Effectiveness of Talc as Adsorbent for Purification and Immobilization of Plant Lipoxygenases

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The present study describes the immobilization of lipoxygenase from *Solanum tuberosum* tuber and *Lupinus albus*, *Cicer arietinum*, and *Pisum sativum* seeds on a magnesium silicate based support, talc. In all cases, an increase in specific activity of many hundreds of units per milligram was obtained and the use of free or immobilized enzymes was quite similar. The adsorption of plant lipoxygenases on talc was a direct, rapid, and inexpensive method to purify and stabilize these enzymes.

Keywords: *Plant lipoxygenases; enzyme adsorption; talc; purification; stabilization*

INTRODUCTION

Lipoxygenases (EC 1.13.11.12) catalyze the oxidation of polyunsaturated fatty acids containing a *Z*-1,*Z*-4-pentadiene moiety and produce *Z*,*E*-conjugated monohydroperoxides as primary products (Pinsky et al., 1971; Veldink et al., 1977).

Lipoxygenases are ubiquitously distributed among plants (Gardner, 1991) and animals (Yamamoto, 1992). Lipoxygenase activities have been demonstrated in a wide range of plant tissues (Mack et al., 1987), but a precise physiological function for plant lipoxygenases has not been identified, although these enzymes have been implicated in growth and development, senescence, wounding, and pest resistance (Gardner, 1991; Siedow, 1991).

Plant lipoxygenases are used as a model to study fatty acid hydroperoxidation because these proteins can be produced in a large amount and have better purity and stability in comparison with animal lipoxygenases (Gardner, 1991; Yamamoto, 1992). However, plant enzymes can lose activity during or after the purification procedure. To overcome this inconvenience, immobilization techniques have been developed (Buchholz and Klein, 1987; Scouten, 1987, Monsan and Combes, 1987).

Some authors have reported previous studies describing adsorption of soybean lipoxygenase to glass and glass wool (Graveland, 1970) and adsorption of wheat lipoxygenase to glutenin and gliadin (Allen, 1968). Recently, Parra-Diaz et al. (1993) have developed a method for covalent immobilization of commercial preparations of soybean lipoxygenase by using a carbonyldiimidazole activated support. Chebli (1993) has studied talc/soybean lipoxygenase interactions, and we have recently described immobilization of *Solanum tuberosum* lipoxygenase on talc (Battu et al., 1994).

The present study describes the immobilization of lipoxygenases from *S. tuberosum* tuber and *Lupinus albus*, *Cicer arietinum*, and *Pisum sativum* seeds on a magnesium silicate based support, talc. These immobilizations were performed as a first step of enzyme purification. The results showed that adsorption on talc could be used to purify and stabilize certain enzymes. Moreover, lipoxygenase adsorption did not modify enzyme specificity.

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Protein adsorption on talc was performed by the creation of ionic interactions between silanol dissociated groups and positive protein charges. Hydrogen bonds were possible with no dissociated silanol groups (Buchholz and Klein, 1987).

MATERIALS AND METHODS

Linoleic acid, arachidonic acid, diethylenetriaminepentaacetic acid (DETAPAC), Brij 99 detergent [poly(oxyethylene ether], and talc were from Sigma Chemical Co.

DEAE-Trisacryl gel was purchased from IBF (France). Dried *C. arietinum* seeds, dried *Lupinum albus* seeds, and potato tuber var. bintje were of commercial origin. *P. sativum* var hortense, subvar. solara seeds were from ITCF (France).

Assay of Lipoxygenase Activity. The standard assay mixture contained the enzyme (free or immobilized) in 3 mL of 0.07 M sodium phosphate buffer at optimum pH, respectively, 6.0 for *C. arietinum* (Sanz et al., 1992), *L. albus* (Najid et al., 1988), and *P. sativum* (Guerdam et al., 1993) lipoxygenases and 6.6 for *S. tuberosum* lipoxygenase (Mulliez et al., 1987).

The reaction was started by adding linoleic acid (ethanolic solution) at a final concentration of 66 μM (Najid et al., 1988). Lipoxygenase activity was measured spectrophotometrically (Perkin-Elmer Lambda 5 UV–vis spectrophotometer) at 20 °C by monitoring the increase in absorbance at 234 nm due to the formation of hydroperoxides ($\epsilon = 26\,500\ M^{-1}\ cm^{-1}$). A unit of enzyme activity was defined as the amount of enzyme that produced 1 nmol of product/min at 20 °C.

The specific activity (units per milligram) was expressed as the number of units per milligram of protein. Protein concentration was determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Enzyme Purification. *C. arietinum.* Peeled dried *C. arietinum* seeds were ground in a rotating knife mill, and 5 g of the resulting mixture was extracted with 30 mL of distilled water and 0.1% Brij 99, at 4 °C for 12 h. After extraction, the mixture was centrifuged at 20000*g* for 30 min at 4 °C.

The resulting supernatant was the crude extract (called EB). Many assays have shown that further purification by ammonium sulfate precipitation, dialysis, and DEAE-Trisacryl column chromatography resulted in a total loss of lipoxygenase activity. To decrease competition between lipoxygenases and other proteins for talc binding sites, low protein concentrations were needed (less than 16 mg/mL). Different immobilization assays were used for different dilutions of crude extract. The best yield of immobilization was obtained for dilution to 1/4. Protein concentration of EB 1/4 was 4.70 mg/mL. This solution was called EB 1/4.

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P. sativum. Dried *P. sativum* seeds were ground in a rotating knife mill, and 5 g of the resulting mixture was extracted with 27 mL of 0.01 M sodium phosphate buffer (pH 4.5) with 0.1% Brij 99, at 4 °C for 12 h. After extraction, the mixture was centrifuged at 20000*g* for 1 h. The resulting supernatant was brought to 60% saturation with ammonium sulfate and agitated at 4 °C for 1 h. The reaction mixture was then centrifuged at 20000*g* at 4 °C for 1 h. The precipitate was dissolved in 5 mL of 0.2 M Tris-HCl buffer (pH 7.4). This fraction was called P_{60} and dialyzed against this buffer at 4 °C for 12 h. After dialysis, P_{60} was concentrated with polyethylene glycol 6000 (PEG 6000). The resulting clear solution was applied to a DEAE-Trisacryl column (40 × 2.5 cm) previously equilibrated with 0.2 M Tris-HCl buffer (pH 7.4). The column was washed with 2 bed volumes (120 mL), and proteins were eluted with a linear gradient (0−1 M) of sodium chloride in 0.2 M Tris-HCl buffer (pH 7.4). The flow rate was set at 27 mL/h, and the effluent was collected in 80 fractions of 4.5 mL. Proteins were monitored at 280 nm, and activity was measured under the same conditions as described before. As for *C. arietinum* lipoxygenase immobilization, different immobilization assays were performed for different dilutions of P_{60}. The best yield of immobilization was obtained for a dilution to 1/2. The final protein concentration was 14.10 mg/mL, and this solution was called P_{60} 1/2.

L. albus. The purification herein described is based on the procedure described by Najid et al. (1988). Dried *L. albus* seeds were ground in a rotating knife mill. The powder (20 g) was extracted with 160 mL of 0.07 M sodium phosphate buffer (pH 8.0) and 0.1% Brij 99, at 4 °C for 12 h. The precipitate was removed by centrifugation at 20000*g* for 10 min, and the resulting supernatant was centrifuged at 20000*g* for 1 h at 4 °C.

In two steps, the resulting supernatant was brought to 30% and 60% saturation with ammonium sulfate. At each step, the reaction mixture was agitated at 4 °C for 1 h and centrifuged at 20000*g* at 4 °C for 1 h. The precipitate between 30% and 60% saturation was dissolved in 10 mL of 0.01 M Tris-HCl buffer (pH 6.8). This fraction was called P_{60} and dialyzed against this buffer at 4 °C for 12 h. After dialysis, P_{60} was concentrated with PEG 6000, and the resulting clear solution was applied to a DEAE-Trisacryl column (40 × 2.5 cm) previously equilibrated with 0.02 M Tris-HCl (pH 6.8). The column was washed with 2 bed volumes (120 mL), and proteins were eluted with a linear gradient (0−0.3 M) of sodium chloride in 0.02 M Tris-HCl buffer (pH 6.8). The flow rate was 60 mL/h, and the effluent was collected in 40 fractions of 2 mL. The active fractions were pooled and dialyzed against 0.02 Tris-HCl buffer (pH 6.8) and concentrated with PEG 6000. Proteins were monitored at 280 nm, and activity was determined under the same conditions as described before. The P_{60} fraction was used for immobilization at a protein concentration of 16.25 mg/mL.

S. tuberosum. Potato lipoxygenase was isolated as described by Mulliez et al. (1987). Peeled potato tubers were ground in a rotating knife mill, and 10 g of the resulting mixture was extracted with 10 mL of 0.1 M sodium acetate/2 mM sodium bisulfite/0.1 M mM DETAPAC buffer (pH 4.5) and 0.1% Brij 99, at 4 °C for 1 h. After extraction, the mixture (11 mL) was filtered through gauze and centrifuged at 20000*g* for 30 min. The resulting supernatant was brought to 50% saturation with ammonium sulfate. The precipitate was dissolved in a minimum of 0.02 M Tris-HCl buffer (pH 7.4). This fraction was called P_{50} and dialyzed against this buffer at 4 °C for 12 h. After dialysis, P_{50} was concentrated with PEG 6000 and the resulting clear supernatant was applied to a DEAE-Trisacryl column (40 × 2.5) previously equilibrated with the above-mentioned Tris-HCl buffer. The column was washed with 2 bed volumes (120 mL), and all proteins were eluted with a linear gradient (0−1 M) of sodium chloride in 0.02 M Tris-HCl (pH 7.4). The flow rate was set at 27 mL/h, and the effluent was collected in 80 fractions of 4.5 mL. The active fractions were pooled and dialyzed against 0.02 M Tris-HCl buffer (pH 7.4) and concentrated with PEG 6000. Proteins were monitored at 280 nm, and activity was determined under the same conditions as described before. P_{50} (protein concentration was 11.5 mg/mL) and Trisacryl chromatography active fractions (protein concentration was 1.4 mg/mL) were used for immobilization.

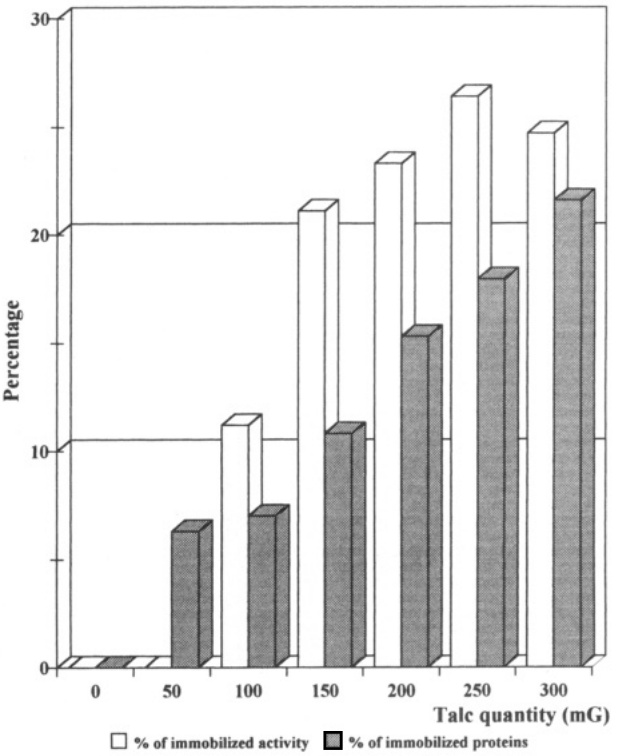

Figure 1. Immobilization isotherm of *P. sativum* lipoxygenase.

Adsorption on Talc. Talc was activated by heating at 130 °C for 2 h, and after cooling, it was dispersed in 2 mL of protein solution with mechanical stirring for 1 min. Adsorption was achieved by continuous and slow agitation of this mixture at 4 °C for 12 h.

After centrifugation for 20 min at 20000*g*, the supernatant was decanted and the talc fraction washed three times with 2 mL of 0.07 mM sodium phosphate buffer at optimal pH for each lipoxygenase. After the last wash, 2 mL of phosphate buffer was added to obtain the final active suspension of talc-adsorbed proteins. The first supernatant was kept at 4 °C to measure residual lipoxygenase activity and protein concentration after immobilization. For each lipoxygenase, the necessary talc quantity was determined by immobilization isotherms.

Immobilization isotherms corresponded in percentage of immobilized activity versus talc quantity used for immobilization under the same conditions of temperature (Figures 1 and 2). In each case, talc quantity was chosen to obtain high immobilized activity and maximal enzyme purification with little residual supernatant enzymatic activity resulting in good specific lipoxygenase talc adsorption. Talc quantities were 100 mg/2 mL of EB 1/4 (*C. arietinum*), 200 mg/2 mL of P_{60} 1/2 (*P. sativum*), 250 mg/2 mL of P_{60} (*L. albus*), 300 mg/2 mL of P_{50}, and 75 mg/1 mL of DEAE-Trisacryl chromatography active fractions (*S. tuberosum*).

Analytical Methods. The enzymatic conversion of linoleic acid by plant lipoxygenases led to the formation of two major compounds, which were 13-hydroperoxyoctadecadienoic acid (13-HPOD) and 9-HPOD. Authentic standards, 9-HPOD and 13-HPOD, were obtained by the action of soybean lipoxygenase on linoleic acid at pH 7 (Gardner, 1991) and were separated by straight-phase HPLC.

In the case of *P. sativum* lipoxygenase, the enzymatic conversion of linoleic acid led to two compounds, 9-HPOD and 2,4-decadienal (Guerdam et al., 1993). The authentic standard, 2,4-decadienal, was obtained by the action of *Vicia sativa* seed lipoxygenase (Andrianarison et al., 1991) on linoleic acid

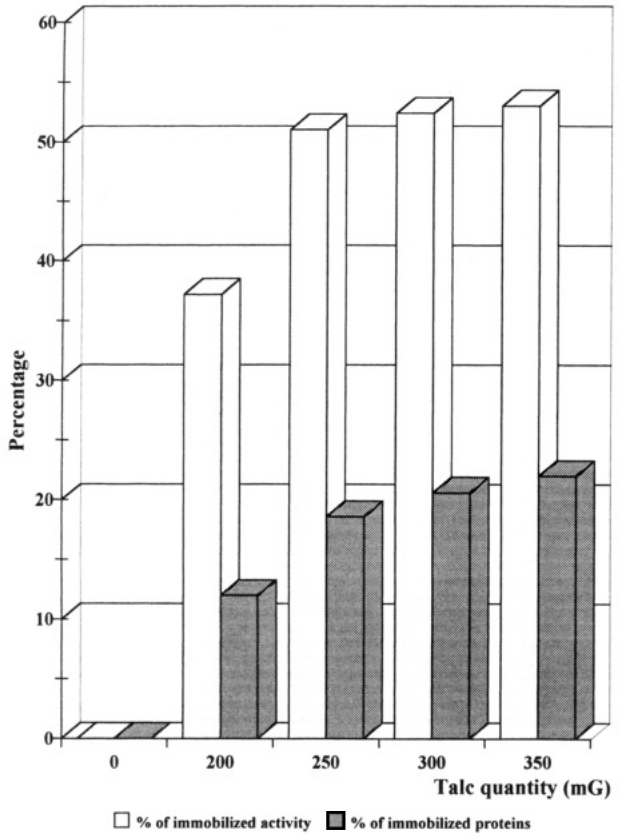

Figure 2. Immobilization isotherm of *S. tuberosum* lipoxygenase.

and purified by reversed-phase HPLC. Absorbance was monitored at 280 nm (Andrianarison et al., 1991).

Reaction products (hydroperoxides) were isolated from the reaction mixture by acidification and extraction with diethyl ether. Straight-phase HPLC was run on a LDC multiple delivery system Model CM 4000. The organic extract was applied to a LDC Milton Roy Spherisorb column and eluted with hexane/ethanol/acetic acid (93/6/1 v/v/v) at a flow rate of 3.5 mL/min. Absorbance was monitored at 235 nm.

Structural determination of reaction products was conducted as previously described (Beneytout et al., 1989). Briefly, hydroperoxides were reduced by $NaBH_4$ and methylated with diazomethane, and the methyl ester derivatives were analyzed by GC/MS after silylation of hydroxyl groups by bis(trimethylsilyl)trifluoroacetamide.

Product isolated from reversed-phase HPLC at 280 nm was analyzed without derivation by GC/MS (Andrianarison et al., 1991).

RESULTS

Purification of Lipoxygenases. Table 1 summarizes the purification steps of each source of lipoxygenase. The purification factor (P) was considered as the increase in enzyme-specific activity relative to that of the crude extract. Immobilization on talc (immobilized enzymes) and DEAE-Trisacryl purification steps resulted in different degrees of enzyme purification.

In two cases (pea and lupin enzyme), Table 2 showed that immobilized and supernatant activities were more than 100%. This observation could be explained by the fact that active enzymatic solution used for immobilization was too concentrated in lipoxygenase activity and immobilization resulted in a partial purification as demonstrated by an increase in activity in the supernatant.

Enzyme Immobilization. The necessary talc quantity used for immobilization was determined by immobilization isotherms (Figures 1 and 2). For each enzyme, the quantity of talc was selected to give high immobilized activity and high enzyme purification with little residual supernatant enzymatic activity indicating specific lipoxygenase talc adsorption (Table 2). These results showed that the purification factor was increased after adsorption of EB 1/4 (*C. arietinum*), P_{60} 1/2 (*P. sativum*), P_{60} (*L. albus*), or P_{50} (*S. tuberosum*) fractions on talc (Table 2). To verify the effectiveness of enzyme purification by talc adsorption, we performed, except for *C. arietinum* (total loss of activity), a DEAE-Trisacryl column chromatography of P_{60} 1/2, P_{60}, or P_{50} fraction and obtained an increase in the purification factor (Table 1).

Active fractions obtained after DEAE-Trisacryl column chromatography purification of P_{50} were used for immobilization on talc. Table 1 shows the increase in purification factor resulting from adsorption of *S. tuberosum* lipoxygenase present in these active fractions.

Long-Term Storage Enzyme Stability. During storage at 4 °C, free lipoxygenases showed a rapid decrease in activity. Immobilization on talc was found to stabilze the enzyme (Table 3).

For *S. tuberosum*, immobilized lipoxygenase activity decreased in the same order as the free enzyme for the first 5 or 10 days and stayed constant for an additional 20 days. On the contrary, free enzyme activity regularly declined and approached zero after 30 days.

For *P. sativum*, we observed the same phenomenon for free enzymes—immobilized enzyme activity decreased slowly during the same time. The same observation was made for *L. albus* activity.

For *C. arietinum*, free enzyme activity was totally lost in <5 days. In comparison, immobilized enzyme activity decreased more slowly.

Reaction Products. HPLC analysis of reaction products (HPODs and 2,4-decadienal) obtained by incubation of linoleic acid with the four enzymes revealed that these products are the same for both free and immobilized enzymes.

For *C. arietinum*, we observed the same proportion of the two major products, 13-HPOD (35%) and 9-HPOD (65%), at pH 6.0, for free or immobilized enzyme.

For *L. albus*, 13-HPOD (96%) was the major product obtained by the action of free or immobilized enzymes, at pH 6.0.

For *S. tuberosum*, we obtained 100% 9-HPOD, at pH 6.6, for free or immobilized enzymes.

For *P. sativum*, we obtained 100% 9-HPOD, at pH 6.6, for free or immobilized enzymes, but we observed that immobilization led to a decrease in lyase activity with a decrease in 2,4-decadienal production (26%).

DISCUSSION

Specific adsorption of enzymes on a support is an essential point to decide whether or not immobilization was effective. Results showed that for all cases we obtained a specific immobilization on talc which was expressed as an increase in specific activity. In the case of *C. arietinum*, *P. sativum*, and *L. albus*, the increases in specific activity, respectively, 298 ($P = 2.15$), 311 ($P = 1.9$), and 324 units/mg ($P = 4.4$), were less than in the case of *S. tuberosum*, which showed a maximum increase in specific activity of 1287 units/mg ($P = 4.3$).

Table 1. Comparison between Results Obtained by Classical Purification Stages and by Talc Immobilization for Each Source of Lipoxygenase

source	classical purification step	immobilized fraction	total protein, mg	total act.	sp act., units/mg	purification (fold), P^a
C. arietinum	crude extract (EB 1/4)		55.9	14422	256	1
		EB 1/4	2.2	1230	554	2.15
P. sativum	crude extract		1927	826683	429	1
	P_{60}		565	315487	558	1.3
	P_{60} 1/2		141	46530	330	0.77
	DEAE eluate		2.11	3157	1496	3.49 (4.5)[b]
		P_{60} 1/2	4.4	2820	641	1.49 (1.9)
L. albus	crude extract		81	5589	69	1
	P_{60}		54	5211	96.5	1.4
	DEAE eluate		0.31	307	990	14.3
		P_{60}	5.3	2240	421	6.10 (4.4)
S. tuberosum	crude extract		196.4	85258	435	1
	P_{50}		115.2	44813	389	0.9
	DEAE eluate		7.2	16034	2227	5.1
		P_{50}	2.56	4291	1676	3.8 (4.3)
		DEAE active fraction	0.608	1726	2839	6.5 (1.3)

[a] P = specific immobilized activity/specific crude extract activity. [b] Numbers in parentheses are the purification factors with respect to solutions used for immobilization.

Table 2. Effectiveness of Talc Adsorption of Different Lipoxygenases[a]

source		initial velocity,[b] nM min^{-1} mL^{-1}	protein, mg/mL	% immobilized act.	% residual act. (supernatant)	sp act., units/mg
C. arietinum	EB1/4	8.00 ± 0.4	4.70			255
	immobilized	4.10 ± 0.17	1.11	51.3		554
	supernatant	0	3.59			0
P. sativum	P_{60} 1/2	31.1 ± 0.3	14.14			330
	immobilized enzyme	9.3 ± 0.5	2.2	29.9		641
	supernatant	29.7 ± 0.5	11.96		95.5	372
L. albus	P_{60}	10.45 ± 0.2	16.3			96.5
	immobilized enzyme (P_{60})	7.5 ± 0.3	2.7	72		421
	supernatant	10.45 ± 0.3	13.6		100	115.4
S. tuberosum	P_{50}	29.9 ± 0.7	11.58			389
	immobilized enzyme	14.30 ± 1.0	1.28	48		1676
	supernatant	3.00 ± 0.3	10.24		10	44

[a] Comparison of lipoxygenase activity initial velocity between solutions used for immobilization (EB 1/4, P60 1/2, P 60 and P50), immobilized enzyme and immobilization resulting supernatant. Increase in specific activity by immobilization. [b] Mean of four experiments.

Table 3. Assay of Storage Stability at 4 °C: Percentage of Lipoxygenase Residual Activity after 5, 10, 20, and 30 Days of Storage at 4 °C for Free and Immobilized Enzyme[a]

source		days of storage 5	10	20	30
S. tuberosum	free	75 ± 3	48 ± 5	27 ± 0.5	0
	immobilized	72 ± 4	64 ± 3	63 ± 4	63 ± 4
P. sativum	free	55 ± 3	35 ± 2	18 ± 0.5	0
	immobilized	96 ± 2	95 ± 1	93 ± 2	91 ± 0.5
C. arietinum	free	5 ± 0.3	0	0	0
	immobilized	80 ± 2.5	65 ± 2	60 ± 3	52 ± 2
L. albus	free	60 ±	41 ±	22 ±	0
	immobilized	90 ± 3	83 ± 2.5	79 ± 3	76 ± 1

[a] Mean of four experiments.

To obtain this specific immobilization on talc, it was necessary to reduce the competition between lipoxygenase and other proteins for talc binding sites. That is why low protein concentrations (less than 16 mg/mL) must be used for the four protein solutions.

Purification of P_{60} 1/2, P_{60}, and P_{50} fractions on DEAE-Trisacryl column chromatography showed the largest increase in purification factor compared to talc adsorption. However, the use of columns was more difficult and time-consuming. Furthermore, after column purification, small quantities of enzymes dispersed in many fractions were obtained and it was necessary to pool and carefully concentrate these active fractions without inactivation of these proteins. Using adsorption, we rapidly obtained purification with an increase in long-term enzyme stability and without modifying the nature or specificity of lipoxygenase activity.

On the other hand, it was possible to compare our technique with the recent work of Parra-Diaz et al. (1993). Immobilization on talc was easier and more rapid, and it was possible to immobilize partially purified lipoxygenases. Immobilized lipoxygenases could be reused several times (Battu et al., 1994). Results showed that in aqueous buffer the activity of immobilized preparation decreased to 84.5% of its original activity after seven cycles. These results did not differ greatly from those of Parra-Diaz et al. (1993) except that we used partially purified *S. tuberosum* lipoxygenase.

CONCLUSION

The adsorption of EB 1/4, P_{60} 1/2, P_{60}, and P_{50} fractions on talc was a direct, simple, rapid, and less expensive method to purify and stabilize lipoxygenases in comparison with classical chromatography purification methods. After immobilization, use of free or immobilized enzymes was quite similar, but in comparison with free enzymes, adsorbed proteins could be

used in a multistep reaction application (Battu et al., 1994). These two procedures of purification were complementary, because classical methods (chromatography columns) are indispensable in obtaining a pure protein which can be used to characterisze and study properties of enzymes.

Adsorption of known lipoxygenases as a first step in purification is a technique to quickly obtain a protein with good stability and purity which can be used for specific applications of immobilized enzymes.

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