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Efficient Degumming of Rice Bran Oil by Immobilized PLA_1 from *Thermomyces lanuginosus*

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Summary

Phospholipase A_1 (PLA_1) immobilized in calcium alginate can effectively overcome the mass transfer resistance at the lipid-water interface making more room for the enzyme to separate itself from the products of reaction and to bind with the next available molecule at the interface. The reaction of an immobilized PLA_1 hydrolase from *Thermomyces lanuginosus* was comparatively faster than of its free form. The rate of phospholipid hydrolysis by PLA_1 was studied in calcium-rich and calcium-depleted environments; and the extent of phosphorus removed from the crude rice bran oil as well as the amount of free fatty acids produced during the reaction were used as indices for analysing the rate of enzymatic hydrolysis under standard conditions of pH, temperature, time of incubation and agitation. The immobilized PLA_1 was found to be superior in removing phosphorus in the presence of 10 mM bivalent calcium ions in a solution. As compared to a maximum of 72.52 % phosphorus removed by 0.01 kg of free enzyme per kg of oil, the same amount of immobilized PLA_1 removed phosphorus from oil by 94.12 % under the same experimental conditions (pH=6, 60 °C, 1-hour incubation). Both the free PLA_1 and its immobilized form had shown extended rates of hydrolysis in a calcium-rich environment. The mass fractions of free fatty acids produced by the free enzyme and by its immobilized form were 14.9 and 14.16 %, respectively, under the above experimental conditions. The removal of phosphorus from oil was accompanied by a significant reduction in colour and restoration of iodine value to the desired level.

Key words: phospholipase A_1, *Thermomyces lanuginosus*, phospholipids, degumming, rice bran oil, immobilization

Introduction

Phospholipases experience enormous mass transfer resistance while catalysing hydrolysis reactions at the lipid-water interface. The effective contact between the enzyme and the substrate phospholipids is hindered by several non-reacting components and products of the reaction as well as by the physicochemical conditions like surface pressure, pH and ions present in the solution (*1*). Phospholipids form aggregates and remain as separate entities in oil. Successive cleavage of glycerol-ester bonds in different phospholipid molecules by a single enzyme becomes difficult due to this adverse environment around the enzyme. Phospholipase reaction in heterogeneous phase largely depends on the concentration of phospholipid aggregates and the amount of enzyme at the lipid--water interface. The rate of phospholipase reaction depends on the fraction of total enzymes that reached the interface from the bulk of aqueous enzyme solution (*2*). The major application of phospholipases in edible oil degumming lies in converting nonhydratable phospholipids (NHPs) into hydratable lysophospholipids. The use of these enzymes in oil degumming is justifiable as they function as alternatives to phosphoric acid degumming. However, the enzymatic process is slower as compared to

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the conventional high-temperature chemical degumming operation. The rate of reaction decreases as most of the enzymes cannot reach the interface from the bulk aqueous phase. Those that do reach cannot hydrolyse the substrate molecules in succession. Most of the studies on mass transfer behaviour of phospholipase reactions so far have been made on phospholipase A_2 (PLA_2) and conclusive outcomes were imposed upon PLA_1 as its catalytic behaviour. PLA_1 enzymes cleave the glycerol-ester bond of phospholipids at *sn*-1 position, while PLA_2 selectively cleave the ester bond at *sn*-2 position. Both PLA_1 and PLA_2 suffer from reduced rate of catalysis in heterogeneous phase comprising lipid and water. The reaction at the beginning follows a simplified Michaelis-Menten kinetics when the enzyme easily finds the substrate as there are enough substrate molecules to undergo catalytic cleavage by the enzymes. The hydrolysis in a specially designed bioreactor is unique, unlike the one that occurs within a cellular environment. Calcium ions are found to be accelerators for many PLA_2 reactions. The role of calcium ion is attributed to the stabilization of anionic phospholipid molecules in the transition state and to its affinity for the conserved aspartic acid residue in many secretory PLA_2 enzymes. It is highly evident from the involvement of bivalent calcium ions and polar water molecules in acyl hydrolase reactions that water not only takes part in the hydrolysis of acyl-ester bonds, but is also required for transferring nucleophilicity of the catalytic site to the substrate. Calcium ion-mediated PLA_1 catalysis has also been found to be effective with edible oil degumming (*3*). The acyl hydrolase reactions, including those of lipases, require a constant supply of water molecules nearer to the active site of the enzyme. Thus, a reduction in the rate of hydrolysis at the oil-water interface may be attributed to a combined effect of three major developments taking place with progress of time, namely (*i*) inhibition due to substrate arrangement (micelle, monolayer or bilayer) and non-reacting components, (*ii*) product inhibition, and (*iii*) inactivation of catalytic site by a water-repellent hydrophobic environment around the enzyme. Among these three constraints of hydrolysis, the third one can be effectively minimized by immobilization of the enzyme within a gel matrix using alginate. The confinement of enzyme and water in an isolated environment within the matrix enhances the rate of reaction by extending the surface of contact between the enzyme and the substrate. However, reactions of immobilized enzymes suffer from diffusion limitations. The possibility of higher amount of substrate remaining unreacted is more pronounced with free enzyme, as part of the latter is inactivated due to its detachment from water molecules in the solution.

Unlike many PLA_2 enzymes, the crystal structure of PLA_1 is still not fully known based on experimental observations. Lipases are active at fairly milder temperature range (40–45 °C) as compared to the temperature optima for many PLA_1 enzymes obtained from different sources. The effective catalysis of acidolysis reactions by PLA_1 enzymes at lower temperatures is indicative of the fact that the nature of the enzyme changes above 45 °C (*4*). A higher temperature and even a higher dose of the enzyme affect the acidolysis process fairly negatively. One cause for such impact on reactivity was attributed to greater solubility of free fatty acids (FFAs) in the oil due to lowering of viscosity of the oil at higher temperatures. Conversely, dispersion of these FFAs after their formation as well as of those present naturally in the crude oil is necessary for achieving a faster rate of hydrolysis with phospholipases. The external mass transfer (film diffusion) with immobilized enzymes in heterogeneous systems depends on the velocity difference between the immobilized beads and the substrate molecules in the solution based on concentration gradient, allowing an effective contact between them. The optimum pH and the K_m (Michaelis-Menten constant) value of immobilized enzymes are significantly altered as compared to the free enzymes. This is due to some conformational changes within the enzyme and redistribution of polarity at the surface when the enzyme is immobilized by bonding to insoluble support, which is not the case when the enzyme is entrapped in polymeric gel matrix like alginate. Moreover, immobilized enzymes are easily recoverable after the reaction. The economic advantage of immobilized PLA_1 lies in the elimination of expensive downstream processing and in easy separation of immobilized beads for further use in an environmentally friendly degumming operation. It has been found that the rate of phospholipase reaction is higher only above the critical micelle concentration of the phospholipids present at the interface (*5*). This phenomenon becomes more pronounced at a later stage of reaction, when the enzymes significantly reduce the number of target phospholipid molecules available at the oil-water interface by converting them into lysophospholipids and FFAs. An increase in the rate of reaction with passage of time under some predetermined set of parameters is impossible unless the mass transfer barriers are overcome by some alternative means.

Rice bran carbohydrates contribute to some lysophospholipids in the crude oil. About 4–5 % phospholipids are present in crude rice bran oil (*6*). The major fatty acids in rice bran oil triacylglycerols (TAGs) are oleic, linoleic and palmitic acids. The phosphorus content varies within 11–25 mg per g of wet bran (14 % moisture basis). Rice bran oil is unique in containing significantly high fraction of FFAs (2–4 %) and that amount increases with prolonged storage. Crude oil with higher amount of NHPs requires their elimination and is subjected to phosphoric acid degumming prior to physical refining, which is always associated with yield loss. The acid treatment is followed by washing with water, which induces emulsification of TAGs in the aqueous phase at moderately higher temperatures. Enzymatic degumming can restore oryzanol (a powerful antioxidant) content of rice bran oil to the maximum (*7*).

Materials and Methods

Characterization of rice bran oil

The crude rice bran oil purchased from local solvent extraction plant was characterized before and after enzymatic treatment. The FFAs of the oil were estimated using 0.005 kg of oil sample. The free acids were titrated with 0.1 M KOH solution using phenolphthalein as an indicator. The FFA content was calculated as percentage of oleic acid using the following equation:

$$w(\mathrm{FFA}) = \frac{V(\mathrm{alkali})/\mathrm{mL}\cdot 0.1\cdot 28.2}{m(\mathrm{oil})/\mathrm{g}}/\,\% \qquad /1/$$

The iodine value (IV) of rice bran oil was estimated using 0.001 kg of sample dissolved in 15 mL of CCl_4. IV was expressed as mass of iodine (in g) absorbed by 0.1 kg of oil (*8*).

The colour of rice bran oil was estimated using photometric colour index (PCI) by measuring absorbances at 460, 550, 620 and 670 nm with a spectrophotometer (Model UV-1800, Shimadzu Corporation, Tokyo, Japan) and calculating the PCI value using the following equation (*9*):

$$PCI=1.29\cdot A_{460\,nm}+69.7\cdot A_{550\,nm}+41.2\cdot A_{620\,nm}-56\cdot A_{670\,nm} \quad /2/$$

Phosphorus content in oil was estimated by molybdenum blue method (*10*). The organic compounds of 0.001 kg of crude rice bran oil were decomposed by zinc oxide, followed by ignition at 550 °C in muffle furnace. The ash thus obtained was dissolved in acid and it reacted with molybdic acid to produce a blue colour. The intensity of colour was proportional to the mass fraction of phosphorus present in the sample and was measured using a spectrophotometer (Model UV-1800, Shimadzu Corporation). The mass fraction of phosphorus in the sample was directly obtained from a calibration curve, which was prepared by reacting different concentrations of KH_2PO_4 solution with molybdic acid. The mass fraction of phosphorus was expressed as mg of phosphorus per kg of oil.

Hydrolysis of oil by free PLA_1

The hydrolysis of oil was performed according to the methods described in literature (*11,12*). The PLA_1 enzyme from *Thermomyces lanuginosus* was purchased from Sigma-Aldrich (St. Louis, MO, USA). A PLA_1 dose of 0.01 kg per kg of oil was employed after dissolving the enzyme in 5 mL of citrate buffers of different pH values (pH=4, 5 and 6). The reactions were performed in 250-mL conical flasks. The volume of the aqueous phase was the same for each set of enzymatic reactions (5 mL per 0.1 kg of oil). The experiment was carried out to estimate the extent of both calcium-dependent and calcium-independent PLA_1-mediated hydrolysis of oil. For PLA_1 hydrolysis under calcium-rich environment, 10 mM solution of $CaCl_2$ was added to the flask. The samples were incubated with 0.01 kg of enzyme per kg of oil at different temperatures (30–60 °C) for 1 h with mild agitation at 75 rpm (0.27×*g*) in a BOD incubator (Model IICIC-07, IIC Industrial Corporation, Kolkata, India). Each hydrolysis was performed in triplicate at three different pH values within the acidic range (pH=4–6). After the reaction was over, the contents of the flask were immediately cooled down to ambient temperature and the organic layer was analysed for its FFA content.

Immobilization of PLA_1

The optimization of matrix concentration was done to achieve a suitable porous membrane and the maximum volume of spherical beads per unit of enzyme activity. For this, a 4.5 % solution of sodium alginate was prepared in demineralised water and the enzyme solution was added and thoroughly mixed. The alginate to enzyme volume ratio was maintained at 2:1 so that the final mass per volume ratio of alginate in the beads reached 3 %. The beads of the immobilized enzyme were dispensed in a 2 % $CaCl_2$ solution (0.18 M) using a syringe and were kept at 8–10 °C in a refrigerator duly immersed in $CaCl_2$ solution for 2 h for hardening. Then the beads (diameter of 2 mm) were washed with citrate buffers of different pH (pH=4, 5 and 6) and dried on Whatman no. 1 filter paper (Whatman, GE Healthcare Life Sciences, Maidstone, UK) at ambient temperature (25 °C). The dried beads had a bulk density of 440 g/L. On an average about 1.8 to 1.9 g of wet beads were produced from 0.001 kg of enzyme. The mass of dried beads was 1.1 to 1.2 g. The beads were used for phospholipid hydrolysis of 100 mL of crude oil. The immobilized beads were added to a 100-mL beaker containing 5 mL of citrate buffer (of pH=4, 5 and 6) and the content was transferred to the oil. The specific gravity of free PLA_1 was found to be 1.14 and 0.001 kg of enzyme was equivalent to 0.88 mL of enzyme. The immobilized PLA_1 was used for hydrolysis of phospholipids under both calcium-rich and calcium-depleted conditions as mentioned above.

Hydration of gum

After the enzymatic reaction, 5 % (by volume) of demineralised water was added to the flask and heated at 60 °C for 10 min with agitation for gum hydration. In the case of immobilized enzyme, the beads were separated from the flask before the oil was subjected to heating with water. The hydrated gum was removed by centrifugation at 6000 rpm (2013×*g*) for 15 min. The supernatant oil was collected for further analysis.

Water degumming

Water degumming was performed on 0.1 kg of oil sample with demineralised water (5 %, by volume) at 80 °C for 15 min and the hydrated gum was removed by centrifugation at 6000 rpm (2013×*g*) for 15 min.

Acid degumming

Acid degumming of crude oil was carried out with 85 % phosphoric acid at 80 °C for 15 min. A mass per volume ratio of 0.05 % H_3PO_4 in oil was employed for the conversion of nonhydratable gums, followed by the hydration of converted gums with 5 % (by volume) of demineralised water at 60 °C for 15 min and centrifugation as mentioned above. The residual acid from oil was removed by washing with water before further analysis. Both the water and the acid degumming were performed on crude rice bran oil to detect the levels of gum removal without the aid of enzyme and to compare the performance of phospholipid hydrolysis by PLA_1 with the conventional degumming operations.

Results and Discussion

In order to verify the efficiency of PLA_1 enzyme, we compared it to Lecitase® Ultra (Novozymes, Bagsværd, Denmark), a PLA_1 enzyme of *Thermomyces lanuginosus* or *Fusarium oxysporium* origin with approximate molecular mass of 35 kDa expressed in genetically modified *Aspergillus oryzae*. This enzyme was found to be calcium-independent and was fairly efficient in removing phospholipids from crude oil at an optimum temperature of 50 °C (*13*). The *Thermomyces lanuginosus* PLA_1 from Sigma-Aldrich is also expressed in *A. oryzae* and is specified by the

manufacturer to have a minimum activity of 10 kLU/g. One lipase unit (LU) is equivalent to the amount of lipase enzyme which releases 1 micromole of butyric acid from tributyrin per minute under the specified conditions of hydrolysis. Lecitase Ultra has closer temperature optima for lipase (40 °C) and PLA_1 (50 °C) activities, while the optimum temperature for Sigma-Aldrich PLA_1 was 60 °C with some observable influence of Ca^{2+} ions on catalysis.

The crude rice bran oil was found to be reddish brown in colour. It originally contained 970.58 mg of phosphorus per kg of oil. The FFA content and IV of crude oil were 5.82 % and 105.7 g per 100 g, respectively (Table 1). The colour of the oil was 94.22 on PCI scale. It was treated with the free PLA_1 enzyme as well as with the same quantity (0.01 kg of enzyme per kg of oil) of immobilized enzyme at different pH (4–6) and temperatures (30–60 °C) for 1 h. The enzyme showed the highest activity at pH=6 and at 60 °C. This was the case for both the free and the immobilized forms of the enzyme (Table 2). In all cases, the immobilized form of PLA_1 was found to be superior in removing phosphatides from the crude rice bran oil (Tables 3 and 4). A steady increase in the amount of phosphorus removed from crude oil was observed between 50 and 60 °C. The enzyme also showed comparatively higher activities in the presence of Ca^{2+}. The IV of the enzyme-treated oil was also satisfactorily high (Table 5). Significant level of colour reduction of crude rice bran oil was observed after the enzymatic treatment.

The Ca^{2+} at the concentration of 10 mM was found to be favourable for the PLA_1 reaction and a maximum of 94.12 % phosphorus was removed by the immobilized PLA_1 as compared to 72.52 % by the free enzyme at pH=6 and 60 °C (Table 4). However, the enzyme activity was not fully dependent on Ca^{2+} and showed significant level of activity even in the absence of calcium ions. The FFA content was measured after the enzymatic hydrolysis with free enzyme (Fig. 1) and with immobilized PLA_1 (Fig. 2). The FFA percentage was slightly higher (14.9 %) when free enzyme was used as compared to that with the immobilized form (14.16 %) at pH=6 and 60 °C. The colour reduction of crude oil was significant and was better with the immobilized PLA_1 (Table 5). When using free enzyme, the reduction in colour was 22 % and when using immobilized enzyme it was 29.42 % at pH=6 and 60 °C. The immobilized enzyme was found to restore a residual activity of 60–65 % with some degree of softening, deformation and breakage of 5–10 % of the beads after the reaction. The immobilized PLA_1 in the two immiscible phases of oil and water was able to distribute itself around the substrate under mildly agitated condition. Apart from the porosity of the immobilized beads, the factor which affects the rate of substrate hydrolysis is the volume of the

Table 1. Comparison of the properties of crude rice bran oil, water-degummed and acid-degummed oil

Sample	w(FFA)/%	IV/(g/100 g)	PCI	w(P)/(mg/kg)
Crude oil	5.82	105.7	94.22	970.58
Water-degummed oil	6.47	103.5	91.67	468.75
Acid-degummed oil	6.03	101.2	89.72	157.56

FFA=free fatty acids, IV=iodine value, PCI=photometric colour index

Table 2. Residual phosphorus in oil after the treatment with free phospholipase A_1 (PLA_1)

pH	Ca^{2+}	w(P)/(mg/kg)			
		Temperature/°C			
		30	40	50	60
4	+	390.5	352.2	338.4	302.5
	–	406.2	372.5	358.8	328.0
5	+	359.6	331.5	320.3	288.7
	–	366.3	337.4	325.5	302.4
6	+	327.7	307.4	295.2	266.7
	–	329.2	306.7	296.8	270.0

+=with Ca^{2+}, –=without Ca^{2+}

Table 3. Residual phosphorus in oil after the treatment with immobilized phospholipase A_1 (PLA_1) in the presence of 10 mM of calcium

pH	w(P)/(mg/kg)			
	Temperature/°C			
	30	40	50	60
4	183.9	168.5	145.4	119.7
5	168.5	152.4	122.5	81.5
6	155.4	139.9	103.6	57.2

Table 4. Percentage of phosphorus removal by free and immobilized enzymes with Ca^{2+} at different pH and temperatures in the presence of 10 mM of calcium

Oil treatment	pH	w(P)/(mg/kg)			
		Temperature/°C			
		30	40	50	60
Free enzyme	4	59.77	63.71	65.13	68.83
	5	62.95	65.84	66.99	70.25
	6	66.24	68.33	69.58	72.52
Immobilized enzyme	4	81.05	82.64	85.02	87.67
	5	82.64	84.30	87.38	91.60
	6	83.99	85.59	89.33	94.12

Table 5. Comparison of the properties of rice bran oil after the treatment with free and immobilized enzyme at pH=6 and at different temperatures in the presence of 10 mM of calcium

Oil treatment	Parameter	Temperature/°C			
		30	40	50	60
Free enzyme	IV/(g/100 g)	103.4	103.29	104.43	105.58
	PCI	87.05	84.85	76.2	73.5
	w(P)/(mg/kg)	327.7	307.4	295.2	266.7
Immobilized enzyme	IV	107.23	111.67	110.02	107.86
	PCI	74.88	68.2	66.8	66.5
	w(P)/(mg/kg)	155.4	139.9	103.6	57.2

IV=iodine value, PCI=photometric colour index

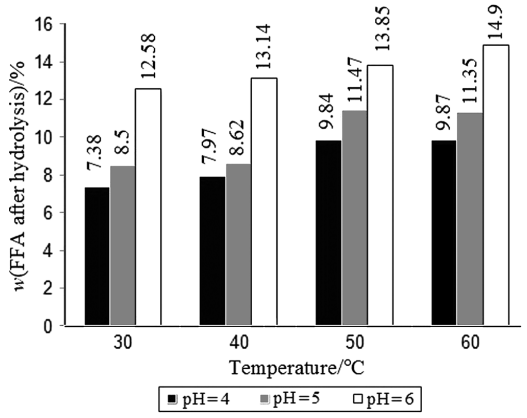

Fig. 1. Free fatty acid (FFA) content of oil after hydrolysis by free phospholipase A_1 (PLA_1)

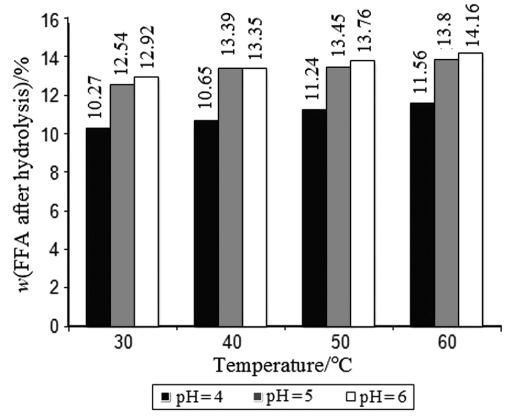

Fig. 2. Free fatty acid (FFA) content of oil after hydrolysis by immobilized phospholipase A_1 (PLA_1)

immobilized beads. The immobilization of enzyme provided extended surface for effective contact between the enzyme and the substrate. The free enzymes were susceptible to exposure to the hydrophobic environment and were inactivated at a faster rate as compared to the immobilized beads.

Conclusions

PLA_1 with pronounced lipase activity shows less uniformity, as can be seen in the difference in FFA content of the final oil at lower temperatures. This effect was significantly lowered at temperatures above 50 °C. The degumming with phosphoric acid (chemical degumming) removed 83.77 % of phosphorus from oil with a residual phosphorus content of 157.56 mg per kg of oil in the acid-degummed oil. Rice bran oil contained higher amount of NHPs and simple water degumming was not helpful in removing most of the phosphatides from the oil. Degummed oil with comparatively better colour and iodine value was obtained by PLA_1 treatment. In the case of catalysis by free enzymes, the aqueous phase carries the enzymes to the interface. Besides representing a medium for a constant supply of the biocatalyst for the phospholipid hydrolysis, the aqueous phase provides better room for the enzyme to move freely within a hydrophobic environment consisting of several FFAs and TAG molecules. This phenomenon is favoured under mild agitation of the reaction mixture. The effective contact between a fraction of the total enzyme in the immobilized form and the substrate was not possible at higher temperatures, as leakage of enzyme from the gel matrix occurred due to increase in the porosity of the beads. However, immobilization with alginate has been found to be effective in many enzymatic reactions due to its greater mechanical stability. Since degumming of edible oil with phospholipases has several advantages over the conventional chemical process, a faster rate of hydrolysis can make them more acceptable to the industries where time required for processing is one of the major criteria for adopting a technique. Immobilized PLA_1 from *Thermomyces lanuginosus* could be one such useful means for edible oil degumming.

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