Effectiveness of Talc as Adsorbent for Stabilization and Expression of *Pisum sativum hortense* Cv. Solara Lipoxygenase−Lyase Coupled Activities

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Pisum sativum hortense cv. Solara lipoxygenase differs from previously described lipoxygenases by the presence, at optimum pH, of dual activities that can convert linoleic acid into two products, 9-hydroperoxyoctadecadienoic acid by a classical lipoxygenase activity and 2,4-decadienal by lyase activity on an intermediate peroxyl radical. This enzyme is a very labile protein and can lose activity during or after the purification procedure. In order to overcome this inconvenience, we immobilized *P. sativum* lipoxygenase−lyase by adsorption on talc and we explored the evolution and stability of both activities after adsorption. For lipoxygenase or lyase activity, we obtained a specific immobilization on talc with an increase in long-term stability at 4 °C in comparison to free enzyme and especially for immobilized lyase activity (60% of activity after 30 days). Immobilized enzymes appeared to be less sensitive to inhibitors than free, but the increase in IC_{50} values for immobilized enzymes was in fact the result of a nonspecific adsorption of inhibitor. Therefore, despite the difference between IC_{50} values, free and immobilized enzyme behavior toward inhibitors were comparable.

Keywords: *Pisum sativum lipoxygenase−lyase; enzyme adsorption; talc*

INTRODUCTION

Lipoxygenases (EC 1.13.11.12) catalyze the oxidation of polyunsaturated fatty acids, such as linoleic and arachidonic acids, containing a (1*Z*,4*Z*)-pentadiene structure to form hydroperoxides with a conjugated *Z*,*E* double bond structure (Veldink et al., 1977). Lipoxygenases are ubiquitously distributed among plants (Gardner, 1991; Siedow, 1991) and animals (Yamamoto, 1992), and lipoxygenase activities have been demonstrated in a wide range of plant tissues (Mack et al., 1987). Plant lipoxygenases are used as a model to study fatty acid hydroperoxidation, but they 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). Adsorption of soybean lipoxygenase has been used for many years (Graveland, 1970; Allen, 1968); more recently, Parra-Diaz et al. (1993) developed a method for covalent immobilization of a commercial preparation of soybean lipoxygenase by using a carbonyldiimidazole-activated support. Recently we described the effectiveness of talc as an adsorbent for purification, immobilization, and stabilization of plant lipoxygenases (Battu et al., 1994a,b).

The present investigation describes the immobilization of a particular lipoxygenase extracted from *Pisum sativum hortense* cv. Solara (*P. sativum*). In a previous paper (Guerdam et al., 1993), we demonstrated that *P. sativum* lipoxygenase differs from previously described lipoxygenases by the presence, at optimum pH, of dual activities that can convert linoleic acid into two products: 9-hydroperoxyoctadecadienoic acid (9-HPOD) by a classical lipoxygenase activity and 2,4-decadienal by lyase activity on an intermediate peroxyl radical. Lipoxygenase and lyase activities were cochromatographed on DEAE-Trisacryl and electrofocusing columns, suggesting that these two enzymatic activities were supported by a single protein (Guerdam et al., 1993). Immobilization was performed to explore the evolution and stability of both lipoxygenase and lyase activities after adsorption.

To complete these investigations, we studied *P. sativum* lipoxygenase and lyase activities in the presence of different lipoxygenase inhibitors on free and immobilized enzymes.

MATERIALS AND METHODS

Nordihydroguaiaretic acid (NDGA), diethylenetriaminepentacetic acid (Detapac), Brij 99 detergent [poly(oxyethylene ether)], and talc were from Sigma. 2-Hydroxycinnamic acid, 3-hydroxycinnamic acid, 4-hydroxycinnamic acid, 3,4-dihydroxyhydrocinnamic acid, and caffeic acid were from Aldrich-Chimie. Linoleic acid was from Fluka. *P. sativum* seeds were from the Institut Technique des Céréales et des Fourrages (ITCF, Paris, France).

Assay of Lipoxygenase and Lyase Activities. The standard assay mixture contained the enzyme in 3 mL of 0.07 M sodium phosphate buffer at pH 7.0 for free enzyme and pH 6.4 for immobilized enzyme.

The reaction was started by adding linoleic acid (ethanolic solution) at a final concentration of 106 μM. Lipoxygenase and lyase activities were measured simultaneously spectrophotometrically (Perkin-Elmer Lamda 5 UV−vis spectrophotometer) at 20 °C by monitoring, respectively, the increase in absorbance at 234 (formation of 9-HPOD, $\epsilon = 26\ 500\ M^{-1}\cdot cm^{-1}$) and at 284 nm (formation of 2,4-decadienal, $\epsilon = 22\ 000\ M^{-1}\cdot cm^{-1}$).

One unit of each enzyme activity was defined as the amount of enzyme that produced 1 μmol of product/min at 20 °C. The specific activity (units/mg) 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 Extraction. Dried *P. sativum* seeds were ground in a rotating knife mill, and 5 g of the resulting powder was

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Table 1. Purification of *P. sativum* Lipoxygenase−Lyase Activities

P. sativum lipoxygenase−lyase act.	classical purification step	immobilized fraction	total protein (mg)	total act.	sp act. (units/mg)	purification (fold), P^a
lipoxygenase	crude extract		1 143.0	310 961	272	1.00
	$P_{60}^{1/2}$		141.0	34 355	244	0.89
		$P_{60}^{1/2}$	5.3	3 763	710	2.61 (2.90)[b]
lyase	crude extract		1 143.0	105 156	92	1.00
	$P_{60}^{1/2}$		141.0	22 983	163	1.77
		$P_{60}^{1/2}$	5.3	1 950	368	4.00 (2.26)[b]

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

Table 2. Activity after Adsorption of *P. sativum* Lipoxygenase−Lyase on Activated Talc

P. sativum lipoxygenase−lyase act.	init. velocity[a] (μmol·L^{-1}·min^{-1})	protein (mg/mL)	% immobil act.	% resid act. (supernatant)	sp act. (units/mg)
lipoxygenase					
$P_{60}^{1/2}$	22.83 ± 0.4	14.08			244
immobilized lipoxygenase	12.58 ± 0.3	2.66	55.1		710
supernatant	6.54 ± 0.4	11.42		28.6	86
lyase					
$P_{60}^{1/2}$	15.23 ± 0.3	14.08			163
immobilized lyase	6.51 ± 0.2	2.66	42.7		368
supernatant	2.12 ± 0.2	11.42		13.9	28

[a] Mean of four experiments.

extracted with 27 mL of 0.07 M sodium phosphate buffer (pH 7.0) 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 0.07 M sodium phosphate buffer (pH 7.0). This solution was called P_{60}. As previously described (Battu et al., 1994b), purification of the P_{60} fraction by DEAE-Trisacryl column chromatography resulted in a larger increase of the purification factor compared to talc adsorption. However, the use of columns was more difficult and time-consuming. Furthermore, after column purification, small quantities of enzyme were dispersed in many fractions and it was necessary to pool and carefully concentrate these active fractions without inactivation of enzymatic protein. Using adsorption, we rapidly obtained purification with an increase in long-term enzyme stability and without modifying the nature or specificity of enzymatic activity. For this study, we used a direct immobilization to obtain sufficient stabilization and partial purification in one step.

Adsorption on Talc. Talc was activated by heating at 130 °C for 2 h and, after cooling, was dispersed in 2 mL (14.08 mg/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 eliminated and the talc fraction washed three times with 2 mL of 0.07 M sodium phosphate buffer at optimal pH. 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 measured residual lipoxygenase and lyase activities and protein concentration after immobilization.

The selected talc quantity was 250 mg/2 mL of $P_{60}^{1/2}$.

Lipoxygenase Inhibitors. Assays were conducted with the addition of variable quantities of inhibitors, 1 μM to 1 mM final concentration in the reaction mixture. Immobilized and free enzymes (40 units) were preincubated 10 min with inhibitors in 3 mL of 0.07 M sodium phosphate buffer at optimum pH, and the reaction was started by the addition of linoleic acid at a final concentration of 106 μM. Inhibitors were predissolved in ethanol, and the final concentration of ethanol did not exceed 1% (v/v) (inhibitor plus substrate) in the assay system.

The initial velocity was directly proportional to lipoxygenase and lyase activities in the incubation mixture. The initial velocity was measured in the presence (VO) and absence (V) of inhibitor. IC_{50} values were calculated from the slope of the plot V/VO versus concentration of inhibitor. Straight lines were obtained by a least-squares method.

Appropriate blanks (heat-denaturated enzyme) and controls (ethanolic buffer instead of tested inhibitors) were run through the same procedure for immobilized and free enzymes.

In order to study the possible adsorption of inhibitors to talc, we tested two representative inhibitors, NDGA and 3-hydroxycinnamic acid at concentrations close to IC_{50} values: 10 μM for NDGA, 50 (lipoxygenase activity) and 100 μM (lyase activity) for 3-hydroxycinnamic acid. Talc was activated by heating at 130 °C for 2 h. After cooling, 250 mg of talc was dispersed in 2 mL of inactive $P_{60}^{1/2}$. After slow agitation of this mixture at 4 °C for 12 h and centrifugation for 20 min at 20000g, the supernatant was eliminated. Talc was resuspended in 2 mL of 0.07 M sodium phosphate buffer (pH 7.0) containing lipoxygenase inhibitor. After 10 min of incubation, the mixture was centrifuged 10 min at 20000*g* and the supernatant (S_A for NDGA and S_B for 3-hydroxycinnamic acid) was kept at 4 °C to measure its inhibitory effect on *P. sativum* lipoxygenase−lyase activities.

Analytical Methods. The enzymatic conversion of linoleic acid by *P. sativum* lipoxygenase−lyase led to two compounds, 9-HPOD and 2,4-decadienal. Separation and structural determination of reaction products were conducted as previously described (Andrianarison et al., 1991).

RESULTS

Purification and Immobilization of Lipoxygenase. Protein preparation of *P. sativum* led to the purification of both lipoxygenase and lyase activities (Table 1). Purification factor (*P*) was considered as the increase in enzyme-specific activity relative to the crude extract, and numbers in parentheses were the purification factors with respect to solutions used for immobilization. The two purifications steps, ammonium sulfate precipitation (to obtain the $P_{60}^{1/2}$ fraction) and enzyme immobilization on talc resulted in different degrees of enzyme purification. The quantity of talc (250 mg/2 mL of $P_{60}^{1/2}$) was selected to give high immobilized lipoxygenase−lyase activities and high enzyme purification with little residual supernatant enzymatic activity indicating specific lipoxygenase−lyase talc adsorption (Table 2).

These results showed an increase in specific lipoxygenase−lyase activities after adsorption of $P_{60}^{1/2}$ on talc (Table 2), resulting in an increased purification factor (Table 1).

Effects of pH. A large variation in the optimum pH value for *P. sativum* lipoxygenase activity was observed.

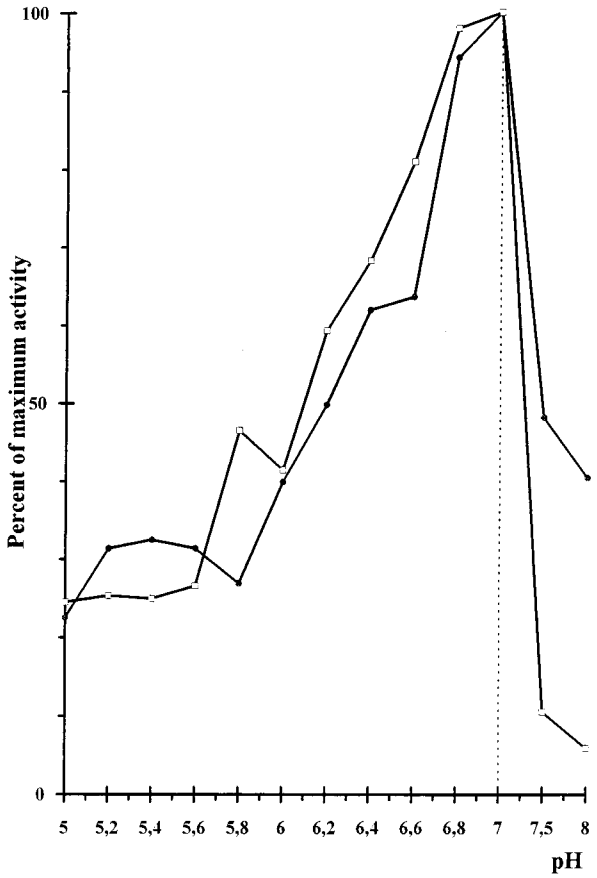

Figure 1. Effect of pH on free *P. sativum* lipoxygenase (●) and lyase (□) activities (mean of four experiments).

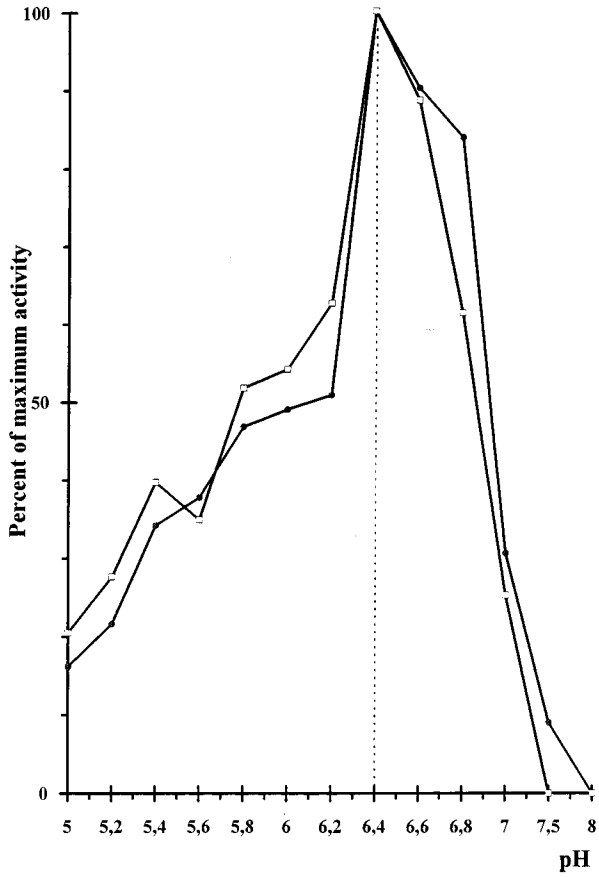

Figure 2. Effect of pH on immobilized *P. sativum* lipoxygenase (●) and lyase (□) activities (mean of four experiments).

Hardy et al. (1991) reported *P. sativum* lipoxygenase activity was optimal near pH 7.0. The optimum pH of pea lipoxygenase was found to be 5.9 by Regdel et al. (1985) and close to 6.5 by Arens et al. (1973) and Chen and Whitaker (1986). Unfortunately, they did not give any information on the species and subspecies of the pea seeds used. Guerdam et al. (1993) reported an optimum pH equal to 5.8, and more recently, we found 6.0 for *P. sativum* (Battu et al., 1994b). Recently, published data from Carrouee et al. (1993) showed a high variation in protein concentration and enzyme activity for the same pea seed species and subspecies. These variations were dependent on climatic, culture, and storage conditions. We have not found a satisfactory explanation for the great variation in optimum pH which is why it is important for us to verify this value for each lot of *P. sativum* seeds studied.

To study the effects of pH on free or immobilized lipoxygenase and lyase activities, the reaction was initiated by addition of linoleic acid at a final concentration of 106 μM to 40 units of enzyme preparation in 3 mL of 0.07 M sodium phosphate buffer for pH 5.0−8.0.

Lipoxygenase and lyase activities demonstrated the same maximal activity at pH 7.0 for free enzymes and 6.4 for immobilized enzymes (Figures 1 and 2).

Long-Term Storage Enzyme Stability. With this lot of *P. sativum* seeds, we observed as expected, during purification and storage at 4 °C, a loss of lipoxygenase and lyase activities which is in agreement with published data (Battu et al., 1994b). Immobilization on talc was found to stabilize enzyme activities (Figures 3 and 4).

Immobilized lipoxygenase activity decreased slowly during the first 10 days and stabilized for an additional 20 days. In contrast, free enzyme activity regularly declined from the first day with more than 75% loss of activity by day 30 (Figure 3).

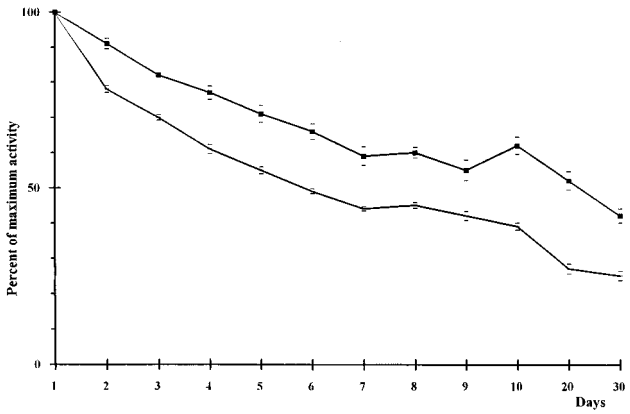

Figure 3. Percentage of maximum activity for free (—) and immobilized (■) *P. sativum* lipoxygenase during storage under the same conditions at 4 °C (data are presented as mean ± SEM, $p < 0.05$).

Immobilized lyase activity decreased similarly to that of free enzyme for the first 3 days but remarkably stabilized at a high level for an additional 27 days. During the same time, free lyase activity regularly declined from the first day with more than 70% loss of activity by day 30 (Figure 4). Data presented in Figures 3 and 4 represent the results of four separate experiments for free or immobilized activities and were tested for statistical significance ($p < 0.05$) using Student's *t*-test. The data are presented as mean ± SEM (Figures 3 and 4).

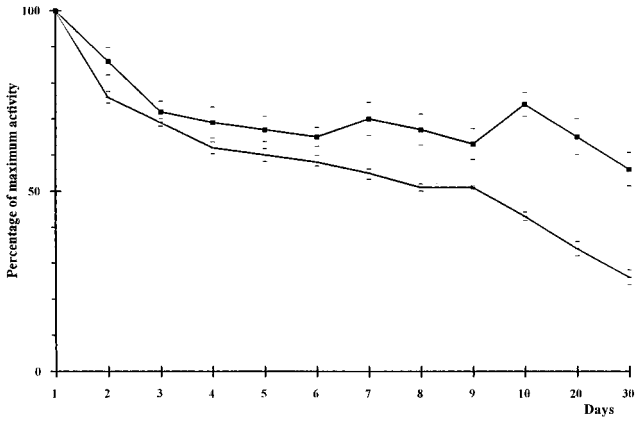

Figure 4. Percentage of maximum activity for free (—) and immobilized (■) *P. sativum* lyase during storage under the same conditions at 4 °C (data are presented as mean ± SEM, $p < 0.05$).

The increases in lyase or lipoxygenase immobilized activity observed for day 10 were not clearly defined, perhaps due to a desorption of immobilized protein. This phenomenon was previously observed with immobilized *Solanum tuberosum* lipoxygenase (Battu et al., 1994a).

These results showed that immobilization increased enzyme storage stability, especially for immobilized lyase activity.

Reaction Products. As previously described (Battu et al., 1994b), the two major products (9-HPOD and 2,4-decadienal) were obtained by action of immobilized enzyme but we observed that immobilization led to a decrease in lyase activity (20%) with a decrease in 2,4-decadienal production (data not shown).

Effects of Lipoxygenase Inhibitors. In order to study *P. sativum* lipoxygenase and lyase sensibilities to inhibitors and the possible role of protein talc adsorption, we tested lipoxygenase inhibitors, such as NDGA (Kemal et al., 1987; Van der Zee et al., 1989), caffeic acid (Koshihara et al., 1984), and derived products of cinnamic acid such as 2-hydroxycinnamic acid, 3-hydroxycinnamic acid, 4-hydroxycinnamic acid, and 3,4-dihydroxyhydrocinnamic acid, that have been previously studied in our laboratory.

Comparison of IC_{50} values of cinnamic acid derivatives on *P. sativum* lipoxygenase (Table 3) showed that free enzyme was apparently more sensitive to inhibitors than immobilized enzyme. Similar results were obtained with free and immobilized lyase activities (Table 3).

These results must be reevaluated with regard to those described in Table 4, which shows a substantial nonspecific adsorption of inhibitor to talc for two representative inhibitors (NDGA, 3-hydroxycinnamic acid).

For example, the percentage of lipoxygenase activity inhibition varied from 51.30% with 10 μM NDGA to 6.31% with S_A (factor 8.13); the percentage of lyase activity inhibition varied from 53.15% with 10 μM NDGA to 5.73% with S_A (factor 9.27). The percentage of lipoxygenase activity inhibition varied from 48.15% with 50 μM 3-hydroxycinnamic acid to 9.37% with S_B (factor 5.14); the percentage of lyase activity inhibition varied from 56.05% with 100 μM 3-hydroxycinnamic acid to 20.72% with S_B (factor 2.70). These factors are in agreement with R_1 and R_2 (Table 3).

Data presented in Table 3 represent the results of four separate experiments and are presented as mean ± SEM in Table 4.

In all cases, NDGA was the most effective inhibitor and 3,4-dihydroxyhydrocinnamic acid was the least effective.

DISCUSSION

Enzyme Immobilization. To obtain a specific talc immobilization, it was necessary to reduce the competition between lipoxygenase—lyase protein and other proteins for talc binding sites. To decrease this competition, low protein concentrations were needed (less than 16 mg/mL), so $P_{60}^{1/2}$ (14.08 mg/mL) fractions were used for immobilization.

The necessary talc quantity used to immobilize *P. sativum* enzyme was determined by immobilization isotherms (Battu et al., 1994b). The quantity of talc (250 mg/2 mL of $P_{60}^{1/2}$) was selected to give high immobilized lipoxygenase—lyase activities and enzyme purification with little residual supernatant enzymatic activity indicating specific lipoxygenase—lyase talc adsorption (Table 2).

Our results showed that, for lipoxygenase or lyase activity, we obtained a specific immobilization on talc that resulted in an increase in specific activity. Talc adsorption of large proteins, i.e., *P. sativum* lipoxygenase [MW 114 000; Guerdam et al. (1994); Hardy et al. (1991)] is less effective than that observed with smaller proteins, i.e., *S. tuberosum* lipoxygenase [MW 92 000; Mulliez et al. (1987)] or *Lupinus albus* lipoxygenase [MW 71 000; Najid et al. (1988)]. This fact could explain the detection of a nonnegligible activity in the immobilization supernatant. This could also be due to the fact that the active enzymatic solution used for immobilization was too concentrated in lipoxygenase activity and immobilization resulted in an effective but partial purification.

For *P. sativum* lipoxygenase and lyase activities, the increases in specific activity (Table 2) were respectively 466 and 205 units/mg. These results showed that it was possible to immobilize and partially purify lipoxygenase

Table 3. Comparison of IC_{50} Values of Various Inhibitors on Lipoxygenase and Lyase Activities for Free and Immobilized *P. sativum* Enzymes

	IC_{50} (μM)					
	free enzyme		immobilized enzyme			
inhibitors	lipoxygenase	lyase	lipoxygenase	lyase	R_1[b]	R_2[c]
2-hydroxycinnamic acid	115 (0.998)	110 (0.994)	445 (0.959)	439 (0.973)	3.9	4
3-hydroxycinnamic acid	57 (0.998)	94 (0.995)	153 (0.995)	181 (0.992)	2.7	1.9
4-hydroxycinnamic acid	144 (0.997)	67 (0.998)	455 (0.975)	138 (0.997)	3.2	2.1
3,4-dihydroxyhydrocinnamic acid	270 (0.990)	468 (0.956)	695 (0.952)	715 (0.950)	2.6	1.5
caffeic acid	62 (0.998)	94 (0.997)	148 (0.993)	164 (0.996)	2.4	1.7
NDGA	9.5 (0.997)	9 (0.999)	82 (0.994)	79 (0.998)	8.6	8.8

[a] Numbers in parentheses correspond to the correlation factors. [b] R_1, IC_{50} (immobilized lipoxygenase activity)/IC_{50} (free lipoxygenase activity). [c] R_2, IC_{50} (immobilized lyase activity)/IC_{50} (free lyase activity).

Table 4. Effects of NDGA and 3-Hydroxycinnamic Acid without or after Talc Adsorption on *P. sativum* Lipoxygenase−Lyase Activities

P. sativum lipoxygenase−lyase act.	initial velocity[a] μmol·L^{-1}·min^{-1}	% inhibition
lipoxygenase		
standard ($P_{60}^{1/2}$)	21.87 ± 0.3	
NDGA (10 μM)	10.65 ± 0.4	51.30
in presence of S_A[b]	20.49 ± 0.4	6.31
3-hydroxycinnamic acid (50 μM)	11.34 ± 0.3	48.15
in presence of S_B[b]	19.82 ± 0.3	9.37
lyase		
standard ($P_{60}^{1/2}$)	15.54 ± 0.4	
NDGA (10 μM)	7.28 ± 0.5	53.15
in presence of S_A[b]	14.65 ± 0.2	5.73
3-hydroxycinnamic acid (100 μM)	6.83 ± 0.4	56.05
in presence of S_B[b]	12.32 ± 0.3	20.72

[a] Mean of four experiments. [b] The supernatants S_A and S_B were obtained as described under Materials and Methods, Lipoxygenase Inhibitors.

and lyase activities at the same time and in the same ratio. As previously described (Battu et al., 1994b), talc immobilization did not modify the specificity of enzymatic activity; we just observed a slight rise in lyase specific activity ($P = 2.26$) in comparison to lipoxygenase specific activity ($P = 2.90$). Lipoxygenase activity utilizes linoleic acid to form an intermediate peroxyl radical which can be metabolized either by a classical lipoxygenase pathway into 9-HPOD or by lyase activity into 2,4-decadienal. Therefore, lyase activity is dependent on lipoxygenase activity (Guerdam et al., 1993). This could explain, despite the presence of dual lipoxygenase−lyase activities on the same protein, the differences between lyase and lipoxygenase activities observed in Tables 1 and 2.

Enzyme Stability. By using talc adsorption, we rapidly obtained an increase in long-term stability at 4 °C in comparison to free enzyme and especially for immobilized lyase activity (60% of activity after 30 days).

In agreement with results obtained by Guerdam et al. (1993), free *P. sativum* lipoxygenase and lyase activities had the same optimum pH and immobilized *P. sativum* lipoxygenase and lyase activities had the same optimum pH as expected for an enzyme with dual activities. Previous results obtained for *S. tuberosum* lipoxygenase (Battu et al., 1994a) showed that the optimum pH was the same for both immobilized and free enzyme because talc did not produce a partitioning effect between solid and liquid phases (Monsan and Combes, 1987). On the contrary, we observed a change in optimum pH for lipoxygenase and lyase activities after immobilization. In our own point of view, this difference could be explained directly by the nature (dual activities) and the molecular weight of this enzyme that modifies interactions between proteins and the talc support.

Lipoxygenase Inhibitors. As shown in Table 3, free lipoxygenase and lyase activities were both strongly inhibited by NDGA. Caffeic acid and cinnamic acid derivatives inhibited lipoxygenase and lyase activities to a lesser extent, but we observed, as for NDGA, an increase in IC_{50} values after immobilization.

These results were not in agreement with those previously described for *S. tuberosum* lipoxygenase (Battu et al., 1994a). Therefore, it was necessary to determine the possibility of nonspecific adsorption of inhibitor to talc. A large quantity of inhibitor (NDGA or 3-hydroxycinnamic acid) was able to adsorb to protein-saturated talc. This phenomenon was responsible for the increase in IC_{50} values after talc enzyme immobilization (Tables 3 and 4). We assume that talc immobilization does not modify enzyme inhibitor sensitivity.

It seems important, for the inhibitory effect, that molecules possess a hydroxyl group in the ortho or meta position in comparison to the para position. The presence of a conjugated hydroxyl group on the aromatic ring, as in caffeic acid, enhanced the inhibitory effect as described for compounds possessing catechol groups (Cashman, 1985). Our results showed that the presence of conjugated trans double bonds on the lateral chain of cinnamic acid-derived compounds was necessary to obtain a good inhibitory effect.

Conclusion. The adsorption of *P. sativum* lipoxygenase−lyase on talc was a direct, simple, rapid, and less expensive method to partially purify and stabilize lipoxygenases. Immobilization on talc greatly enhanced the long-term stability, did not modify enzyme activity, and conserved dual activities without changing the nature or specificity of lipoxygenase or lyase activities, in particular for inhibitory effects.

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