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Immobilization of an enzymatic extract from *Penicillium camemberti* containing lipoxygenase and hydroperoxide lyase activities

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Abstract

An enzymatic extract from *Penicillium camemberti*, containing lipoxygenase (LOX) and hydroperoxide lyase (HPL) activities, was immobilized on oxirane acrylic beads, Eupergit C and Eupergit C250L-iminodiacetic acid (IDA). The optimum pH for LOX activity was determined to be 4.0 and 6.0 for the free enzyme extract and 6.0 for the immobilized one, whereas that for the HPL activity was 4.0 and 6.0 for the immobilized and free extracts. The optimal reaction temperature for LOX activity was 30 and 55 °C for the free and immobilized enzyme extracts, respectively, whereas the HPL activity showed its optima at 45 and 30 °C, for the free and immobilized extracts, respectively. The immobilization of the enzymatic extract dramatically enhanced the thermostability of LOX and HPL activities. In term of enzymatic stability, the lyophilized immobilized extract showed that its HPL activity at 4 °C was more stable than that of LOX. The results indicated a decrease and an increase in enzyme efficiency for LOX and HPL activity, respectively, upon immobilization.

Keywords: Lipoxygenase; Hydroperoxide lyase; *Penicillium camemberti*; Immobilization; Eupergit

1. Introduction

Lipoxygenase (LOX) (linoleate: oxygen oxidoreductase; EC 1.13.11.12) catalyzes the dioxygenation of various polyunsaturated fatty acids (PUFAs) containing a 1(*Z*),4(*Z*)-pentadiene moiety into various regio-isomers of hydroperoxides of PUFAs, which can be subsequently cleaved by hydroperoxide lyase (HPL) into aldehydes and alcohols [1]. The sequential action of LOX and HPL enzymes results in the bioconversion of PUFAs acids into a wide variety of flavor compounds. Although there has been an increasing interest in the LOX/HPL biocatalyzed production of aroma compounds [2,3], the limited stability of these enzymes has restricted their biotechnological applications [4].

Immobilization offers a substantial enzymatic stabilization as well as the possibility of the reuseability of the biocatalyst in continuous packed-bed reactors [4]. Further benefits of immobilization, include the easy separation of the enzyme from its end products, which minimizes downstream processing costs [5]. The immobilization of an enzyme to a support is contingent on chemical bond formation between the functional groups of the immobilization support and those of the enzyme. Several types of immobilizations exist, including adsorptive, entrapment and crosslinking [5]. Covalent supports form bonds at a variety of attachment points on the enzyme, including $–NH_3^+$, $–COO^-$ and –SH groups [6,7]. Oxirane acrylic resins possess many oxirane groups that bind to the primary amines of the enzyme protein molecule [8]. Eupergit® supports are among the most used types of covalent supports and have been employed in the stabilization of different types of enzymes, often by multipoint attachment between the enzyme and the support [8]. Several studies have been carried out for the immobilization of enzymatic extracts, containing LOX [9,10] and HPL activities [6,7].

The overall objective of this study was to investigate the effect of immobilization on a LOX/HPL enzymatic extract, from *Penicillium camemberti*, using different supports. The specific objectives were to characterize the LOX/HPL activity in free and immobilized extracts, in terms of optimum pH,

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reaction temperature, thermostability, long-term stability and other kinetic parameters.

2. Materials and methods

2.1. Materials

Linoleic acid (*cis*-9, *cis*-12-octadecadienoic acid) was purchased from Nu-Chek Prep (Elysian, MN). Silica gel support was purchased from Silicycle (Quebec City, Qc), whereas Eupergit® C and Eupergit® C250L (oxirane acrylic beads) were offered as gifts from Rohm Pharma (Darmstadt, Germany). Dowex® 50WX4-200 (anionic ion-exchange resin), ethylenediamine (EDA) and iminodiacetic acid (IDA) as well as trishydroxy methylaminomethane (TRIS) were obtained from Aldrich (Milwaukee, WI). Xylenol orange [(3,3′-*bis*(*N*,*N*-di(carboxymethyl)aminomethyl)-*o*-cresol)] was purchased from Sigma Chemical Co. (St. Louis, MO). Mono- and dibasic potassium phosphate were purchased from Fisher Scientific (Fair Lawn, NJ).

2.2. Culture growth and preparation of the enzymatic extract

P. camemberti was induced to sporulate, and the resultant spore suspension was counted, using a Neubauer Counting Chamber (Hausser Scientific, Horsham, PA) for the inoculation of liquid medium, according to the procedure outlined by Perraud and Kermasha [11]. After harvesting the biomass after 10 days of fermentation, it was filtered through cheesecloth and the mycelia were washed (2× 50 mL) with cold water (4 °C) followed by potassium phosphate buffer solution (pH 6.5, 0.1 M). The recovered mycelia were blended (5 mL of the phosphate buffer per 1 g biomass) and homogenized, using 0.45–0.50 mm diameter glass beads, in an MSK cell homogenizer (Braun, Melsungen, Germany) for 2× 2 min. The LOX/HPL enzymatic extract was recovered by centrifugation (12,000 × *g*, 15 min) and concentrated by ultrafiltration (Amicon, 30 kDa NMWCO, 40 psi). All subsequent steps were performed at 4 °C, unless otherwise stated.

2.3. Preparation of oxirane acrylic supports

The investigated supports, including the covalent oxirane acrylic supports Eupergit® C and Eupergit® C250L, which were used as unmodified and modified supports with EDA and IDA, were prepared according to the procedure outlined by Mateo et al. [12]. The modification involved the suspension of the support (1 g wet weight) in 10 mL of EDA (5%, w/v) or 5 mL of IDA (1.8 M); the suspension was subjected to gentle stirring at 25 °C for 15 min and 5 h, respectively. The modified EDA and IDA supports were washed with deionized water.

2.4. Immobilization of LOX and HPL

The immobilization of LOX and HPL, expressed in the enzymatic extract from *P. camemberti*, was conducted at 4 °C, using 40 mg protein/g wet support. Potassium phosphate buffer solution (pH 6.5, 0.1 M) was used for all steps of immobilization, unless otherwise indicated. The immobilization on unmodified and modified supports was carried out, in conical 5 mL screw-cap tubes under mild agitation, using the phosphate buffer solution at 1.0 and 0.1 M concentration, respectively. After 18 h, the agitation was halted and the supernatants were recovered for protein determination. The supports, containing the immobilized enzymatic extract, were washed with 1× 15 mL of deionized water and 2× 15 mL of the phosphate buffer, where each wash solution was recovered for protein determination. The washed supports, containing the immobilized enzymatic extract, were re-suspended in the phosphate buffer solution (0.1 g wet support/mL) and assayed for LOX and HPL activities. Protein immobilization yield (%) was defined as the ratio of protein, immobilized onto a support (mg), divided by the initial protein content (mg) multiplied by 100. The retention of enzyme activity (%) was defined as the specific activity of LOX or HPL of the immobilized enzyme extract, divided by the specific activity of LOX or HPL of the free extract and multiplied by 100.

2.5. Substrate preparation

For LOX studies, linoleic acid was used as substrate; linoleic acid stock solution was prepared at a concentration of 4.0 mM in the appropriate buffer solutions (0.1 M), according to the procedure outlined by Perraud et al. [13]. For HPL studies, 10-hydroperoxide of octadecadienoic acid (10-HPOD) was used as substrate; 10-HOPD was obtained by the photo-oxidation of linoleic acid and purified by solid phase extraction followed by a prepative normal phase high-performance liquid chromatography (NP-HPLC), according to the procedure outlined by Kermasha et al. [14].

2.6. LOX assay of free and enzymatic extracts

For the LOX assay of the free enzymatic extract, it was initiated by the addition of 180 μL of the enzyme suspension (1.5 mg protein/mL) to 0.6 mL of substrate solution (4.0 mM), and the total volume was adjusted 1.5 mL with sufficient quantity of the buffer solution. The LOX assay for the immobilized enzymatic extract was initiated by the addition of 0.6 mL of immobilized enzyme suspension (0.1 g support/mL) to 1 mL of substrate (4.0 mM), and the total volume was adjusted 2.6 mL with sufficient quantity of the buffer solution.

The LOX assays for the free and immobilized enzyme extracts were carried out at 25 °C, under mild stirring, for 12 and 35 min, respectively. Aliquots of the reaction homogenate (0.1 mL) were taken at selected time intervals and were immediately added to 1 mL of xylenol orange reagent solution, which was prepared as a mixture of deionized/degassed water, ferrous sulfate (0.25 mM), perchloric acid (85.0 mM) and xylenol orange salt (0.1 mM) [15]. The absorbance of the reaction mixture was measured after 20 min of color development at 560 nm (10-HPOD; MEC 18,765 M^{-1} cm^{-1}), using a Beckman DU-650 spectrophotometer (Beckman Instruments Inc.; San Raman, CA). LOX specific activity was defined as nmol of conjugated

diene linoleic acid hydroperoxide per mg protein per min. All LOX assays were performed in duplicate in tandem with a blank trial, containing all components of the enzymatic assay with the exception of that LOX extract was thermally inactivated (95 °C, 1 h).

2.7. HPL assay of free and immobilized enzymatic extracts

The HPL assay was carried out with 10 nmol of 10-HPOD as substrate, containing Tween 80 (polyoxyethylene 20 sorbitan mono-oleate) (0.3 μL per 0.9 μmol), introduced into 5 mL thermostated tubes with 10 μL of the appropriate buffer solution (0.1 M). The mixture was sonicated for 3 min according to the procedure outlined by Kermasha et al. [3]. The HPL reaction was initiated by the addition of 15 μL of the free enzyme extract (1.5 mg protein/mL) or the immobilized enzymatic extract (0.1 g support/mL), and proceeded under gentle agitation for 5 min. The HPL reaction was halted by the addition to the reaction homogenate 2 mL of xylenol orange reagent. The HPL specific activity was defined as nmol of 10-HPOD per mg protein per min. The specific activity was determined from several trials, using serial dilutions of the HPL extract. All HPL assays were carried in duplicate in tandem with a blank trial, containing all components with the exception of that HPL extract was thermally inactivated (95 °C, 1 h).

2.8. Effect of pH on enzymatic activities

The effect of pH on LOX/HPL activities of free and immobilized enzymatic extracts was investigated, using a wide range of buffer solutions (0.1 M), including citrate phosphate for the pH range of 3.0–5.5; potassium phosphate for the pH range of 6.0–8.0 and glycine–NaOH for the pH values of 8.5 and 9.0.

2.9. Effect of reaction temperature on enzymatic activities

The effect of reaction temperature on LOX/HPL activities of the free and immobilized enzymatic extracts was performed at a wide range of temperatures (5–75 °C).

2.10. Thermostability of enzymatic activities

Free and immobilized enzymatic extracts were subjected to an extended thermal treatment at 25 °C (0–84 h), using a reciprocal shaking-bath (Model 25; Precision Scientific, Chicago, IL); the residual LOX/HPL activities were measured at determined interval times. The concentration of the free enzymatic extract was 1.50 mg protein/mL, whereas that of the immobilized one was 0.15–0.50 mg protein/mL.

2.11. Kinetic parameters of enzymatic activities

The effect of linoleic acid concentration on the specific activity of the free and immobilized LOXs was investigated, using substrate concentrations ranging from 1.5 to 12×10^3 μM and 1.5 to 77×10^3 μM, respectively. The effect of 10-HPOD concentration on the specific activity of the free and immobilized HPLs was investigated, using substrate concentrations of 0.8 to 25×10^3 μM and 0.7 to 100×10^3 μM, respectively.

3. Results and discussion

3.1. Selection of appropriate supports for the immobilization of the enzymatic extract

Table 1 summarizes the experimental data for the immobilization of the enzymatic extract from *P. camemberti*, containing LOX/HPL activities, using selected supports. The results indicate that for LOX activity, a wide range of protein immobilization yield from 10.4 to 32.9% was obtained with the use of Eupergit® C-EDA, Eupergit® C-IDA, Eupergit® C250L and Eupergit® C250L-EDA. The protein immobilization yield was higher for Eupergit® C (28.4%) than that for Eupergit® C250L-IDA (10.1%), where the retention of LOX activity was 366.6 and 422.5%, respectively. These results suggest that the greater number of reactive epoxides in Eupergit® C (~600 μmol oxirane functionalities/g dry weight support) were effective at covalently binding amino, hydroxyl and thiol residues in LOX [16]. Table 1 also shows that the immobilization of the enzymatic extract on several investigated supports, including Silica, Eupergit® C-EDA, Eupergit® C-IDA, Eupergit® C250L and Eupergit® C250L-EDA, resulted in a complete inactivation of LOX activity; these results may be due to conformational changes in the protein during immobilization, steric hindrance at the active site and/or substrate diffusion limitations [12,16], as well as to the interference of the support itself with the Xylenol assay [8]. Vega et al. [10] reported that the protein immobilization yield for soybean LOX type-1B on Eupergit® C250L-IDA was 29.3%, which is greater than that determined (10.13%) for the LOX from *P. camemberti* (Table 1). The overall results suggest that Eupergit® supports could have differential immobilization yields, depending on the source and concentration of the enzyme [8]. The potential oxidative interference effect of oxirane acrylic beads on the substrate was reported by Carmen Pinto and Macías [9]; this interference was avoided by the reduction of the excess of unreacted oxirane groups of the support, upon its overnight incubation in 2-mercaptoethanol (5%, w/v). The retention of enzyme activity for the immobilized LOX from *Pisum sativum* was 290% [17]. The experimental findings (Table 1) suggest that the period of 18 h is the optimal immobilization time of Eupergit® supports for the immobilization of the enzymatic extract of *P. camemberti*; however, shorter immobilization time for ionic or adsorptive supports of 1–3 h has been reported [3,18]. Most covalent supports require immobilization time of 12–24 h [10,17]. The optimum ratio of enzyme to support for the immobilization yield of the enzymatic extract from *P. camemberti*, was 40 mg protein/g wet support (data not shown). Vega et al. [10] used 100 mg protein/mL wet support for Eupergit® supports for the immobilization of soybean LOX type-1B. Carmen Pinto et al. [19] reported that 0.7 mg protein/mL wet support was used for the immobilization of potato LOX on Eupergit®. On the basis of the experimental findings (Table 1), Eupergit® C was selected for further investigation for the immobilization

Table 1
Summary of the immobilization parameters of the enzyme extract from *Penicillium camemberti* containing lipoxygenase (LOX) and hydroperoxide lyase (HPL) activities

Support	Enzymatic activity[a] (nmol HPOD/(g support min))	Protein immobilization yield[b] (%)	Retention of activity[c] (%)
LOX[d]			
Eupergit® C	33.1	28.4	366.6
Eupergit® C-EDA[e]	nd[f]	15.9	0
Eupergit® C-IDA[g]	nd[f]	10.4	0
Eupergit® C250L	nd[f]	16.3	0
Eupergit® C250L-EDA[e]	nd[f]	32.9	0
Eupergit® C250L-IDA[g]	16.5	10.1	422.5
Silica	nd[f]	20.7	0
Dowex 50WX4-200	14.9	26.8	281.2
HPL[h]			
Eupergit® C	50.1	18.1	145.0
Eupergit® C250L	213.6	12.7	472.5
Eupergit® C250L-EDA[e]	284.6	36.7	197.5
Eupergit® C250L-IDA[g]	151.5	12.4	356.0
Silica	nd[f]	24.2	0
Dowex 50WX4-200	nd[f]	30.3	0

[a] Activity per gram support was defined as nmol produced (LOX) or consumed (HPL) hydroperoxide per gram wet support per min.
[b] Protein immobilization yield percentage was defined as ratio of immobilized protein relative to the initial amount of free protein.
[c] Retention of activity percentage was defined as the specific activity of either immobilized LOX or HPL divided by the specific activity of free LOX or HPL and multiplied by 100.
[d] LOX activity was determined using linoleic acid as substrate.
[e] The Eupergit® supports were modified with ethylene diamine (EDA).
[f] Not detected.
[g] Iminodiacetate (IDA).
[h] HPL activity was determined using the 10-hydroperoxide of linoleic acid as substrate.

of the enzymatic extract from *P. camemberti*, containing LOX activity.

The immobilization of enzymatic extract from *P. camemberti*, containing HPL activity, on various supports shows (Table 1) protein immobilization yield ranging from 12.5 to 36.7%, which is close to that for LOX; however, HPL activity per gram support (50.1–284.6 nmol HPOD/(g support min) was much greater than that obtained for LOX (14.9–33.1 nmol HPOD/(g support min)). Similar protein immobilization yields were reported for HPL from potato (18.9%) [17] and from mung bean (26%) [7]. Although the highest retention of HPL activity was achieved with the use of Eupergit® C250L and Eupergit® C250L-IDA, the protein immobilization yield obtained with those supports were only 12.7 and 12.4%, respectively. However, Silica, Dowex® 50WX4-200, Eupergit® C250L and Eupergit® C250L-EDA, showed large interference with the xylenol orange assay; this interference may be due to structural denaturation at/or near the active site of HPL. Based on the overall results for HPL activity (Table 1), the immobilization of the enzymatic extract from *P. camemberti* on Eupergit® C250L-IDA was considered for further investigation; this choice of support was due to the low of its interference with the xylenol orange assay as well as to the level of enzyme activity/g support, with a value of 151.5 nmol of 10-HPOD/(g support min). The retention of enzymatic activity, determined for HPL from *P. camemberti*, ranged from 145.0 to 472.5%; these results suggest that the immobilization enhanced the HPL activity. These experimental findings are in agreement with those reported [17] for the immobilized HPL from *P. sativum*, which showed 226% retention of enzymatic activity [17]. Decrease in HPL activity for the immobilized extract as compared to the free one was also reported [6] for that from *Chlorella* sp., which was covalently bound on a variety of supports, including Affi-Gel 10 and Affi-Gel 501, with 55.3 and 67.1% residual specific activity, respectively. HPL from mung bean, immobilized on Ultralink Iodoacetyl, retained 47% of its residual activity after immobilization [7].

3.2. Effect of pH on LOX/HPL activities

LOX of the free enzymatic extract and that immobilized one on Eupergit® C was determined to show its optimum at pH at 6.0 (Fig. 1A). These results are close to the pH optimum of 6.5, reported by Perraud and Kermasha [11] for *P. camemberti* LOX. However, no optimum at pH 8.0 was determined in the current study; instead a minor optimum at pH 4.0 was obtained. The pH optimum for the HPL activity of the free enzymatic extract from *P. camemberti* was determined to be 6.0, whereas that of the immobilized one on Eupergit® C250-IDA was most active at pH 4.0, with a minor optimum at 8.0 (Fig. 1B). Kermasha et al. [3] reported that the free HPL from *P. camemberti* has relatively higher activity at pH 6.5. The pH shifts, associated with the immobilization on Eupergit® C250-IDA, may be attributed to the polycationic nature of the support, which attracts more OH^- ions around the immobilized enzyme, thus making the pH of the enzyme's micro-environment higher than the bulk solution [8]. The immobilized enzyme, therefore, requires a lower pH for its optimal activity than the free one. The optima pH for LOX from potato [19], were 6.0 and 6.5 for the free and the immobi-

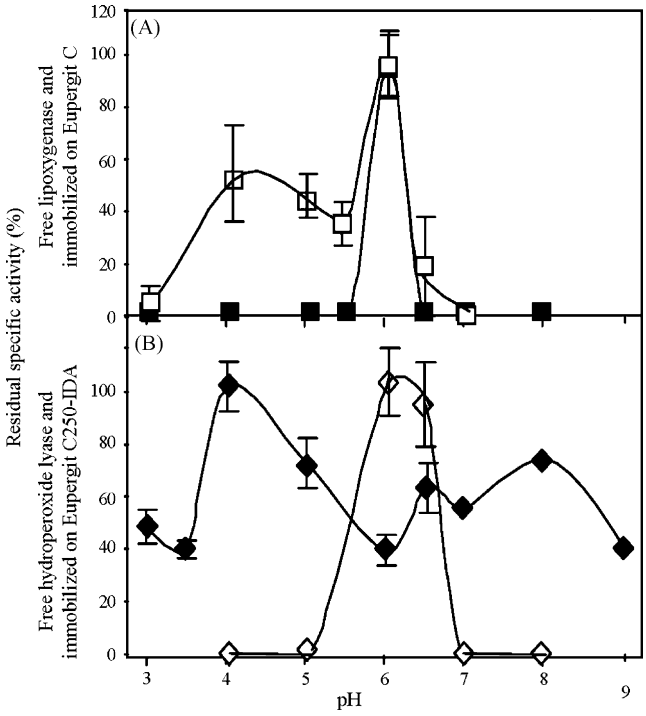

Fig. 1. The pH profiles of lipoxygenase (LOX) and hydroperoxide lyase (HPL) activities in the enzymatic extract from *Penicillium camemberti*, where the residual (%) specific activity of LOX was: (A) (□) free, and (■) immobilized and that of HPL was (B) (◊) free and (♦) immobilized, respectively.

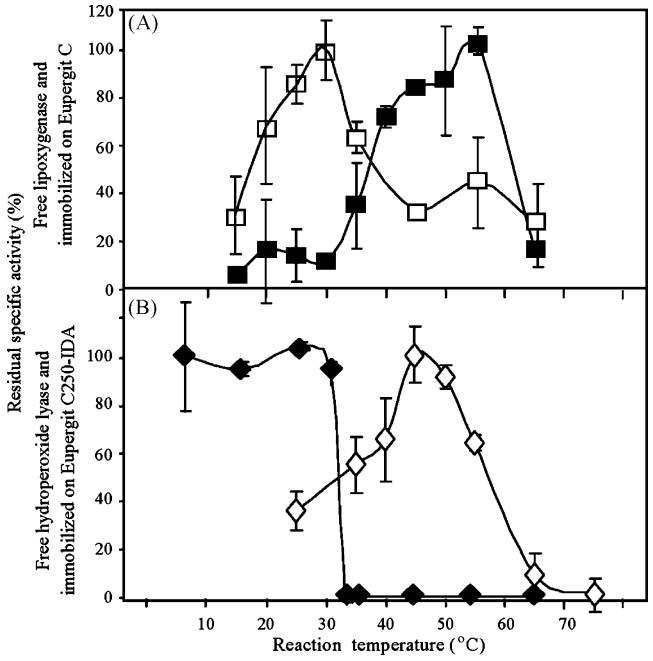

Fig. 2. The reaction temperature profiles of lipoxygenase (LOX) and hydroperoxide lyase (HPL) activities in the enzymatic extract from *P. camemberti*, where the residual (%) specific activity of LOX was: (A) (□) free and (■) immobilized and that of HPL was (B) (◊) free and (♦) immobilized, respectively.

lized one, respectively. Soybean LOX type-1B was reported [4] to have a pH optimum at 9.0 for both free and sol–gel immobilized one, whereas free and immobilized HPLs, from *Chlorella* sp. [6], showed the same pH optimum at 6.4 [6]. The free LOX and HPL, from *P. sativum*, were reported [17] to share a pH optimum at 7.0; however, their immobilization on talc changed their optimum to 6.4. The pH optimum of Eupergit® C250-IDA immobilized HPL, from *P. camemberti* was relatively low (4.0); however, other HPLs have been reported to show acidic pH optima, including bell pepper [20], cucumber [21] and olive fruits [22].

3.3. Effect of reaction temperature on LOX/HPL activities

LOX activity of free enzymatic extract and that immobilized on Eupergit®C is shown (Fig. 2A) over a wide range of reaction temperatures, from 10 to 65 °C, whereas the HPL activity of free enzymatic extract and that immobilized on Eupergit®C250L-IDA was investigated at 5 to 75 °C (Fig. 2B). The results (Fig. 2A) show that the optimum temperature for LOX activity of the free enzymatic extract was 30 °C, whereas that of the immobilized one was 55 °C. The optimal temperature for HPL activity of the free enzymatic extract was 45 °C, whereas that of the immobilized one was relatively constant from 5 to 30 °C, after which a sharp decrease in HPL activity was monitored. The increase in the optimum temperature for LOX activity indicates a concomitant increase in the enzyme stability upon its immobilization on Eupergit C. However, the decrease in the optimum temperature for HPL activity, upon immobilization on Eupergit®C250L-IDA, could be attributed to conformational changes in the enzyme structure [16]. Many free LOXs, characterized in terms of reaction temperature, have optima in the range of 40–60 °C, including banana leaf (40 °C) [23], *Thermoactinomyces vulgaris* (50 °C) [24] and *Gaümannomyces graminis* (60 °C) [25]. Several LOXs from different sources showed reaction temperature optima close to those determined in this study, including *Gersemia fruticosa* (15 °C) [26] and *Pleurotus pulmonarius* (25 °C) [27]. Carmen Pinto and Macías [9] reported that soybean LOX, immobilized on polyacrylamide gel, showed an optimum reaction temperature of 35 °C, whereas its free counterpart was at 30 °C. Several free HPLs have been reported to have reaction temperature optimum of 30 °C, including *Oscillatoria* sp. [28] tomato leaf [29] and cucumber fruit [21]. Limited studies have endeavored to investigate the optimal temperature for HPL activity; instead, these studies opted to investigate dual LOX/HPL activities at the LOX temperature optimum [17]. Nevertheless, higher HPL activity was reported from the immobilized enzymatic extracts at 20 °C for *P. sativum* [17] as well as at 25 °C for mung bean [7] and for *Chlorella* sp. [6].

3.4. Thermostability of LOX/HPL

Thermostability profiles of LOX and HPL activities of the free and immobilized enzymatic extracts at 25 °C are displayed in Figs. 3 and 4, respectively. The thermal inactivation of LOX/HPL activities followed first-order kinetics (data not shown). The free enzymatic extract displayed a dramatic decrease in LOX activity, upon thermal treatment (25 °C), with a residual specific activity of 95.5, 58.8, 27.2 and 0% after 4, 12, 24 and 36 h, respectively (Fig. 3A); the enzymatic extract,

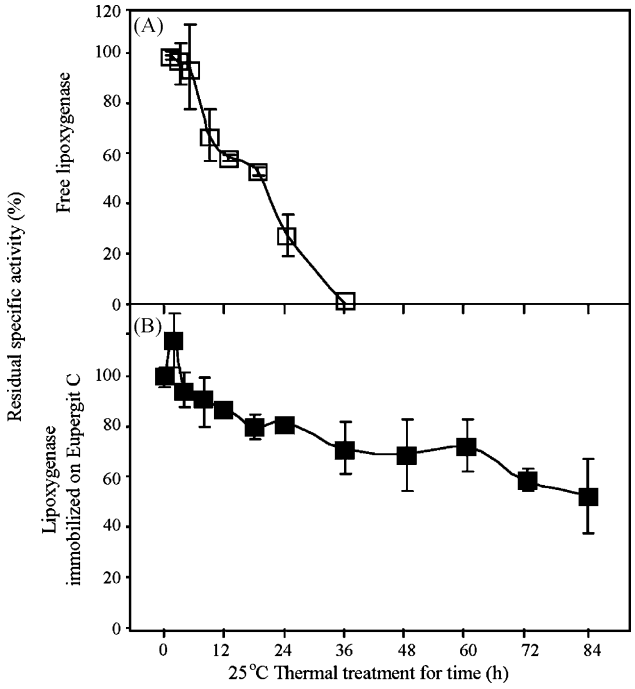

Fig. 3. The thermostability profiles, at 25 °C, of lipoxygenase (LOX) activity in the enzymatic extract from *P. camemberti* for the: (A) (□) free and (B) (■) immobilized preparations, respectively.

immobilized on Eupergit® C, showed LOX residual specific activitiy of 95.3, 88.4, 82.2 and 72.4%, also after 4, 12, 24 and 36 h, respectively. After 84 h of storage, more than 50% of the LOX residual activity of the immobilized enzyme extract was maintained (Fig. 3B). The HPL activity of the free enzymatic extract displayed a steady decrease in residual specific activity (%) throughout the thermal treatment (25 °C), with values of 96.3, 78.1, 80.1, 35.1 and 27.3% after 1, 2, 3, 4 and 5 h, respectively (Fig. 4A). The HPL activity of the enzymatic extract, immobilized on Eupergit®C250-IDA, showed ∼100% of residual specific activity over the duration of the thermal treatment (25 °C) (Fig. 4B).

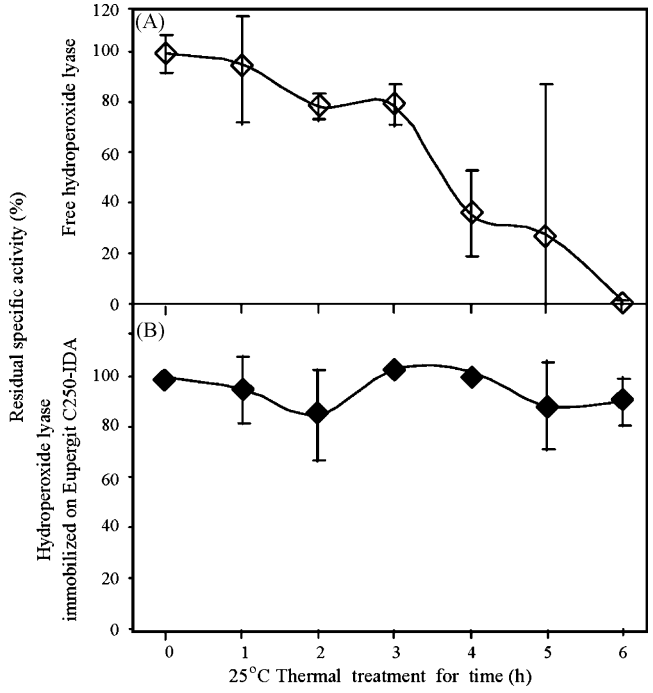

Fig. 4. The thermostability profiles, at 25 °C, of hydroperoxide lyase (HPL) activity in the enzymatic extract from *P. camemberti* for the: (A) (◊) free and (B) (♦) immobilized preparations, respectively.

Both free LOX and HPL activities for the free enzymatic extract were relatively unstable, with respect to the thermal treatment; however, the LOX of the free enzymatic extract demonstrated a higher thermostability than the HPL (Figs. 3A and 4A). The immobilization of the enzymatic extracts, onto their respective supports, provided higher thermostability for both LOX and HPL activities (Figs. 3B and 4B). These results may indicate a higher resistance of immobilized enzyme extract to thermal denaturation than that of the free ones; this resistance may be due to the multipoint covalent attachment between the enzyme and the support [16]. Knezevic et al. [30] investigated the enhanced thermostability (75 °C, 10 h) of Eupergit®C immobilized lipase; these authors indicated that the free enzyme showed a linear inactivation profile, whereas that of the immobilized one was linear at temperatures ranging from 37 to 75 °C and biphasic above 75 °C. Knezevic et al. [30] suggested that this may be due to a variety of subpopulations of multipoint immobilized enzyme molecules onto the Eupergit® support [30]. In the current study, LOX/HPL of both free and immobilized enzymatic extracts showed linear inactivation rate constants (data not shown); however, the thermal inactivation temperature of 25 °C was below that one associated with the biphasic inactivation profiles in other studies [30]. The experimental findings (Fig. 4) also indicate a higher instability, at 25 °C, of HPL of the enzymatic extract from *P. camemberti* as compared to that in other sources [5,21]; however, an enhanced thermostability of HPL activity was obtained by the immobilization of the enzymatic extract on Eupergit®C250-IDA (Fig. 4).

3.5. Long-term stability of LOX/HPL

Fig. 5 shows that throughout the first 8 weeks of storage at 4 °C, the residual LOX activity of the enzymatic extract, immobilized on Eupergit®C, was 77.2, 69.1, 53.5 and 12.6%, after 2, 4, 6 and 8 weeks, whereas no residual activity was determined after the 9th week of storage. In addition, the results (Fig. 5) also indicate that when the enzymatic extract, immobilized on Eupergit® C250L-IDA, was stored at 4 °C, the HPL residual activity was relatively maintained throughout the 8 weeks of storage.

Most of LOX long-term stability studies [17,19] have focused on the storage at ∼4 °C of enzymatic extract suspensions. When the free enzymatic extract from *P. sativum* (0.07 M, pH 6.4) was stored at 4 °C for 30 days, it maintained 25% of its residual LOX activity, whereas that immobilized on talc maintained ∼50% of its residual LOX activity [17]. Carmen Pinto et al. [19] indicated that the free and polyacrylamide immobilized soybean LOX (0.05 M, pH 9.0) maintained 28% and 60% of its activity, respectively, after a storage at 4 °C for 45 days. Hsu et al. [31]

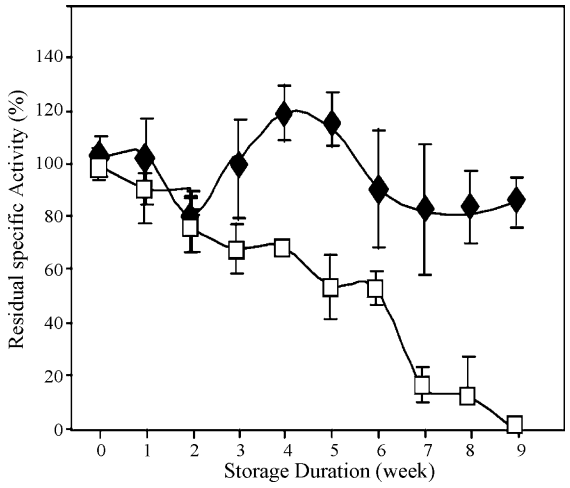

Fig. 5. The long-term stability profiles, at 4 °C, of (□) lipoxygenase (LOX) and (♦) hydroperocxide lyase (HPL) activities in lyophilized immobilized enzymatic extracts from *P. camemberti*, at 4 °C.

reported that the storage of sol–gel immobilized soybean LOX type-1B (0.2 M, pH 9.0) lost ~50% of its activity after 168 h of storage at 25 °C.

HPL studies [17] indicated that the immobilization of enzymatic extracts contributed to its stability for many weeks of storage. The storage of free enzymatic extract *P. sativum* at 4 °C for 30 days (0.07 M, pH 6.4) resulted in the maintenance of ~25% of its residual HPL specific activity, whereas upon its immobilization on talc the HPL residual specific activity was ~75% [17]. Affi-Gel 10 immobilized enzymatic extract from *Chlorella* sp. [6], containing HPL activity (0.05 M, pH 7.0) was stable for 4 months at 4 °C, whereas the immobilized enzymatic extract from mung bean on UltraLink Iodoacetal maintained the stability of its HPL activity (0.1 M, pH 6.5) after 18 days at 4 °C [7]. The experimental findings (Fig. 5) are similar to those reported for the long-term stability of HPL activity of other sources, suggesting hence that the immobilization of the enzymatic extract on Eupergit®C250L-IDA resulted in an appropriately long-term stability.

3.6. Determination of kinetic parameters of LOX/HPL

Table 2 summarizes the kinetic parameters (V_{max} and K_m) for the LOX activity of both free enzymatic extract and the immobilized one on Eupergit®C, as well as the HPL activity of both free enzymatic extract and the immobilized one on Eupergit®C250L-IDA. The results show that the LOX activity of the immobilized enzymatic extract showed a higher V_{max} value than that of the free one, with 46.9 and 12.2 nmol HPOD/(mg protein min), respectively. In addition, the results showed that the K_m value for the LOX activity of the free enzymatic extract (57.3 μM) is much lower than that for the immobilized one (58.5 mM); the low substrate affinity for the immobilized enzymatic extract may be due to the substrate partitioning and/or the conformational changes in enzyme structure [32]. As a result, the LOX enzymatic catalytic efficiency value for the free enzymatic extract (2.13×10^{-4}) is much higher than (8.00×10^{-7}) that for the immobilized one.

The results (Table 2) also show that V_{max} value for the HPL activity of the immobilized enzymatic extract was higher than that for the free one, with 1383.07 and 54.17 nmol HPOD/(mg protein min), respectively. In addition, the results indicate that the K_m values for the HPL activity of the free enzymatic extract (0.16 mM) is lower than (0.25 mM) that of the immobilized one, indicating hence a higher substrate affinity of the HPL of the free enzymatic extract. As a result, the HPL enzymatic catalytic efficiency value of the immobilized enzymatic extract (5.55×10^{-3}) is higher than (3.31×10^{-4}) that of the free one.

The LOX enzymatic catalytic efficiency for the free enzymatic extract was close to that (1.19×10^{-4}) reported for the partially purified LOX fraction from *P. camemberti* [11]. The literature [17,19] indicated that the effect of the immobilization of enzymes on their kinetic parameters generally resulted in an increase in K_m and a decrease in V_{max} values. The immobilization of potato extract containing LOX activity, with oxirane acrylic, resulted in a K_m value of 0.31 mM versus 0.15 mM for the free one [19]. The results (Table 2) suggest lower enzymatic catalytic efficiency and substrate affinity for the LOX of the

Table 2
Kinetic parameters of lipoxygenase (LOX) and hydroperoxide lyase (HPL) in an enzyme extract from *Penicillium camemberti* in the free preparation and that immobilized on Eupergit® C and Eupergit® C250L-IDA, respectively

	LOX[a]			HPL[b]		
	K_m[c]	V_{max}[d]	Enzymatic catalytic efficiency[e]	K_m[c]	V_{max}[d]	Enzymatic catalytic efficiency[e]
Free	0.057	12.17	2.13×10^{-4}	0.16	54.17	3.31×10^{-4}
Immobilized	58.49	46.95	8.00×10^{-7}	0.25	1383.07	5.55×10^{-3}

[a] Lipoxygenase specific activity was defined as nmol produced hydroperoxides/(mg protein min) and was determined from the plot of the residual hydroperoxide produced vs. enzyme concentration using the xylenol orange assay (molar exctinction coefficient 18,765 M^{-1} cm^{-1}, 560 nm). All LOX trials were performed in duplicate series run in tandem with a blank, at pH 6.0, at 30 and 55 °C, for the free extract and the immobilized one, respectively.

[b] Hydroperoxide lyase specific activity of was defined as nmol consumed 10-hydroperoxide of linoleic acid/(mg protein min) and was determined from the plot of the residual 10-hydroperoxide substrate vs. enzyme concentration using xylenol orange assay (molar exctinction coefficient 18,765 M^{-1} cm^{-1}, 560 nm). All HPL trials were performed in duplicate series run in tandem with a blank, at pH 6.0, 45 °C and pH 4.0, 30 °C, for the free extract and the immobilized one, respectively.

[c] The K_m values were defined as mM of substrate.

[d] The V_{max} values were defined as substrate produced or converted/(mg protein min), for LOX and HPL, respectively.

[e] The enzymatic catalytic efficiency was defined as the ratio of V_{max} to K_m.

immobilized enzymatic extract as compared to those of the free one; these findings may be due to a diffusion limitation of the substrate into the micro-environment of the immobilized preparation [3,17]. Several studies also indicated a relative increase in K_m and a decrease in V_{max} upon immobilization of extract containing HPL activity [7,17]; however, an enhanced enzyme velocity with a concomitant increase in K_m for the HPL of the immobilized enzymatic are shown (Table 2); these results suggest a high selectivity of the Eupergit®C250L-IDA for the immobilization of the enzymatic extract, containing HPL activity.

4. Conclusion

The overall findings show that the enzymatic extract from *P. camemberti* was stabilized most effectively by its immobilization on Eupergit®C for LOX and on Eupergit®C250L-IDA for HPL. Parameters of optimum pH, reaction temperature and kinetic parameters indicated that the LOX and HPL activities share many similarities to those from other sources. The LOX and HPL activities of the immobilized enzymatic extracts were more thermostable than those of the free counterparts, showing hence higher residual activity. The enhanced stability of the immobilized enzymatic extracts provides a better use of these activities in biotechnological application, in particular for the production of flavor precursors and flavor compounds.

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