Investigation of Crude Latex from Various *Carica papaya* Varieties for Lipid Bioconversions

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ABSTRACT: The protein contents in crude latices from various varieties of papaya *(Carica papaya)* and their catalytic activities in proteolysis, lipolysis, and interesterification reactions were studied with regard to the variety, the geographic location of cultures, and the frequency of fruit tapping. Biocatalytic activities of these raw materials were compared to several commercially available crude and purified preparations of papain. These investigations were carried out in order to have a better physicochemical characterization of these raw materials, to select the adequate papaya latex for protein or lipid bioconversions, and to valorize them on an industrial scale. For the purified preparations of papain, only proteolytic activity was obtained. All crude papaya latices exhibit proteolytic, lipolytic, and interesterification activities, and no relationship between the proteolytic and lipolytic activities was observed. The high multiple correlation coefficient (R) on the order of R = 0.93–0.99, obtained from the regression analysis for the lipolytic and interesterification activities for all crude papaya latices investigated suggested that there was a correlation between these enzyme activities. However, for the same lipase preparation, the interesterification activity differed substantially depending on the type of interesterification reaction.

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KEY WORDS: Acidolysis, *Carica papaya* latex, interesterification, lipolysis, plant lipase, proteolysis, transesterification.

Plant enzymes, such as lipases, may have an advantage over animal or microbial enzymes because of their availability, their lower cost, their apparent relative ease of purification, and their particular specificities (1). Today one of the most important plant extracts exploited in various industries is crude papain. This is the commercial name given to the spray-dried latex obtained by tapping the green fruits or stems and trunk of papaya *(Carica papaya)*. This latex principally contains cysteine thiol-proteases (2) such as chymopapain (EC 3.4.22.6) and papain (EC 3.4.22.2), which are widely used in the food and beverage industries because of their high proteolytic activities (2,3). Crude *C. papaya* latex (CPL) also exhibits good lipase activity (4) in comparison to other plant lipases and, moreover, shows particular lipase selectivities (*sn*-3 stereoselectivity) for lipid modifications (4–8). Several authors demonstrated that CPL could be used as a biocatalyst for various enzymatic processes involving oils and fats (9–17).

In this work we studied the effect of the plant variety, the geographic location of cultures, and the frequency of fruit incisions on the biocatalytic activities of crude CPL preparation. Proteolysis, lipolysis, and interesterification activities of CPL preparations freshly collected from each plant variety were compared with several commercially available crude and purified preparations of papain. These investigations were carried out in order to have a better physicochemical characterization of these raw materials, to determine the relationship between the different catalytic activities of CPL, to select the adequate CPL preparation for protein or lipid bioconversions, and to try to valorize them on an industrial scale.

Initially, the ability of CPL to catalyze proteolysis and lipolysis reactions was determined by the pH-Stat method, which is a rapid hydrolysis test. Then transesterification-specific activities of CPL were investigated according to the method developed by Graille *et al.* (18) and Muderhwa *et al.* (19). Finally, interesterification activities of CPL were further evaluated in different subreactions of interesterification, such as transesterification and acidolysis, by a new method using a homogeneous triacylglycerol (TAG) and various acyl donors.

EXPERIMENTAL PROCEDURES

Chemicals. All solvents were of analytical reagent quality and purchased from Sigma (St. Louis, MO). The 1,4-dithio-DL-threitol (DTT), 99% purity, was purchased from Lancaster (Eastgate, White Lund, Morecambe, England). EDTA was purchased from Fluka Chemie AG (Buchs, Switzerland).

Substrates. Casein [from bovine milk, 90% (w/w) protein, Ref. C-0376] was purchased from Sigma. The natural copra TAG were from commercial copra oil; this TAG mixture was purified by column chromatography on activated aluminum oxide (150 mesh; Sigma) following the procedure mentioned in a previous paper (17). Capric acid, stearic acid, and the homogeneous TAG (purity >99%), namely, tributyrin, tricaproin, tricaprin, and trilaurin, were purchased from Sigma.

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The capric and stearic acids were converted to methyl caprate and methyl stearate, respectively, by derivitization with a $MeOH/H_2SO_4$ mixture (95:5; vol/vol) by boiling for 30 min.

Enzymes. Several commercially available crude or purified preparations of papain were purchased from Merck (Darmstadt, Germany) (water-soluble papain, EC 3.4.22.2, from *C. papaya* latex, Ref. Art 7144); Reco Industry Ltd. (Kampala, Uganda) (refined papain, EC 3.4.22.2, from *C. papaya* latex, Ref. RILS 029905SDP); Valley Research Co. (South Bend, IN) (validase-purified papain "conc. sulfite free," EC 3.4.22.2, from *C. papaya* latex, Ref. DP-477); and Sigma (crude papain, from *C. papaya* latex received from Uganda, Ref. P-3375).

Fresh CPL preparations from the Deshaies plant variety and Martinique–number two-(MTQ2) plant variety were collected in Guadeloupe, French department. Fresh CPL preparation from the Madagascar plant variety was collected in the Tamatave countryside, Madagascar. All fresh latices were collected in the early morning by tapping the green fruits (2- to 4-mon-old) of both sexes (female and hermaphrodite) following the optimal collection procedure described by Madrigal *et al.* (20): fresh latices were obtained by making three longitudinal incisions (depth 2–3 mm) on the green fruit epidermis using a stainless steel blade. Umbrella-like devices were attached around the trunks to collect the exuded latex, which was then dried at 55–60°C for approximately 20 h in an oven. This tapping procedure was repeated at 5-d intervals on the same fruits and fresh latices. This second fruit tapping was further dried under the same conditions. All dried latices were stored at 4°C and ground before use. Protein contents (%prot) in enzyme preparations were determined according to the Kjeldahl assay ($N \times 6.25$) and the water activity (a_w) was measured at 25°C with an FA-ST/1 instrument (GBX Scientific Instrument, Romans, France). Water content (%) of the CPL preparations was calculated after determination of dry materials in enzyme preparations: samples were dried at 103°C for 24 h in an oven.

Proteolysis reactions. A solution of casein (5.55 g) in 50 mL of distilled water, with or without activators, was used to determine proteolytic activities. The two classical activators of cysteine thiol-proteases added in the reaction medium were DTT as a reducing agent (2.0 mM) and EDTA as a chelating agent of possible metallic cations (10.0 mM). The reaction mixture was agitated by magnetic stirring at 300 rpm. Reactions were carried out at optimal conditions for proteolysis reactions catalyzed by CPL, namely, 45°C for 10 min of incubation time and constant pH 7.5 (2). After incubation for 10 min under these conditions, proteolysis reactions were carried out with an enzyme preparation/protein ratio of 0.5% (w/w), i.e., with 25 mg enzyme preparation and 5 g protein from casein. This quantity was weighed precisely with a Mettler balance, type H35AR, fine precision (Mettler, Viroflay, France). The cleaved peptide bonds were automatically titrated with a 0.5 M NaOH aqueous solution by using a pH-Stat (736 GP-Titrino, Metrohm, Switzerland) equipped with a pH glass electrode (KCl/3 M) and a thermostatted vial (Ikamag-Ret; Janke & Kunkel, Staufen, Germany).

Lipolysis reactions. Tributyrin (500 mg) was emulsified mechanically in 40 mL of NaCl solution (0.15 M) by the use of a magnetic stirrer at 300 rpm (Ikamag-Ret; Janke & Kunkel). Reactions were carried out at optimal conditions for lipolysis catalyzed by CPL, namely, 50°C for 10 min of incubation time and constant pH 8.0 (4,17). After incubation for 10 min under these conditions, lipolysis reactions were carried out with an enzyme preparation/TAG ratio of 1% (w/w), i.e., with 5 mg enzyme preparation (precisely weighed) and 500 mg substrate. No emulsifier was added to the reaction mixture in order to prevent any interaction between emulsifier and protein, and to get the intrinsic enzyme activity. The released free fatty acids (FFA) were automatically titrated with a 0.1 M NaOH aqueous solution by using the above pH-Stat equipped with the same pH glass electrode and thermostatted vial.

Determination of transesterification specific activity (TSA). Transesterification, which is one of the subreactions of interesterification, was carried out with 5 g (7.8 mmol) of copra TAG and 114 mg (0.38 mmol) of methyl stearate, i.e., in the appropriate molar ratio of 20 to 1 according to Graille *et al.* (18) and Mudherwha *et al.* (19). The substrates were placed in a vial without solvent, and each reaction was catalyzed by CPL preparation (10% w/w of total lipids, i.e., with 512 mg CPL preparation and 5.12 g substrate). The sealed vials were placed in an oven at 50°C and reaction mixtures were agitated by magnetic stirring at 300 rpm throughout the reaction. Over the time course of the reactions, samples (50 mg of total lipids) were removed periodically from the reaction medium. After dilution with 1 mL *n*-hexane, they were filtered (Millex 0.5 µm, Millipore, Bedford, MA). Then an aliquot (400 µL) containing approximately 20 mg of total lipids was analyzed by thin-layer chromatography (TLC) as follows. The sample was applied with an automatic applicator (CAMAG Linomat III, Camag Ltd., Muttenz, Switzerland) to a preparative plate (20 × 10 cm, Silica gel 60 F_{254}, 0.25 mm layer thickness, Merck). Development was carried out in an *n*-hexane/diethyl ether/acetic acid mixture (80:20:1; vol/vol/vol). The band corresponding to the fatty acid methyl esters (FAME), having an $R_f = 0.83$, was detected by transparency and separated from the TAG ($R_f = 0.54$). These FAME were extracted twice from silica with 3 mL of diethyl ether and recovered by stripping off the solvent using nitrogen at room temperature. Then the FAME were dissolved in 2 mL *n*-hexane, and aliquots (0.5 µL) were analyzed by gas–liquid chromatography (GLC) in a Carlo Erba instrument model HRGC (Erba Science, Paris, France) with cold on-column capillary injector and an Rtx-1 dimethyl polysiloxane capillary column (3 m × 0.32 mm i.d. × film thickness 0.25 µm, Restek, Bellefonte, PA). The chromatography conditions were flame-ionization detection at 370°C and He carrier gas at 5.5 $mL \cdot min^{-1}$. Separations were made using the following oven temperature profile: initial temperature 70°C for 1 min, 70 to 220°C at $20°C \cdot min^{-1}$, and final time 3 min.

Interesterification reactions. Three types of interesterifica-

tion reactions were investigated using the same homogeneous TAG and various acyl donors such as another TAG, a fatty acid (FA) ester, or an FFA. The first subreaction was carried out between 100 µmol of trilaurin (LaLaLa) and 100 µmol of tricaprin (CCC), the second between 100 µmol of LaLaLa and 300 µmol of methyl caprate. Strictly speaking, those two reactions are transesterification reactions. Then the last reaction, commonly called acidolysis, was carried out between 100 µmol of LaLaLa and 300 µmol of capric acid. All reactions were initiated by the addition of 12 mg CPL preparation (approximately 10% w/w of total lipids) to each vial containing 10 mL *n*-hexane and substrates. The sealed vials were placed in an oven at 50°C, and reaction mixtures were agitated by magnetic stirring at 300 rpm throughout the reaction. Over the course of the interesterification reactions, samples (50 µL) containing approximately 0.6 mg of lipids were removed periodically from the reaction medium. After dilution with 2 mL of *n*-hexane, they were filtered (Millex 0.5µm, Millipore), and a known amount of internal standard (125 µg of tricaproin dissolved in 25 µL *n*-hexane) was added to each sample in order to quantify the newly formed TAG. No fractionation by TLC was required, and an aliquot of the mixture (0.4 µL) was directly analyzed by GLC as described above. Separations were made using the following oven temperature profile: initial temperature 70°C for 1 min, 70 to 200°C at 20°C·min^{-1}, 200 to 330°C at 10°C·min^{-1}, and final time 2 min.

RESULTS AND DISCUSSION

Comparison of different CPL preparations in crude papain yields and protein contents. Data given in Table 1 indicate that no appreciable differences in crude papain (or dried crude CPL preparation) yields were encountered using fresh latices obtained by the first or the second fruit tapping of *C. papaya* (at 5-d intervals). For example with the Madagascar variety of *C. papaya*, the amount of fresh latex obtained by the first fruit tapping was 7.0 g/kg fruit, and after the drying process the amount of crude papain obtained was 1.4 g/kg fruit. During the second fruit tapping, the amount of fresh latex collected was 5.6 g/kg fruit and the crude papain yield was 1.3 g/kg fruit. The protein contents (Table 1) in crude CPL preparations from the different plant varieties were similar (66–71% protein) irrespective of the geographic locations of cultures and fruit incisions tested. In comparison to these crude CPL preparations, the commercially available crude preparation of papain studied had a lower protein content (about 60% protein), suggesting that all crude CPL preparations from the various plant varieties investigated should be considered as appropriate enzyme preparations for a valorization in biocatalysis.

Hydrolysis reaction kinetics. The pH-Stat is an efficient method to determine proteolytic and lipolytic activities of a biocatalyst. This method is based on the principle that pH is kept constant during hydrolysis of the substrate by means of automatic titration with a base. During the hydrolysis of a protein macromolecule, namely, casein, the peptide bonds of the protein are cleaved along with the uptake of H_2O. By the cleavage of one equivalent peptide bond, one equivalent of the α-carboxyl groups as well as of the α-amino groups is formed. Provided that the average p*K* value of the α-amino groups is constant, there will exist a proportionality between the consumption of the base (equal to the liberation of protons) and the number of peptide bonds cleaved. This proportionality factor corresponds to the degree of dissociation (α) of the α-amino groups. In our experimental conditions, namely, digestion of casein at pH 7.5 and 45°C, the α value was 0.66. During the hydrolysis of a TAG, such as tributyrin, the consumption of the base (µmol·min^{-1}) corresponds to the amount of butyric acid released per minute.

The time courses for the consumption of the base in proteolysis (hydrolysis of casein) and lipolysis (hydrolysis of tributyrin) reactions with a commercially available crude preparation of papain as biocatalyst are shown in Figure 1. The initial slopes of the kinetic curves were determined by polynomial regression ($y = ax^2 + bx + c$) of the first 10 min of

TABLE 1
Physical and Chemical Characteristics of Several Commercially Available Crude or Purified Preparations of Papain and Crude *Carica papaya* Lipase (CPL) Preparations from Various Plant Varieties

Enzyme preparation	Solubility in water[a]	Water content (%[b])	Dried material content (%[b])	Protein content (%[b])
Refined papain	Soluble	1.5	98.5	84.6
Water-soluble papain	Soluble	0.9	99.1	50.0
Purified papain	Soluble	4.6	95.4	66.7
Crude papain	Not soluble	7.8	92.2	60.7
Crude CPL—first tapping				
Variety MTQ2	Not soluble	4.5	95.5	68.7
Variety Deshaies	Not soluble	3.8	96.2	70.6
Variety Madagascar	Not soluble	4.4	95.6	69.9
Crude CPL—second tapping (day +5)				
Variety Deshaies	Not soluble	2.8	97.2	71.1
Variety Madagascar	Not soluble	6.6	93.4	66.3

[a]Test of solubility was carried out at 25°C with an enzyme solution in distilled water (10 mg/L).
[b]Percentage (w/w) to total enzyme preparation (or total crude plant extract).

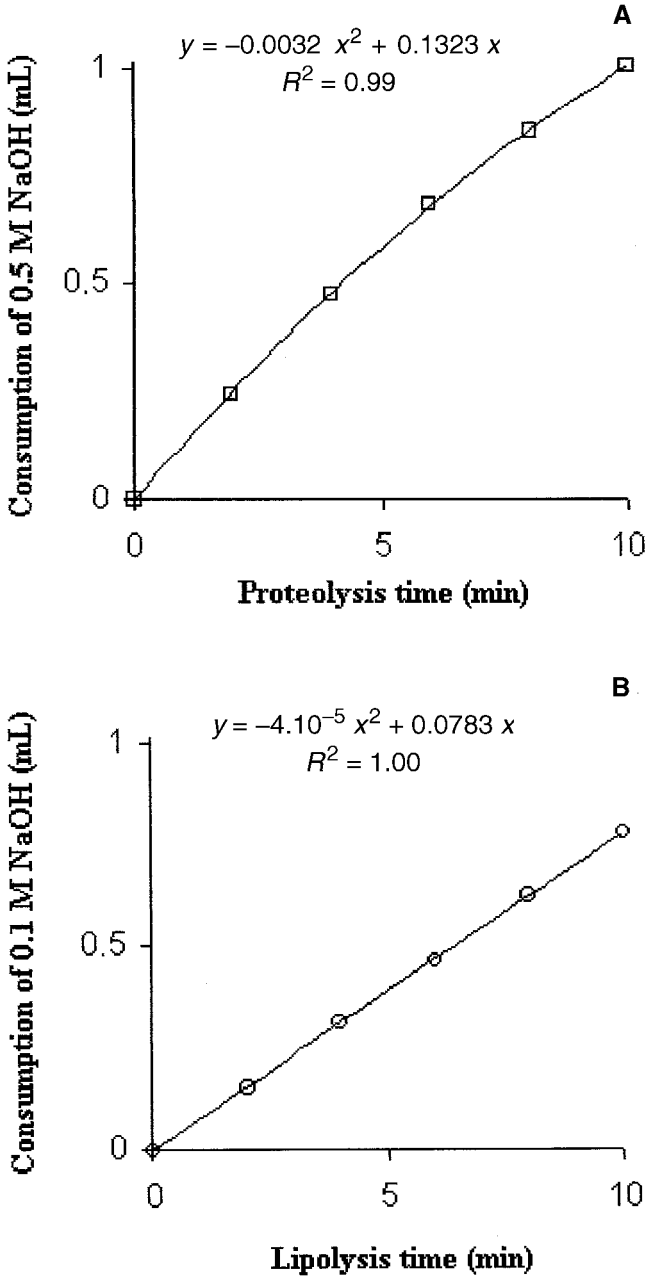

FIG. 1. Time course for the proteolysis and lipolysis reactions using crude papain as biocatalyst. (A) The proteolysis reaction was carried out with casein as substrate at constant pH 7.5 (with 2 mM of 1,4-dithio-DL-threitol and 10 mM of EDTA) at 45°C using 25 mg of crude papain as given in the Experimental Procedures section. The cleaved peptidic bonds were titrated with a 0.5 M NaOH solution. (B) The lipolysis reaction was carried out with a tributyrin emulsion at constant pH 8.0 and 50°C using 5 mg of crude papain as given in the Experimental Procedures section. The released butyric acid was titrated with a 0.1 M NaOH solution.

reaction. The coefficient *b* corresponds to the initial velocity of the reaction, and it is expressed as milliliters of base consumed per minute. The proteolytic and lipolytic activities were derived from these initial velocities. Proteolytic activity was expressed as protease units (PU) per gram of enzyme preparation. One PU corresponds to one microequivalent of peptide bond cleaved per minute. Lipolytic activity was expressed as international units (IU) per gram of enzyme preparation. One IU corresponds to one micromole of butyric acid released per minute.

Comparison of different CPL preparations in proteolysis and lipolysis. Data given in Table 2 show the proteolytic and lipolytic activities of crude CPL preparations from various plant varieties as compared to those of several commercially available crude and purified preparations of papain. Data were arithmetic means of three determinations. Results show that proteolytic and lipolytic activities of dried crude CPL differ substantially depending on the variety and the geographic location of cultures as well as the frequency of fruit tapping.

In proteolysis, all crude CPL preparations from the different plant varieties exhibit very high activities in comparison to the commercially available crude or purified preparations of papain tested. Only one commercially available purified preparation of papain displayed a substantially higher activity than the several crude CPL preparations investigated. For example, the proteolytic activity of the commercial crude preparation of papain investigated was only 39% of the proteolytic activity of the crude CPL from the Deshaies plant variety collected in Guadeloupe. The proteolytic activities of crude CPL from the Madagascar plant variety were less than those from the two plant varieties collected in Guadeloupe, namely the Deshaies and MTQ2 varieties. Differences in activity with the frequency of fruit tapping were not appreciable for each plant variety investigated. Crude CPL preparations obtained by the first and the second fruit tapping (at 5-d intervals) exhibit virtually the same proteolytic activity. In short, the dried crude CPL from the Deshaies, MTQ2, and Madagascar plant varieties could be advantageously exploited on an industrial scale for classical enzymatic reactions of protein transformation.

Lipolysis data given in Table 2 indicate that none of the commercially available purified preparations of papain have any lipolytic activity, because the lipolytic enzymes contained in the crude *C. papaya* latex were eliminated during the purification process for the isolation of the proteases. These results corroborated the ones obtained in a previous study by Giordani *et al.* (4), who have shown that the two proteases purified from the *C. papaya* latex, namely papain and chymopapain, as well as the other water-soluble enzymatic constituents extracted from this latex, were unable to catalyze lipolysis reactions. Only the crude latex was shown to efficiently catalyze lipolysis reactions. It seems probable therefore that the lipolytic enzymes are tightly associated with the particulate fraction of the crude latex. In lipolysis reactions catalyzed by crude CPL preparations, the results given in Table 2 show that CPL from the MTQ2 (587 IU/g) and Deshaies (322–814 IU/g) plant varieties collected in Guadeloupe exhibit appropriate activities, even though these activities were usually less than that of the commercially available crude preparation of papain (1,567 IU/g). Lipolytic activities of CPL from the Madagascar plant variety were very low (only 9% of that from the commercially available crude

TABLE 2
Proteolytic and Lipolytic Activities of Crude CPL Preparations from Various Plant Varieties in Comparison with Commercially Available Crude or Purified Preparations of Papain[a]

Enzyme preparation	Proteolytic activities (PU/g)[b]		Lipolytic activities (IU/g)[b]
	Without activator	With activators	
Refined papain	10046 ± 364[c] (3.6%[d])	11352 ± 58[c] (0.5%[d])	No activity
Water-soluble papain	3379 ± 99[c] (2.9%[d])	7381 ± 227[c] (3.1%[d])	No activity
Purified papain	1842 ± 144[c] (7.8%[d])	5919 ± 68[c] (1.1%[d])	No activity
Crude papain	11.02 ± 28[c] (2.6%[d])	3842 ± 229[c] (6.0%[d])	1567 ± 35[c] (2.3%[d])
Crude CPL—first tapping			
Variety MTQ2	4532 ± 367[c] (8.1%[d])	8711 ± 230[c] (2.6%[d])	587 ± 18[c] (3.0%[d])
Variety Deshaies	5515 ± 93[c] (1.7%[d])	9794 ± 191[c] (2.0%[d])	814 ± 38[c] (4.7%[d])
Variety Madagascar	3752 ± 279[c] (7.4%[d])	6474 ± 155[c] (2.4%[d])	145 ± 17[c] (11.8%[d])
Crude CPL—second tapping (day +5)			
Variety Deshaies	6316 ± 383[c] (6.1%[d])	9602 ± 112[c] (1.2%[d])	322 ± 10[c] (3.1%[d])
Variety Madagascar	3549 ± 159[c] (4.5%[d])	5695 ± 433[c] (7.6%[d])	68 ± 5[c] (6.7%[d])

[a]Proteolytic activities were determined with casein at constant pH 7.5 with or without activator (2 mM dithiothreitol and 10 mM EDTA) and 45°C. One protease unit (PU) corresponds to one microequivalent of peptide bond cleaved per minute. Lipolytic activities were determined with tributyrin at constant pH 8.0 and 50°C. One international unit (IU) corresponds to one micromole of butyric acid released per minute.
[b]Values of activity were arithmetic means of three determinations.
[c]Standard deviation.
[d]Relative standard deviation (%). For other abbreviation see Table 1.

preparation of papain). Moreover, for each plant variety tested, lipolytic activity of CPL differed substantially depending on the frequency of fruit tapping. Thus, the lipolytic activity of crude CPL preparations obtained by the second fruit tapping was always less (generally 50%) than the one collected during the first fruit tapping. The crude CPL preparations obtained by the first fruit tapping from the two plant varieties collected in Guadeloupe, namely, Deshaies and MTQ2, could be exploited for modifying the FA composition of several oils and fats, particularly the FA composition in the *sn*-3 position, by selective hydrolysis, due to their high lipolytic activities and the particular *sn*-3 stereoselectivity of the lipase of *C. papaya*.

The analysis of activities of all crude CPL preparations investigated (Fig. 2) indicated that proteolytic activity yield cannot always be taken as a guide for lipolytic activity yield. For example, the proteolytic and lipolytic activities of crude CPL from the MTQ2 plant variety were substantially higher than those from the Madagascar plant variety, but if the proteolytic activity of crude CPL from the MTQ2 plant variety also exceeds the one of the commercially available crude preparation of papain, its lipolytic activity is only 37% that of the abovementioned commercial crude preparation of papain. The multiple correlation coefficient (R) obtained from this regression analysis for the proteolytic activity and lipolytic activity was very small ($R = -0.34$, with a confidence interval 95%). Therefore, both these hydrolytic activities should be determined independently in order to evaluate aptitudes of the biocatalyst to catalyze proteolysis and/or lipolysis reactions.

Comparison of different CPL preparations in transesterification. The TSA of each CPL preparation was determined by following the incorporation of lauric acid ($C_{12:0}$), which is the main FA of copra oil, into the fatty acid methyl ester (FAME) fraction during the transesterification of copra TAG with methyl stearate. The time course of incorporation of $C_{12:0}$ during the transesterification reaction using a commercially available crude preparation of papain as biocatalyst is shown in Figure 3. The TSA was expressed as micromoles of $C_{12:0}$ incorporated in the FAME fraction per hour and per gram of biocatalyst and derived from the initial reaction velocity. This initial velocity, expressed as the mole percentage (mol%) of $C_{12:0}$ incorporated per hour, was calculated from the initial slopes of the kinetic curve (curve of $C_{12:0}$ incorporated) by polynomial regression of the first 48 h of reaction. During this time period, the reaction velocity was linear (Fig. 3). The conversion rates were calculated from the x/a ratio, where x was the experimental mol% of $C_{12:0}$ incorporated at a considered time and a the theoretical mol% incorporated at equilibrium. A theoretical value of 32 mol% of $C_{12:0}$ incorporated at equilibrium was determined by Graille *et al.* (18) and Muderhwa *et al.* (19) for the transesterification reaction between copra TAG and methyl stearate, in a 20:1 molar ratio. The $C_{12:0}$ concentration in methyl stearate was, obviously, nil. The initial copra TAG contained 32.8 mol% of $C_{12:0}$ in each external position of glycerol. Using the following formula these authors calculated the theoretical mol% (a) of $C_{12:0}$ incorporated in methyl esters at equilibrium:

$$a = [(32.8 \times 2 \times 20) + 0] / [(200 \times 20) + 100] = 0.32 \text{ or } 32 \text{ mol\%} \quad [1]$$

Table 3 reports the TSA of crude CPL preparations from the various plant varieties investigated in comparison to a commercially available crude preparation of papain. The a_w values correspond to the thermodynamic water activity measured at 25°C of each crude CPL preparation after they have been ground. In order to study the synthesis activities of all crude CPL preparations investigated and to compare them to the commercially available crude preparation of papain, it is

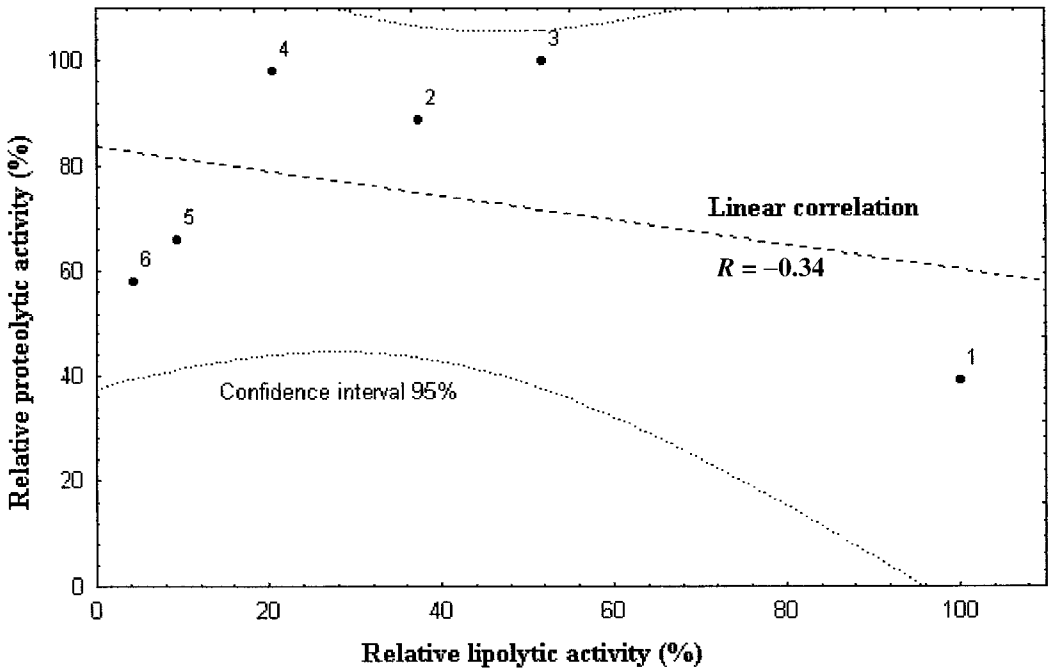

FIG. 2. Proteolytic activity as a function of lipolytic activity for crude *Carica papaya* lipase (CPL) preparations. The highest proteolytic activity exhibited by one of the crude CPL preparations was taken as 100% for the calculation of relative proteolytic activity, and it is the same for the calculation of relative lipolytic activity. Abbreviations: *R,* multiple correlation coefficient (linear regression with a confidence interval 95%); no. 1, commercially available crude preparation of papain; no. 2, crude CPL from MTQ2 plant variety (first tapping), Guadeloupe; no. 3, crude CPL from Deshaies plant variety (first tapping), Guadeloupe; no. 4, crude CPL from Deshaies plant variety (second tapping), Guadeloupe; no. 5, crude CPL from Madagascar plant variety (first tapping), Madagascar; no. 6, crude CPL from Madagascar plant variety (second tapping), Madagascar.

very important to specify the initial a_w of each lipase preparation, because this parameter has great influence on the rates of synthesis. The effect of initial a_w and water content of crude CPL on the rates of synthesis during different CPL-catalyzed interesterification and esterification reactions will be studied in a forthcoming investigation. The conversion rates (*x/a* ratio) obtained at various reaction times and TSA values, given in Table 3, show that the commercially available crude preparation of papain displayed the best synthesis activity. However, transesterification-specific activities of all crude CPL preparations, calculated by the method described above, remain less than one unit, and differences in activity are not appreciable. We attempted to apply another method for the determination of interesterification activity of these enzyme preparations in different types of interesterification reactions.

TABLE 3
Conversion Rates and Transesterification Specific Activities (TSA) of Crude CPL Preparations from Various Plant Varieties in Comparison with Commercially Available Crude Preparation of Papain

		Conversion rates (%)					
Enzyme preparation	a_w	Time (h) 0	24	48	96	168	TSA[a]
Crude papain	0.57	0.0	14.4	26.9	45.9	57.5	0.72
Crude CPL—first tapping							
Variety MTQ2	0.43	0.0	12.2	20.3	29.1	35.9	0.63
Variety Deshaies	0.70	0.0	9.7	15.3	21.9	29.7	0.51
Variety Madagascar	0.45	0.0	6.8	9.7	11.9	13.8	0.37
Crude CPL—second tapping (day +5)							
Variety Deshaies	0.56	0.0	10.3	16.8	22.8	29.7	0.54
Variety Madagascar	0.52	0.0	6.3	8.8	10.3	12.5	0.35

[a]TSA was expressed as the initial rate of lauric acid ($C_{12:0}$) incorporation into the fatty acid methyl esters (FAME) per hour and per gram of enzyme preparation. Reactions were carried out without solvent at 50°C between copra triacylglycerols (TAG) and methyl stearate (20:1; mol/mol). The conversion rate (%) corresponds to the ratio between the experimental concentration of $C_{12:0}$ incorporated into the methyl esters and the theoretical concentration of $C_{12:0}$ incorporated at the equilibrium reaction (32 mol%). Abbreviation: a_w = thermodynamic water activity. For other abbreviation see Table 1.

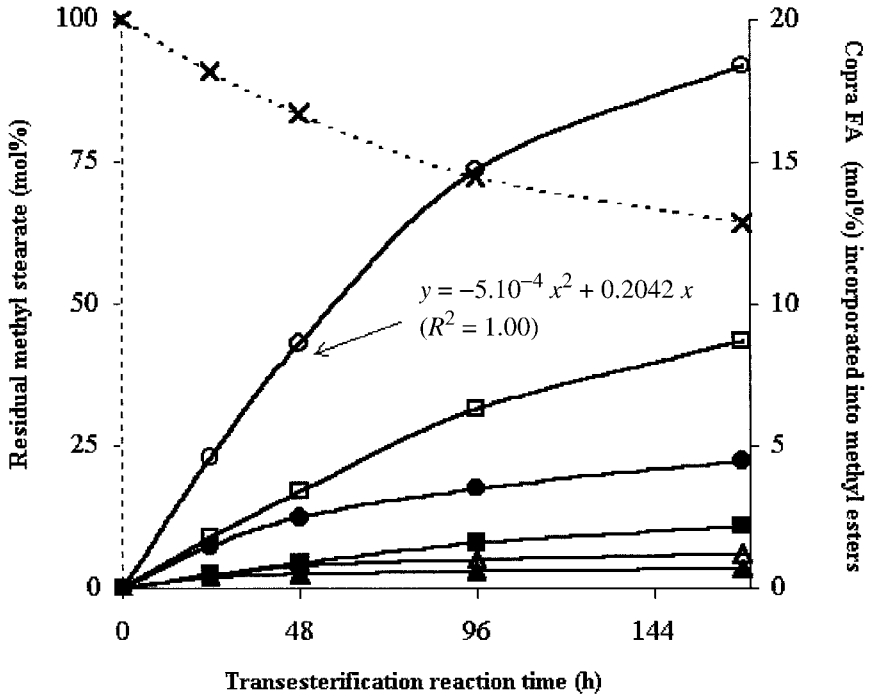

FIG. 3. Time course for the incorporation of copra fatty acids (FA) into fatty acid methyl esters (FAME) during the transesterification of copra triacylglycerols (TAG) with methyl stearate using crude papain as the biocatalyst. The reaction was carried out with copra TAG and methyl stearate at 50°C (without solvent) using 512 mg of crude papain. The incorporation of copra FA into the FAME was followed by gas–liquid chromatography (GLC), after a fractionation by thin-layer chromatography (TLC) as given in the Experimental Procedures section (■ $C_{8:0}$; ▲ $C_{10:0}$; ○ $C_{12:0}$; □ $C_{14:0}$; ● $C_{16:0}$; △ $C_{18:1}$, and × $C_{18:0}$).

Kinetic model for the determination of interesterification activity. Interesterification is an umbrella term encompassing the reactions of transesterification, acidolysis, and alcoholysis. We have applied a simple method to evaluate by polynomial regression the interesterification activity of lipase preparations in three types of interesterification reactions between a reference TAG (trilaurin, which is the main TAG of copra oil) and various FA donors, i.e., another TAG (tricaprin), a FAME (methyl caprate), or an FFA (capric acid). Lipase catalyzes acyl exchanges between the two substrates, and each acyl exchange is characterized by the formation of new TAG, which have a total carbon number different from those of the two initial substrates. The concentrations of the newly formed TAG are measured by GLC using an internal standard method.

Figure 4 depicts the transesterification reaction between trilaurin and tricaprin using the commercially available crude preparation of papain as the biocatalyst, producing dilauroyl-caproyl glycerol (LaLaC), dicaproyl-lauroyl glycerol (CCLa), and acylglycerols such as diacylglycerols (DAG) and monoacylglycerols (MAG). Throughout the reaction, the proportion of new TAG with two caproyl residues (CCLa) was higher than new TAG with two lauroyl residues (LaLaC). These results are in accordance with the ones obtained in a previous study (17), in which it was demonstrated that during a CPL-catalyzed transesterification reaction between two different homogeneous TAG, the proportion of new TAG with two short-chain FA residues remains higher than new TAG with two long-chain FA residues. The transesterification reaction studied (Fig. 4) reached equilibrium after 48 h, and the total DAG concentration did not exceed 14 mol% of the initial TAG concentration. Initial velocity of this subreaction of interesterification could be calculated from the initial slopes of kinetic curves of the newly formed TAG (LaLaC and CCLa) by polynomial regression of the first 8 h of reaction. In this period the reaction velocity (4.07 μmol newly formed TAG/h) was linear.

Figure 5 depicts the time course of transesterification of trilaurin with methyl caprate and acidolysis of trilaurin with capric acid, both catalyzed by a commercially available crude preparation of papain and producing 1,2-dilauroyl-3-caproyl glycerol (LaLaC), 1,3-dicaproyl-2-lauroyl glycerol (CLaC), tricaprin (CCC), and acylglycerols. Reactions approached equilibrium after 48 h. The total DAG concentration did not exceed 29 and 14 mol% of the initial trilaurin concentration, respectively, after 78 h of transesterification and acidolysis reaction. In both cases, initial velocity of reactions could be calculated from the initial slopes of kinetic curves of the newly formed TAG (LaLaC, CLaC, and CCC) by polynomial regression of the first 8 h of reaction. For example, the initial reaction velocity was only 0.44 μmol newly formed TAG/h during the CPL-catalyzed transesterification reaction of trilaurin with methyl caprate (Fig. 5A) and 5.7 μmol during the CPL-cat-

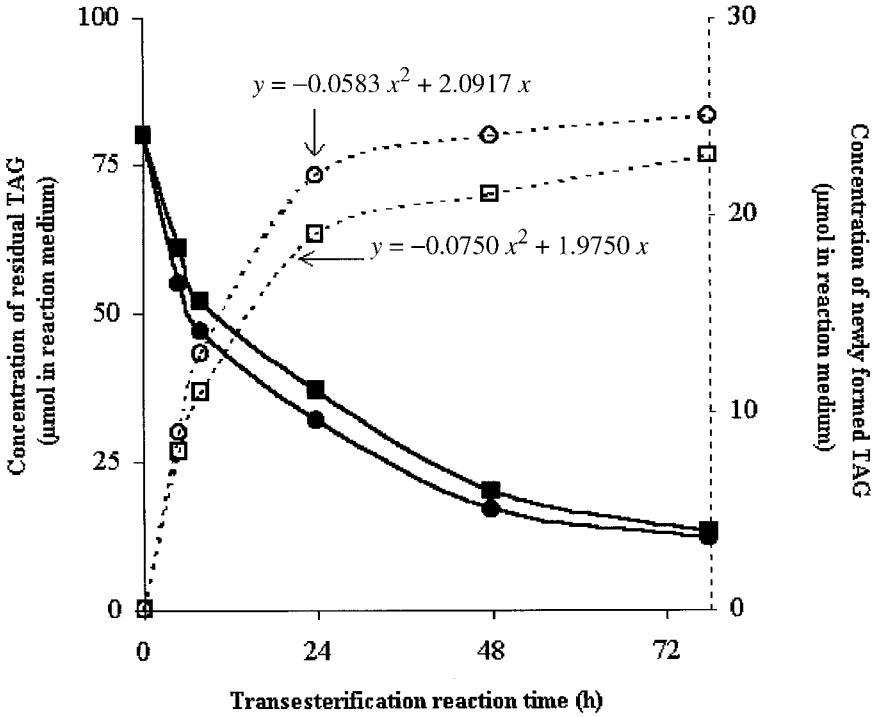

FIG. 4. Time course for the production of dilauroyl-caproyl glycerol (LaLaC) and dicaproyl-lauroyl glycerol (CCLa) during transesterification of trilaurin with tricaprin using crude papain as biocatalyst. The reaction was carried out between trilaurin and tricaprin (1:1; mol/mol) at 50°C in *n*-hexane using 12 mg of crude papain. The formation of new TAG in the reaction medium was followed by GLC. (■ LaLaLa; ● CCC; □ LaLaC; ○ CLaC). For abbreviations see Figure 3.

alyzed acidolysis of trilaurin with capric acid (Fig. 5B).

We showed that in all interesterification reactions investigated, the initial reaction velocity could be evaluated by polynomial regression and expressed as total amount (µmol) of the new TAG formed in the reaction medium per hour. Consequently, in the above subreactions of interesterification, we could use a common interesterification activity and express it as interesterification units (ItU) per gram of biocatalyst, where one ItU corresponds to one micromole of new TAG formed in the reaction medium per hour.

Comparison of different CPL preparations with regard to interesterification activity. Experiments (data not shown) indicated that none of the commercially available purified preparations of papain had any interesterification activities. For reaction periods of up to 78 h, only crude CPL preparations were able to catalyze classical interesterification reactions. These results indicated that only the lipolytic enzymes contained in the crude papaya latex displayed an ability to catalyze interesterification reactions. Our results corroborated the results obtained by Mukherjee and Kiewitt (12,13) who showed that chymopapain and papain, both proteases purified from the *C. papaya* latex, were unable to catalyze an esterification reaction between 1-butanol with various FA; only the crude preparation of CPL was able to catalyze this esterification reaction.

Table 4 reports the interesterification activities of crude CPL preparations from the various plant varieties investigated in comparison to that of the commercially available crude preparation of papain. This commercial crude papain displayed the best overall interesterification activity (339 ItU/g in transesterification between TAG, 37 ItU/g in transesterification between TAG and FAME, and 517 ItU/g in acidolysis between TAG and FFA). All crude CPL preparations from the Madagascar plant variety showed very poor interesterification activities, but the crude CPL preparations from the MTQ2 and Deshaies plant varieties exhibited good interesterification activities. No appreciable differences in interesterification activity were encountered in the transesterification reaction between TAG, or between TAG and FAME, using crude CPL preparations obtained by the first or the second fruit tapping. However, the interesterification activity in the acidolysis reaction between TAG and FFA of these CPL preparations differed substantially depending on the frequency of fruit tapping. Crude CPL preparations obtained by the first fruit tapping showed a better interesterification activity in acidolysis. In short, the crude CPL preparations obtained by the first fruit tapping from the two plant varieties collected in Guadeloupe have great potential as a biocatalyst for the selective modification of oils and fats due to their high interesterification activities and the particular *sn*-3 stereoselectivity of lipase of *C. papaya*. For instance, they could be used to modify the FA composition and the distribution of

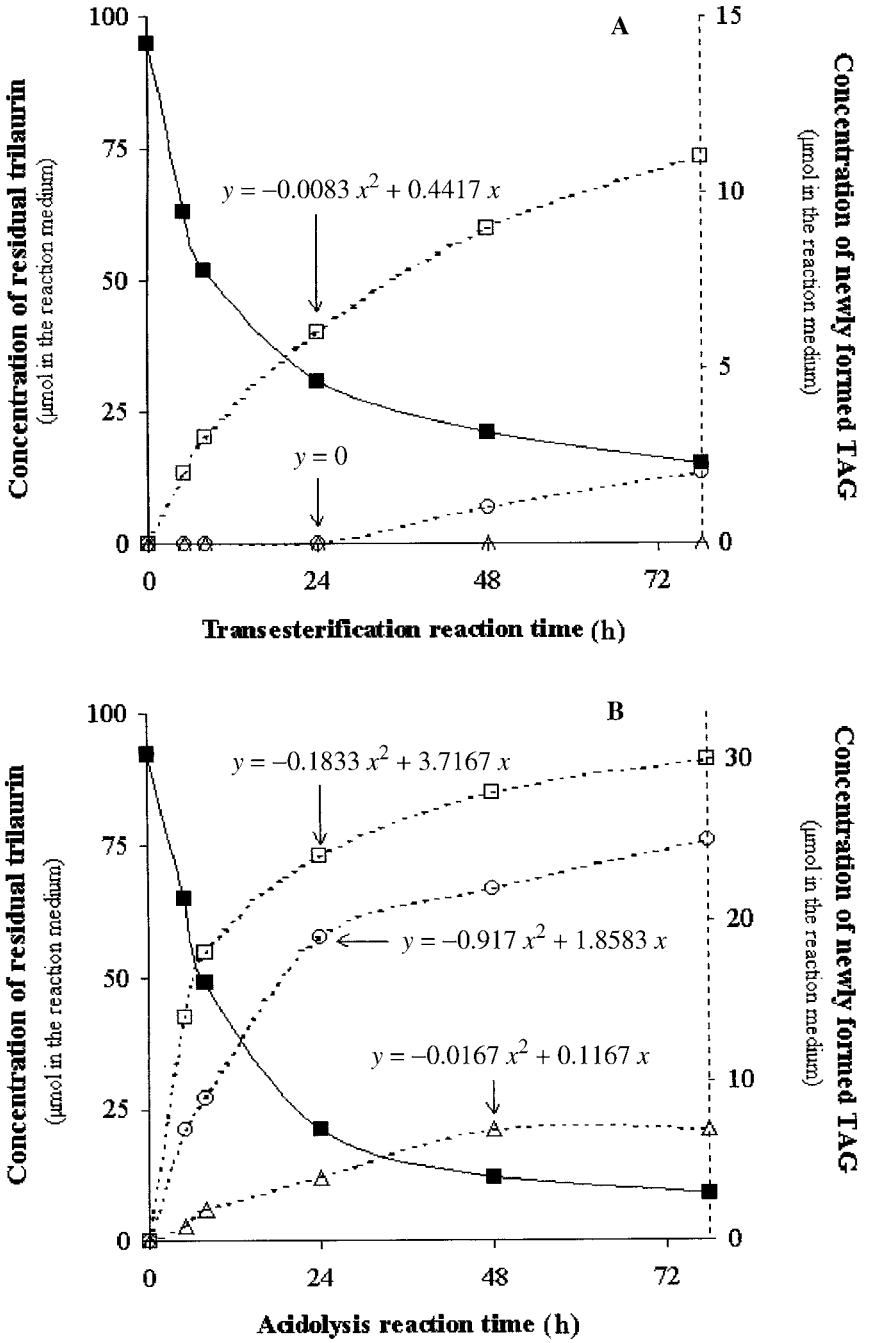

FIG. 5. Time course for the production of newly formed 1,2-dilauroyl-3-caproyl glycerol (LaLaC), 1,3-dicaproyl-2-lauroyl glycerol (CLaC), and tricaprin (CCC) during transesterification or acidolysis of trilaurin with methyl caprate or capric acid, respectively, using crude papain as biocatalyst. (A) The transesterification reaction was carried out between trilaurin and methyl caprate (1:3; mol/mol) at 50°C in *n*-hexane using 12 mg of crude papain. The formation of new TAG in the reaction medium was followed by GLC. (■ LaLaLa; □ LaLaC; ○ CLaC; △ CCC). (B) The acidolysis reaction was carried out between trilaurin and capric acid (1:3; mol/mol) at 50°C in *n*-hexane using 12 mg of crude papain. The formation of new TAG in the reaction medium was followed by GLC. (■ LaLaLa; □ LaLaC; ○ CLaC; △ CCC). For abbreviations see Figure 3.

TAG in particular oils and fats in order to obtain new products with desired physical and chemical properties (medium-chain triacylglycerols, long-chain fatty acid esters, etc.).

Bearing in mind the fact that the lipolytic and interesterification activities of a lipase preparation are often independent from each other (19), we decided also to evaluate the inter-

TABLE 4
Interesterification Activities of Crude CPL Preparations from Various Plant Varieties in Comparison with Commercially Available Crude Preparation of Papain

	Mol% of newly formed TAG (into the TAG fraction)											
	Transesterification (TAG + TAG)				Transesterification (TAG + FAME)				Acidolysis (TAG + FFA)			
Enzyme preparation	8 h	24 h	48 h	(Activity[a])	8 h	24 h	48 h	(Activity[a])	8 h	24 h	48 h	(Activity[a])
Crude papain	19.5	37.3	54.9	(339)	5.5	16.2	32.3	(37)	37.2	69.1	82.6	(517)
Crude CPL—first tapping												
Variety MTQ2	11.0	24.8	44.0	(201)	1.4	3.5	6.4	(18)	14.1	39.2	53.6	(220)
Variety Deshaies	9.2	23.9	39.1	(120)	1.3	2.7	4.9	(14)	15.7	38.3	62.9	(238)
Variety Madagascar	2.5	7.4	16.3	(50)	<0.1	<0.1	<0.1	(0)	2.2	6.4	11.5	(29)
Crude CPL—second tapping (day +5)												
Variety Deshaies	8.9	22.4	36.4	(120)	1.4	3.2	5.7	(14)	11.3	31.1	51.9	(149)
Variety Madagascar	0.8	1.9	4.0	(15)	<0.1	<0.1	<0.1	(0)	1.4	4.3	7.1	(17)

[a]Interesterification activities were determined by following the initial formation of new TAG in the reaction medium by gas–liquid chromatography. These activities were expressed as initial interesterification unit (ItU) per gram of enzyme preparation. One ItU corresponds to one micromole of new TAG formed in the reaction medium per hour. The first transesterification reaction was carried out between trilaurin and tricaprin, the second one between trilaurin and methyl caprate. The acidolysis reaction was carried out between trilaurin and capric acid. All reactions were carried out in *n*-hexane at 50°C and initiated by 10% (w/w) of enzyme preparation to total substrates. Abbreviation: FFA = free fatty acid. For other abbreviations see Tables 1 and 3.

esterification activity as a function of lipolytic activity for all crude CPL preparations investigated (Fig. 6). The result shows that for lipase of *C. papaya*, there was a correlation (R = +0.93 to +0.99 with a confidence interval 95%) between the lipolytic and the interesterification activity for the six crude CPL preparations investigated. The enzyme preparation that displayed the best lipolytic activity also displayed the best interesterification activity. However, data indicated that the interesterification activity of all crude CPL preparations differed among the different types of interesterification reactions studied. Thus, values of interesterification activity obtained in transesterification between TAG or between TAG and FAME, or in acidolysis between TAG and FFA, differ substantially with the same CPL preparation. Therefore all lipase preparations should be carefully pretested for the interesterification reaction in question before being used for specific applications in oil and fat bioconversions.

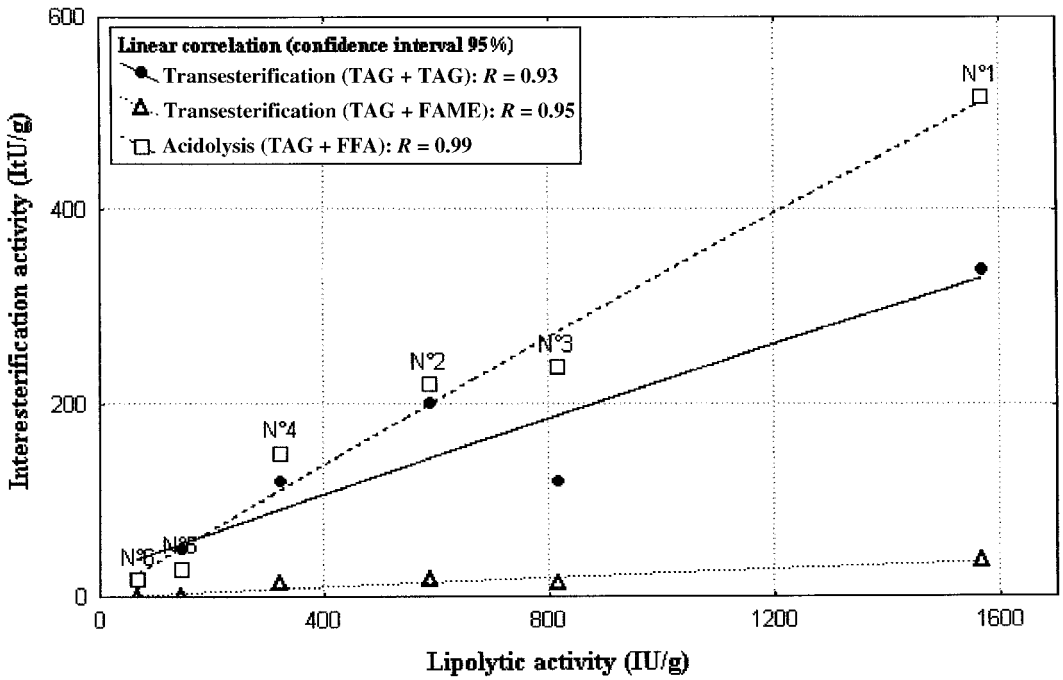

FIG. 6. Interesterification activity as a function of lipolytic activity for crude CPL preparations. Interesterification activity was expressed as interesterification unit (ItU) per gram of enzyme preparation and one ItU corresponds to one micromole of new TAG formed in the reaction medium per hour. Lipolytic activity was expressed as international units (IU) per gram of enzyme preparation and one IU corresponds to one micromole of butyric acid released per minute. Abbreviation: FFA, free fatty acid. For other abbreviations see Figures 2 and 3.

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