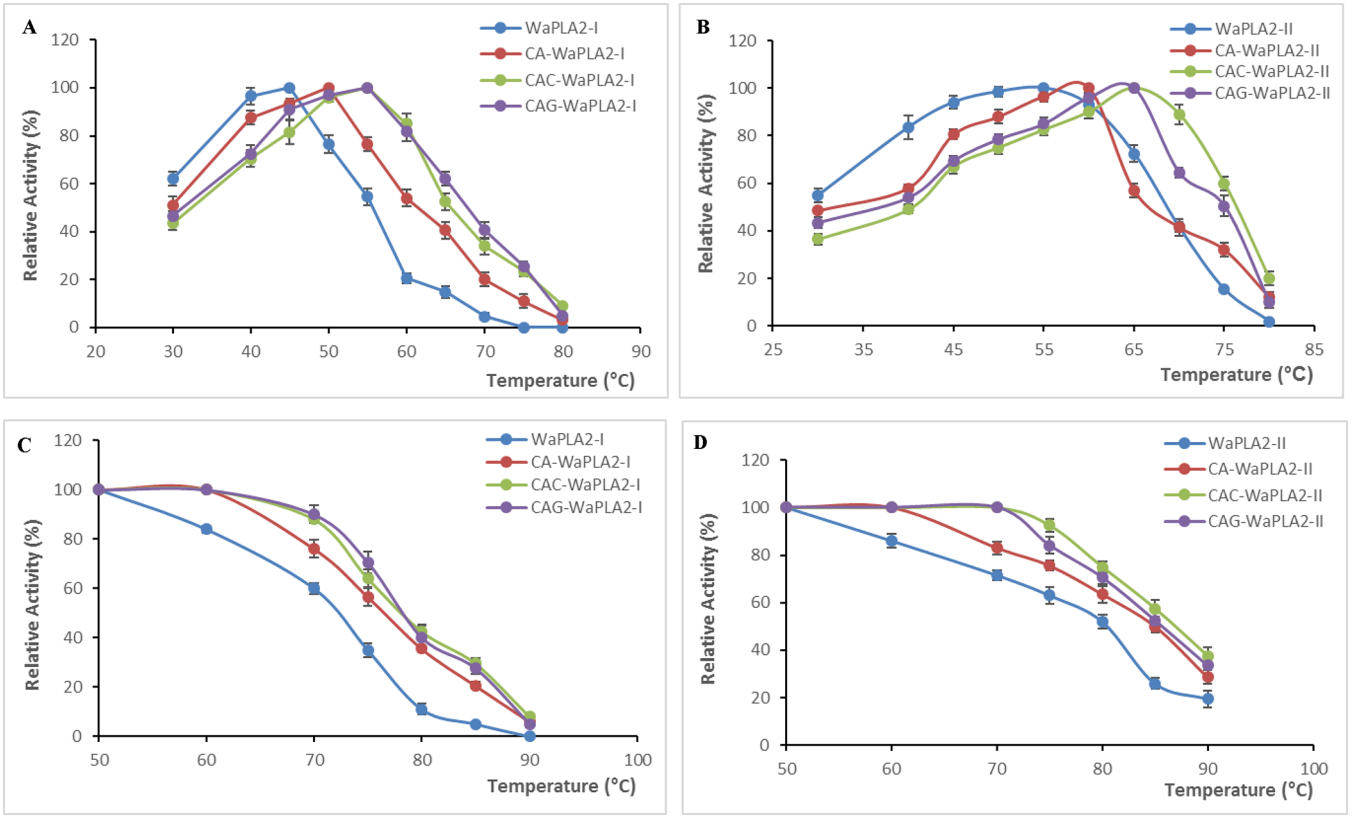

Figure 2. Effect of temperature on the activity and the stability of free and immobilized WaPLA$_2$-I (A, C) and WaPLA$_2$-II (B, D). The results are presented as means ± SD of three independent experiments.

product was obtained when CA was combined with gelatin or chitosan.

2.2. Effect of pH and Temperature on the Activity and the Stability of Free and Immobilized WaPLA$_2$s. Upon phospholipase immobilization, the optimum temperature and pH were found to remarkably increase. As shown in Figure 1A,B, free WaPLA$_2$-I and WaPLA$_2$-II displayed their maximal activities at pH 8.5 and 9.5, while the optimum pH values of 9 and 10 were recorded for CA− immobilized WaPLA$_2$-I and WaPLA$_2$-II, respectively. In the same line, optimum pH values of 9.5 and 10.5 were indicated for CAC−/CAG−WaPLA$_2$-I and CAC−/CAG−WaPLA$_2$-II, respectively.

This observation could be supported by previous studies, which reported that electrostatic charges could be influenced by immobilization, resulting in the optimal pH shift to a slightly alkaline region.[35,36] Likewise, WaPLA$_2$-I and WaPLA$_2$-II stability at extreme pH values was also enhanced after immobilization expanding the pH range 3−12, compared to pH 5−11 for both free enzymes. Interestingly, even after incubation for 1h at pH 12, CA−, CAC−, and CAG−*Wa*PLA$_2$ enzymes retained 50.5−62.5, 66−72.75, and 65.25−68.05% of their relative activities, respectively, while the free enzymes activity was reduced to 30.5 and 37% for WaPLA$_2$-I and WaPLA$_2$-II, respectively (Figure 1C,D).

Similarly, the optimum temperature increased from 45 to 50 or 55 °C and from 55 to 60 or 65 °C after immobilization of WaPLA$_2$-I and WaPLA$_2$-II in CA or CAC and CAG, respectively (Figure 2A,B). It is worthy to note that at pH 12 or 65 °C, all the immobilized forms of WaPLA$_2$-I retained more than 35% of their initial activities while the free WaPLA$_2$-I activity was reduced to less than 15%. Interestingly, at pH12 or 75 °C, CAC− and CAG−WaPLA$_2$-II displayed more than 50% of their full activities, whereas the free enzyme activity was reduced up to less than 30% indicating that the immobilization allowed a broader pH and temperature profiles probably due to greater pH and temperature stability. However, at higher temperatures, enzyme activity was markedly reduced due to PLA$_2$ thermal unfolding and consequently its inactivation.

Figure 2C,D also demonstrates that immobilization significantly improved the thermal stability of WaPLA$_2$ enzymes as well. Indeed, after 1 h of incubation at 85 °C, all the immobilized WaPLA$_2$-I and WaPLA$_2$-II retained more than 20 and 48% of their catalytic activities, respectively, against only 5 and 26% for their respective free forms, when incubated under the same conditions. Furthermore, CAC−PLA$_2$-I and CAC−PLA$_2$-II displayed the highest thermal stability retaining 29.5 and 57.5% of their initial activities at 85 °C, respectively. CA− and CAG−WaPLA$_2$-II also exhibited a remarkably important thermal stability since their initial activities were reduced to only 48.5 and 52.5% at 85 °C, respectively, compared to the free WaPLA$_2$-II, which retained only 26% of its full activity under the same conditions (1 h at 85 °C). A similar behavior was observed with WaPLA$_2$-I, which was, however, less stable against heat compared with WaPLA$_2$-II.The improvement in thermal stability upon immobilization indicating reduced enzyme denaturation could be attributed to the attachment of PLA$_2$ enzymes to the support materials, which partially compensate for the activity loss observed for the free enzyme. Overall, these findings were in accordance with previous reports on PLA$_2$ showing enhanced enzyme performance and increasing tendency of pH and temperature after immobilization by different techniques[19,36] and therefore suggested the potential

https://doi.org/10.1021/acsomega.4c01558
ACS Omega 2024, 9, 21322−21332

biotechnological applications of the immobilized $WaPLA_2$ enzymes in several industrial and pharmaceutical processes.

2.3. Storage Stability of Free and Immobilized $WaPLA_2$s. One of the most important factors in estimating the enzyme's characteristics is its storage stability, which can make an enzyme immobilized rather than free more useful and applicable. Free-form enzyme storage is generally less appropriate for long-term use than an immobilized system. In this context, the enzyme activity of free and immobilized beads, which were stored at room temperature (25 °C) or at 4 °C in buffer A, was followed over a 120 days period (Table 2). One could easily see that immobilized enzyme activity showed a slower decrease compared to that of its respective free form. After 120 days at 4 °C, residual enzyme activities of 33−36, 58−60.5, 65−72, and 63−72% were recorded for free enzymes and immobilized CA, CAC, and CAG beads, respectively. On the other hand, after 80 days incubation at 25 °C, residual enzyme activities of 5−11, 22−38, 30−45, and 34−47% were indicated for free enzymes and immobilized CA, CAC, and CAG beads, respectively. Notably, our findings indicated that CAC immobilized beads are the most stable and advantageous.

2.4. Organic Solvent Tolerance. Due to the easy solubility of nonpolar molecules, organic solvents are used in the majority of synthetic processes conducted on an industrial scale. Indeed, the thermal stability of enzymes and the solubility of nonpolar substrates could be extremely improved by using organic solvents, which, in addition, might eliminate microbial contamination or decrease water-dependent side reactions.[37] In the presence of water-miscible organic solvents, both free $WaPLA_2$-I and $WaPLA_2$-II displayed significant stability as they maintained almost 100% of their initial activities after exposure, for 4 h at room temperature (25 °C), to acetone (50%), acetonitrile (50%), diethyl ether (50%), ethanol (50%), or methanol (50%) (Table 3). However, acetic acid was the most harmful among all tested organic solvents probably due to its acidity inactivating the free $WaPLA_2$-I and $WaPLA_2$-II up to 50%, which was reduced to less than 30%, upon immobilization. Interestingly, compared to controls, the addition of 50% ethanol, 50% acetonitrile, or 50% methanol caused more than 15, 25, or 30% immediate increases of the CA−, CAG−, or CAC−$WaPLA_2$-I and CA−, CAG−, or CAC−$WaPLA_2$-II activities, respectively. Hence, the stability of $WaPLA_2$-I and $WaPLA_2$-II in organic solvents enabled their commercial application in a variety of useful reactions, such as esterification.

2.5. Calcium and Bile Salt Dependence. Numerous enzymes, including $sPLA_2$, are known to be induced, stabilized, and protected against conformational changes by the presence of calcium ions (Ca^{2+}).[38] Meanwhile, tensioactive agents such as bile salts were well-known to be crucially involved in solubilizing phospholipids and the hydrolysis reaction products.[39] Current study confirmed that both $WaPLA_2$-I and $WaPLA_2$-II also required Ca^{2+} and bile salts to express their catalytic activities since the hydrolysis rates increased with increasing Ca^{2+} or sodium taurodeoxycholate (NaTDC) concentrations as depicted in Figure 3. At 8 mM Ca^{2+} and 4 mM NaTDC, maximal $WaPLA_2$-I and $WaPLA_2$-II activities were reached under optimal reaction conditions. Comparable findings were observed with the three immobilized $WaPLA_2$-I and $WaPLA_2$-II, implying that the affinity for Ca^{2+} and NaTDC was the same for both free and immobilized enzymes. Additionally, a strict dependence on Ca^{2+} was observed for all tested enzymes, which were completely inactive without added Ca^{2+} and the presence of EDTA or EGTA as chelator agents (Figure 3C,D). These findings were

Table 2. Long-term Stability of Free and Immobilized $WaPLA_2$-I and $WaPLA_2$-II Stored at Room Temperature (25 °C) or in a Refrigerator (4 °C)[a]

	$WaPLA_2$-I		CA-$WaPLA_2$-I		CAC-$WaPLA_2$-I		CAG-$WaPLA_2$-I		$WaPLA_2$-II		CA-$WaPLA_2$-II		CAC-$WaPLA_2$-II		CAG-$WaPLA_2$-II	
days	4 °C	25 °C	4 °C	25 °C	4 °C	25 °C	4 °C	25 °C	4 °C	25 °C	4 °C	25 °C	4 °C	25 °C	4 °C	25 °C
0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
10	100 ± 0	65 ± 5.7	100 ± 0	88 ± 4.9$^{\#}$	100 ± 0	92 ± 4.9$^{\#}$	100 ± 0	96 ± 3.8$^{\#}$	100 ± 0	81 ± 3.8	100 ± 0	95 ± 6.5	100 ± 0	100 ± 0	100 ± 0	100 ± 0
20	100 ± 0	47 ± 4.3	100 ± 0	75 ± 5.3$^{\#}$	100 ± 0	83 ± 5.3$^{\#}$	100 ± 0	81 ± 4.5$^{\#}$	100 ± 0	67 ± 4.3	100 ± 0	83 ± 4.8	100 ± 0	94 ± 4.9	100 ± 0	91 ± 4.5
40	80 ± 4.2	33 ± 2.8	98 ± 6.5*	53 ± 4.7	100 ± 0*	61 ± 2.8$^{\#}$	100 ± 0*	65 ± 5.2$^{\#}$	85 ± 5.3	45 ± 3.5	100 ± 0*	68 ± 3.2	100 ± 0*	74 ± 3.8$^{\#}$	100 ± 0*	76 ± 5.2
50	73 ± 3.5	20 ± 1.7	92 ± 7.2	36 ± 3.7	100 ± 0*	50 ± 3.7	100 ± 0*	52 ± 4.7	77 ± 2.8	27 ± 2.1	100 ± 0*	59 ± 4.3$^{\#}$	100 ± 0*	63 ± 4.7$^{\#}$	100 ± 0*	65 ± 6.1$^{\#}$
60	62 ± 2.8	12 ± 0.8	85 ± 4.3*	30 ± 2.3	95 ± 5.2**	38 ± 1.7	94 ± 2.8**	40 ± 3.5	68 ± 3.7	18 ± 1.5	91 ± 4.5	47 ± 2.8$^{\#}$	96 ± 3.5	51 ± 4.7$^{\#}$	100 ± 0**	50 ± 4.9$^{\#\#}$
80	55 ± 4.7	5 ± 0.5	75 ± 4.9*	22 ± 1.4$^{\#\#}$	83 ± 3.5**	30 ± 1.5$^{\#\#}$	85 ± 3.7**	34 ± 2.7$^{\#\#}$	57 ± 4.3	11 ± 1.2	80 ± 5.1*	38 ± 2.1$^{\#}$	88 ± 4.9**	45 ± 3.5$^{\#\#}$	92 ± 3.8**	47 ± 3.5$^{\#\#}$
100	45 ± 2.8	0 ± 0	66 ± 3.7	15 ± 0.7$^{\#\#}$	72 ± 5.3*	22 ± 2.1$^{\#\#}$	76 ± 2.1*	23 ± 1.1$^{\#\#}$	49 ± 3.2	8 ± 0.3	71 ± 6.8*	26 ± 1.7$^{\#}$	82 ± 2.8**	30 ± 2.4$^{\#\#}$	85 ± 4.2**	29 ± 1.7$^{\#}$
120	33 ± 3.2	0 ± 0	60 ± 2.8*	9 ± 0.2$^{\#\#}$	65 ± 4.9*	13 ± 1.1$^{\#\#}$	63 ± 3.7*	11 ± 0.7$^{\#\#}$	36 ± 2.5	0 ± 0	60.5 ± 1.2	10 ± 0.7$^{\#}$	72 ± 4.9	19 ± 1.4$^{\#\#}$	72 ± 2.8	16 ± 1.2$^{\#\#}$

[a]Assays were performed under standard conditions as described in the main text. The results are presented as means ± SD of three independent experiments, at 4 °C $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus control values (free $WaPLA_2$-I and $WaPLA_2$-II), and at 25 °C $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus control values (free $WaPLA_2$-I and $WaPLA_2$-II).

https://doi.org/10.1021/acsomega.4c01558
ACS Omega 2024, 9, 21322−21332

Table 3. Stability of Free and Immobilized $WaPLA_2$-I and $WaPLA_2$-II in Several Organic Solvents[a]

	$WaPLA_2$-I	CA-$WaPLA_2$-I	CAC-$WaPLA_2$-I	CAG-$WaPLA_2$-I	$WaPLA_2$-II	CA-$WaPLA_2$-II	CAC-$WaPLA_2$-II	CAG-$WaPLA_2$-II
none	100	100	100	100	100	100	100	100
2-propanol	90 ± 2.5	99 ± 1.5	107 ± 2.2	110 ± 2.7	95 ± 3.2	103 ± 0.7	119 ± 1.2	110 ± 2
ethanol	101 ± 1.5	118 ± 4.5	132 ± 2.3*	124 ± 1.9*	103 ± 2.8	118 ± 4.5	145 ± 4.8*	130 ± 3.3*
methanol	98 ± 2.4	116 ± 3.2	135 ± 1.9*	126 ± 2.1*	94 ± 1.2	110 ± 3.6	130 ± 2.1*	121 ± 3.1*
acetonitrile	93 ± 2.7	109 ± 1.1	126 ± 3.3*	118 ± 1.4*	97 ± 3.5	113 ± 0.8	138 ± 1.2**	127 ± 4.3**
acetone	91 ± 3.1	100 ± 0.6	105 ± 3.6	106 ± 2.3	95 ± 2.7	105 ± 2.3	108 ± 2.8	102 ± 1.3
acetic acid	50 ± 2	72.1 ± 2.5*	78 ± 2.1*	81.6 ± 1.1**	55 ± 3.4	79 ± 3.7*	82 ± 2*	85 ± 2.1**

[a]Free and immobilized $WaPLA_2$-I and $WaPLA_2$-II were incubated in each organic solvent (50%) for 4 h at 25 °C. Values represent the means of three replicates. The results are presented as means ± SD of three independent experiments. *$p < 0.05$; **$p < 0.01$ versus control values (free $WaPLA_2$-I and $WaPLA_2$-II).

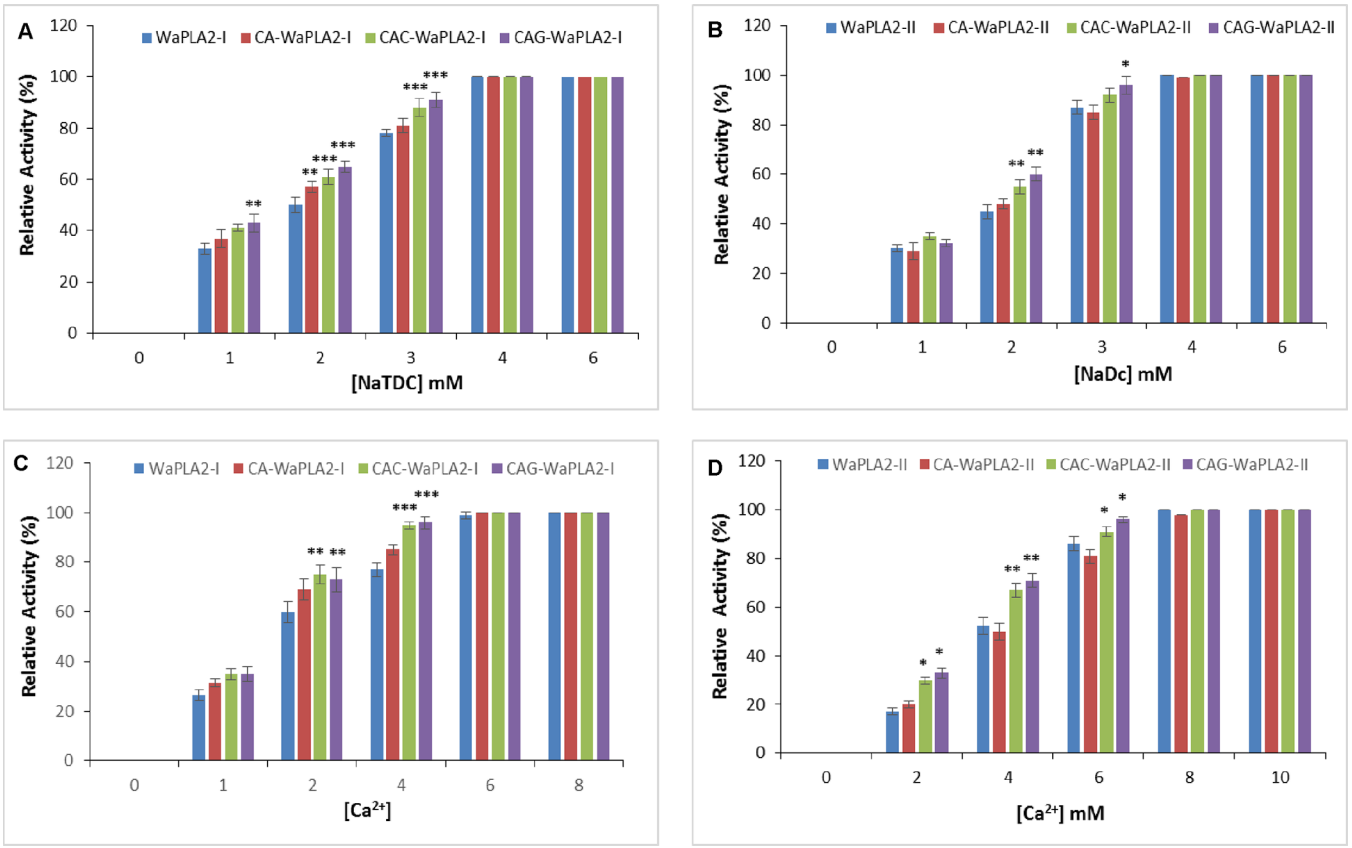

Figure 3. Effect of NaTDC and calcium on free and immobilized Wa-PLA_2-I (A, C) and Wa-PLA_2-II (B, D). The results are presented as means ± SD of three independent experiments, *$p < 0.05$; **$p < 0.01$; ***$p <$ 0,001 versus control values (free $WaPLA_2$-I and $WaPLA_2$-II).

consistent with those of earlier investigations on PLA_2 from various sources, therefore emphasizing the crucial role of Ca^{2+} in stabilizing the enzyme−substrate interactions during the catalysis mechanism.[3,37,40]

2.6. Application of $WaPLA_2$s in Soybean Oil Degumming. Crude vegetable oils contain a high level of phospholipids causing oil darkening and subsequent off-flavors when they are stored and therefore creating constraints on the processing of the oil downstream.[41] Consequently, eliminating these phospholipids is crucial to generate high-quality finished oil with a phosphorus content of less than 10 mg/kg.[42] Hydratable phospholipids are removed, during the industrial oil refining process, by water degumming, whereas chemical or enzymatic treatment are performed to eliminate nonhydratable phospholipids. Enzymatic degumming, which is commonly conducted with phospholipases, exhibited numerous advantages, for instance, ameliorating the process yield, decreasing the level of wastewater, alkali, and acid, and reducing the industrial operating costs, and negative impacts on the environment.[3,5,7] Among phospholipases, $sPLA_2$ are among the most effective degumming candidates as they convert nonhydratable PLs into their hydratable forms (lysoPLs).[1,8,9]

Soybean oil, the most widely consumed vegetable oil, was used in this study to assess the performance of free and immobilized $WaPLA_2$-I and $WaPLA_2$-II in the degumming process by measuring the phosphorus content. Additionally, the reaction time effect on the residual levels of phosphorus was evaluated under the optimized temperature and pH of each PLA_2 as determined above. At 1 h intervals, aliquots of the degumming oil were taken to analyze the residual phosphorus concentrations (Figure 4). As shown in Figure 4A,B, as the time of the $WaPLA_2$ catalyzed reaction increased, the phosphorus content steeply dropped particularly by $WaPLA_2$-II and to lesser extent by $WaPLA_2$-I. Indeed, the phosphorus level was reduced

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ACS Omega 2024, 9, 21322−21332

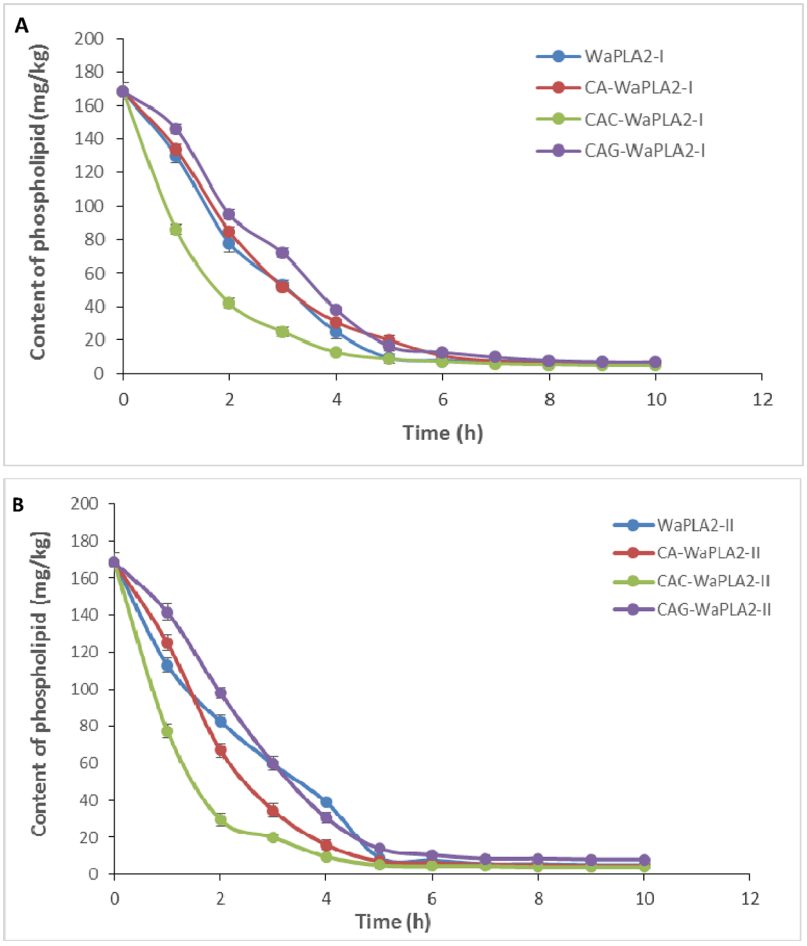

Figure 4. Degumming soybean oil by free and immobilized WaPLA$_2$-I (A) and WaPLA$_2$-II (B). The results are presented as means ± SD of three independent experiments.

by both free enzymes to less than 10 or 7 mg/kg after 5 or 10 h of reaction, respectively, which is adequate for soybean oil physical refining. Likewise, all immobilized WaPLA$_2$ displayed comparable profiles; however, the most effective degumming process was achieved by using CAC−WaPLA$_2$-II and CA−WaPLA$_2$-II; the residual phosphorus concentrations were diminished up to 5 mg/kg over 5 and 7 h reaction against only 9 and 7.5 mg/kg with CAC−WaPLA$_2$-I and CA−WaPLA$_2$-I, respectively, under the same conditions. In contrast, a phosphorus content of 14 or 16.5 mg/kg was reached after 5 h of CAG−PLA$_2$-II or CAG−PLA$_2$-I catalyzed reactions, respectively. These findings were in line with previous studies on immobilized pancreatic PLA$_2$, showing that the phosphorus content was lowered up to 10 mg/kg over 5 or 9 h using the free and CAC−PLA$_2$ or CA−PLA$_2$, respectively. In contrast, only 15 mg/kg of phosphorus was achieved over a 10 h CAG−PLA$_1$-catalyzed reaction.[19] Yu et al.[18] also reported that the immobilized CAC−PLA$_1$ was the best immobilized enzyme complex for the continuous phospholipids' hydrolysis in crude soybean oils. Moreover, Qu et al.[36] demonstrated a residual phosphorus content of 9.8 mg/kg was obtained by the enzymatic degumming with PLA$_2$-Fe_3O_4/SiOx-g-P(GMA) over 5 h.

2.7. Reusability of Immobilized WaPLA$_2$s. Besides enhanced catalytic activity and stability, several methods used for enzyme immobilization permit easy separation, recovery, and reuse of PLA$_2$. In fact, one of the major obstacles in biocatalysis is the removal and recycling of enzymes from the reaction media, which directly affects economic feasibility. To investigate the reusability of all immobilized WaPLA$_2$ in soybean oil degumming, the degumming process was carried out recurrently in 15 rounds (for 75 h), and the recovered conjugated PLA$_2$ was abundantly washed with buffer A and reused for the next degumming reaction cycle. The residual activities of each immobilized WaPLA$_2$ were determined after each 5 h reaction cycle and plotted versus cycle numbers as shown in Figure 5. After eight degumming cycles, CA−, CAC−, and CAG−WaPLA$_2$-I or WaPLA$_2$-II retained 55, 82, and 87% or 50, 77, and 85% of their relative activities, respectively (Figure 5A,B). However, after 12 cycles, CA−WaPLA$_2$-I and CA−WaPLA$_2$-II residual activities dropped up to 21 and 15%, respectively, while both CAC− and CAG−WaPLA$_2$-I or CAC− and CAG−WaPLA$_2$-II still maintained more than 45% of their maximal activities. Yu et al.[19] evaluated the reusability of the CA−, CAC−, and CAG− immobilized PLA$_2$ from mammal pancreas for soy oil degumming and found that, after seven cycles, the three immobilized enzymes maintained 30 to 60% of their original activities. Whereas, Qu et al.[36] found that magnetic immobilized PLA$_2$ retained more than 80% of its initial activity even after five cycles of soybean oil degumming. Another study by Liu et al.,[43] described the immobilization of Lecitase ultra (IM-LU) onto macroporous resin for the synthesis of diacylglycerols from soybean oil. The authors reported that about 32% of diacylglycerol might be produced even after 28 glycerolysis reaction runs. Interestingly, at 55 °C, PLA$_1$ immobilized on magnetic nanoparticles exhibited more than

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ACS Omega 2024, 9, 21322−21332

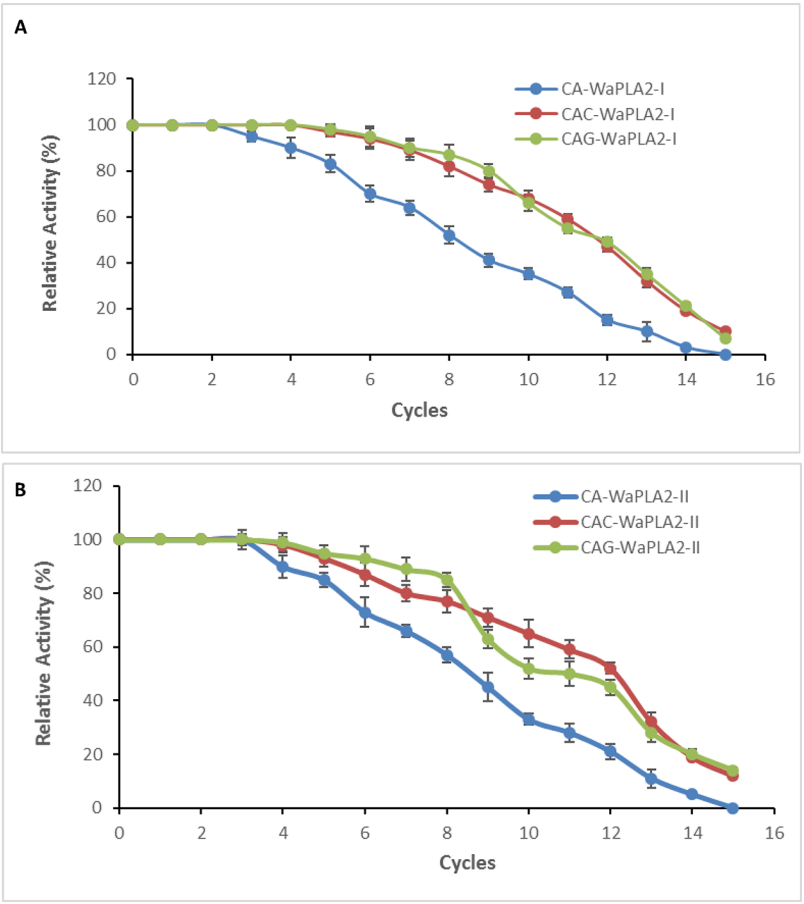

Figure 5. Effect of immobilized $WaPLA_2$-I (A) and $WaPLA_2$-II (B) reuse on the soybean oil degumming process. The results are presented as means ± SD of three independent experiments.

80% of its initial activity even after 10 runs of soybean oil degumming reaction.[19]

3. MATERIALS AND METHODS

3.1. Enzymes. *W. aegyptia* group-I ($WaPLA_2$-I) and $WaPLA_2$-II were purified as previously described by Bacha et al.[44] and Abid et al.,[45] respectively.

3.2. Immobilization of $WaPLA_2$s. Immobilization of $WaPLA_2$-I and -II on silica gel, $CaCO_3$, or Celite 545 supports was carried out according to Rosu et al.'s[46] protocol. Briefly, 2 g of each support was mixed with each PLA_2 solution (5 mL) and incubated at 4 °C for 2 h with stirring (200 rpm). Meanwhile, PLA_2 enzymes were immobilized in CA following the protocol described by Ateş and Mehmetoğlu[47] but slightly modified. The PLA_2 solution (5 mL) was added to a mixture of 50 mL of sodium alginate (2.0%)/acetic acid (5.0%) under stirring (200 rpm). A 10 mL sterile hypodermic syringe needle was used to extrude the homogeneous mixture at 5 drops/s into a 0.2 M $CaCl_2$ solution allowing 2 mm diameter beads formation. Sun et al.'s[21] method with slight modification was used to prepare CAC−PLA_2. Five mL of PLA_2 solution was added to a mixture of 25 mL of chitosan (4%)/$CaCl_2$ 0.2 M/acetic acid (5%) followed by a gentle dropping of 25 mL of sodium alginate (4%)/acetic acid (5%) to form beads. In addition, preparation of PLA_2−CAG was performed following Zhu et al.'s[48] protocol but slightly adjusted. First, 25 mL of gelatin (8 wt %) was added to a mixture of 25 mL of sodium alginate (4%)/acetic acid (5%). Thereafter, 5 mL of PLA_2 solution was mixed with the resulting alginate−gelatin solution (50 mL) and 0.2 M $CaCl_2$ solution was subsequently dropped into the mixture under continuous stirring (200 rpm) to form beads. Finally, after hardening with glutaraldehyde solution (0.4%, 5 mL), washing, and freeze-drying of all formed beads, the PLA_2 activity was determined as described below.

3.3. Enzyme Activity Assay. PLA_2 activity was measured titrimetrically on phosphatidylcholine (PC) as substrate according to Abousalham and Verger,[49] in the presence of different concentrations of Ca^{2+} and NaTDC and at the indicated temperatures and pH values. One unit of PLA_2 activity corresponds to 1 μmol of fatty acid released under optimized reaction conditions (pH and temperature).

3.4. Thermal, pH, and Storage Stability of Free and Immobilized $WaPLA_2$. The thermal stability of the free and immobilized PLA_2 was studied by incubating the enzymes in buffer A (50 mM Tris HCL buffer, pH 8) at different temperatures spanning from 50 to 90 °C for 1 h. Meanwhile, the pH stability was investigated by incubating each enzyme at ambient temperature for 1 h in different buffers (200 mM): sodium acetate buffer (pH 3−5); potassium phosphate buffer (pH 6−7); tris−HCl buffer (pH 8−9); glycine-NaOH buffer (pH 10−12). In addition, in order to evaluate their storage stability, the relative activities of free and immobilized enzymes stored at room temperature (25 °C) or 4 °C were also measured over 120 days. After each incubation, the enzyme samples were rapidly cooled and centrifuged and the residual activities were determined in the resulting supernatants under optimal reaction

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conditions. The recorded activities were compared with the corresponding initial activities (defined as 100%) to calculate the relative activity.

3.5. Batch Degumming of Soybean. Free and immobilized WaPLA$_2$ enzymes were evaluated for oil degumming under the optimized reaction conditions. A mixture of soybean oil (200 mL) and citric acid (45%, 130 mL) was heated to 80 °C with stirring for 5 min at 900 rpm. After cooling to 60 °C, the oil was adjusted to the appropriate pH using 1 M NaOH followed by addition of water (3 mL) under continuous stirring at 30 rpm for 20 min. Then, the free or immobilized WaPLA$_2$ (200 U/kg oil mass) was added and the mixture was stirred (30 rpm) at 60 °C for 10 h. Samples for phosphorus analysis were withdrawn from the reaction mixture at 1 h intervals and centrifuged at 7000*g* to remove mucilaginous gums and the immobilized enzymes. Residual phosphorus content in the resulting oil phase was performed according to the AOCS method ca. 12−55.[50]

3.6. Reusability of Immobilized WaPLA$_2$s. Reusability of immobilized PLA$_2$ was measured through monitoring the hydrolysis of the soybean oil phospholipids at 50−65 °C over 15 cycles. After each reaction run, CA, CAC, and CAG beads were removed, washed with buffer A to remove any remaining substrate within the beads, and reused in a fresh reaction medium. The residual PLA$_2$ activity was determined under the optimal reaction conditions defined and compared to the initial activity defined as 100%. This process was repeated for 15 cycles.

3.7. Statistical Analysis. All of the assays were done in biological triplicates with three technical replicates, and data are given as mean ± standard deviation (SD). The statistical analysis was carried out through one-way analysis of variance (ANOVA) as well as Duncan's post hoc test using GraphPad Prism version 9. A p-value of <0.05 was regarded as significant, and asterisks are used to indicate significance.

4. CONCLUSIONS

Here, a successful immobilization of WaPLA$_2$-I and WaPLA$_2$-II onto CA, CAC, and CAG matrices was described, and their efficiency in soybean oil degumming was evaluated. These supports maximized the stability, efficacy, performance, and reusability of the immobilized WaPLA$_2$, which showed higher tolerance to high temperatures and acidic and basic pH, as well as better stability in the presence of organic solvents compared to free enzymes. In the batch oil degumming, all immobilized WaPLA$_2$ displayed comparable profiles; however, the most effective degumming process was achieved by using CA− and CAC−WaPLA$_2$-II reducing the phosphorus content up to 5 mg/kg within 5 and 7 h reactions, respectively. Although CAG−WaPLA$_2$-I and CAG−WaPLA$_2$-II were less efficient at hydrolyzing phospholipids compared to free enzymes, they have the advantage of being reusable enzymes and could be, together with other immobilized Wa-PLA$_2$, excellent candidates for industrial applications.

■ ASSOCIATED CONTENT

Data Availability Statement

"Not applicable"

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Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analyses were performed by M.A. and A.B.B. The first draft of the manuscript was written by A.B.B. All authors have read and agreed to the published version of the manuscript.

Funding

This research received no external funding.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors extend their appreciation to the researchers' Supporting Project number (RSP2024R237), King Saud University, Riyadh, Saudi Arabia, for funding this work.

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