Characterization of Purified Lipase Fractions from *Rhizopus niveus*

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Lipase from *Rhizopus niveus* (FI) was fractionated by gradual precipitation with ammonium sulfate at 0−40 (FIIa), 40−60 (FIIb), and 60−100% (FIIc) of saturation. The partially purified enzyme (FIIb) was further purified by successive ion-exchange and size exclusion chromatographies, using the Fast Protein liquid chromatography system. Ion-exchange chromatography of fraction FIIb resulted in one fraction (FIII) with a specific activity of 29.9×10^3 units/mg. The results also demonstrated that size exclusion chromatography of fraction FIII resulted in one major enzymatic fraction (FIVa) which possessed 93% of the total lipolytic activity as well as a specific activity of 36.1×10^3 units/mg. The electrophoregrams of native polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate−PAGE showed that the purified enzymatic fraction FIVa exhibited a major protein band with a relative molecular mass of 26 ± 3 kDa. Optimum pH values for lipase activity were 7.0 for fraction FI and 7.7 for fractions FIIb, FIII, and FIVa. The V_{max} values for fractions FI, FIIb, FIII, and FIVa were 0.83×10^3, 0.98×10^3, 1.30×10^3, and 1.61×10^3 units/mg, respectively, with corresponding K_m values of 0.46, 0.40, 0.34, and 0.29 mg of butter fat/mL.

Keywords: *Lipase; Rhizopus; purification; characterization*

INTRODUCTION

Extracellular microbial lipases (glycerol ester hydrolases EC 3.1.1.3) catalyze the hydrolysis of triacylglycerols into free fatty acids and glycerols, as well as mono- and diacylglycerols (Macrae, 1983). However, the enzymatic reaction could also be reversible in organic solvent media in which these enzymes can catalyze the synthesis of ester bonds as well as the trans- and interesterification of fatty acids in lipids (Bello et al., 1987).

The first crystallization of lipase from *Aspergillus niger* was achieved by Fukumoto et al. (1962). Tsujisaka et al. (1973) also obtained a crystallized lipase from *Geotricum candidum*. Kohno et al. (1996) reported the crystallization of lipase from *Rhizopus niveus*. In addition, Iwai and Tsujisaka (1974) showed that *Rhizopus delemar* could produce large amounts of multiform lipases, such as A-, B- and C-lipases, in its culture fluid. Over the years, many microbial lipases have been successfully purified to homogeneity by the utilization of technical advances in enzyme isolation including chromatographic and electrophoretic procedures (Borgstrom and Brockman, 1984).

Lipases from *Rhizopus niveus* have been shown to be useful in food applications (Matsuo et al., 1980). The use of specific lipases to catalyze the interesterification reaction in triacylglycerols has received considerable attention due to certain advantages over those of chemical catalysts (Thomas et al., 1988; McDougall, 1989). The positional specificity of 1,3-specific lipases from *Mucor miehei*, *Aspergillus niger*, and *Pseudomonas fluorescence* was investigated in the absence (Kalo et al., 1988) and presence of organic solvent media, such as isooctane (Kalo et al., 1989) and hexane (Kalo et al., 1990), using butter fat as substrate.

Milk fat contains appreciable amounts (27.8%) of oleic acid (Robertson, 1987), which is randomly distributed at the three positions on the triacylglycerol molecules (Nawar, 1985). Hayes et al. (1991) reported that the interchange of palmitic acid with oleic acid at the 2-position of the triacylglycerol molecule suppressed the cholesterol-raising potential of milk fat. Selected commercial microbial lipases obtained from *M. miehei*, *Mucor javanicus*, *R. niveus*, and *R. delemar* were investigated in our laboratory in terms of their interesterification specificity (Safari and Kermasha, 1994); the results indicated that lipase N (Amano), obtained from *R. niveus*, showed the most appropriate specificity in changing the positional distribution of selected fatty acids (C18:1 and C16:0) within the triacylglycerol molecules of butter fat. Recently, Kermasha and Safari (1996) reported that the use of the purified lipase fraction from *R. niveus* in the interesterification of butter fat increased the acyl-exchange reactions of hypercholesterolemic fatty acids, located originally at the *sn*-2 position of the triacylglycerol molecule, by hypocholesterolemic fatty acids, located originally at the *sn*-1,3 positions.

This study is part of ongoing research (Safari et al., 1993, 1994a,b; Kermasha et al., 1995; Pabai et al., 1995a,b, 1996) aimed at the optimization of interesterification of selected fatty acids in butter fat using organic solvent media. The objective of this work was to purify and characterize the purified enzymatic fractions of lipase from *R. niveus* in terms of their molecular weights and electrophoretic profiles as well as optimum pH and K_m and V_{max} values.

MATERIALS AND METHODS

Partial Purification of Lipase. The partial purification of commercial lipase N (Amano Pharmaceutical Co. Ltd.,

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Table 1. Purification Scheme of Lipase Extract from *R. niveus*

fraction	total protein[a] (mg)	specific activity[b] (units/mg) × 10^3	total activty[c] (units) × 10^{-6}	recov (%)	purifn (fold)
crude extract (FI)[d]	27700	11.7	323.25	100.00	1.00
ammonium sulfate precipitation (FII)[e]					
0−40% (FIIa)	512	6.7	3.43	1.06	0.57
40−60% (FIIb)	1186	27.6	32.73	10.12	2.36
60−100% (FIIc)	1789	11.7	20.93	6.47	1.00
ion-exchange chromatography (FIII)[f]	670	29.9	20.03	6.19	2.55
size-exclusion chromatography (FIV)[g]					
FIVa	427	36.1	15.41	4.77	3.09
FIVb	39	1.5	0.06	0.02	0.13
FIV[c]	55	1.2	0.07	0.02	0.10

[a] Protein concentration was measured according to the method of Hartree (1972), using bovine serum albumin as standard. [b] Specific activity is defined as micromoles of free fatty acids produced per milligram of protein per minute. [c] A unit of activity is defined as the formation of 1 μmol of free fatty acid/min. [d] Commercial crude lipase N (Amano), obtained from *R. niveus*. [e] Partially purified enzyme, fractionated by gradual precipitation with ammonium sulfate. [f] Purified enzymatic fraction obtained by preparative ion-exchange chromatography on a Mono Q 10 column, using the FPLC system. [g] Purified enzymatic fractions obtained by size exclusion chromatography on a Superdex 75 column, using the FPLC system.

Nagoya, Japan), obtained by a unique fermentation process of a selected strain of *R. niveus*, was performed by suspending 100 g of the commercial crude enzyme extract (FI) in 700 mL of sodium phosphate buffer solution (0.1 M, pH 7.0). Fractionation of the enzyme extract was performed by the addition of solid ammonium sulfate at 0−40 (FIIa), 40−60 (FIIb), and 60−100% (FIIc) of saturation, followed by centrifugation (10000*g*, 15 min). The precipitates were resolubilized in the minimum amount of sodium phosphate buffer solution and desalted by ultrafiltration (Minitan ultrafiltration system, Millipore Corp., Bedford, MA), using a YM3 membrane with a 10000 Da molecular weight cutoff (Amicon, Beverly, MA). The desalted protein fractions were then subjected to lyophilization.

Ion-Exchange Liquid Chromatography. The partially purified enzymatic fraction (FIIb), precipitated by ammonium sulfate at 40−60% of saturation, was further purified by preparative ion-exchange chromatography on a Mono Q HR 10/10 column (Pharmacia, LKB Biotechnology, Uppsala, Sweden), using the Fast Protein liquid chromatography (FPLC) system. The column was equilibrated with 2 column volumes of sodium phosphate buffer solution (0.01 M, pH 7.7). A sample volume of 500 μL of enzyme solution (10 mg of protein) was injected. A gradient elution system was used and consisted of eluant (A), sodium phosphate buffer solution (0.01 M, pH 7.7), and eluant (B), the same buffer as in A but containing 2 M NaCl. The elution was performed at a flow rate of 1 mL/min for 35 min with a gradient system starting with 100% buffer A, followed by a gradual increase to 100% buffer B within a period of 23 min, and ending by an isocratic elution using 100% B. The eluted protein fractions were collected (1 mL/tube) and monitored at 280 nm and tested for lipase activity.

Size Exclusion Liquid Chromatography. Fraction FIII, obtained from ion-exchange liquid chromatography, was further purified by size exclusion liquid chromatography on a Superdex 75 HR 10/30 column (Pharmacia), using the FPLC system. The column was equilibrated with 2 column volumes of sodium phosphate buffer solution (0.1 M, pH 7.7). A sample volume of 200 μL of enzyme suspension (10 mg) was injected. The elution was performed using a sodium phosphate buffer solution (0.1 M, pH 7.7) at a flow rate of 0.7 mL/min. The eluted protein fractions were collected (3 mL/tube) and monitored at 280 nm and tested for lipase activity.

Electrophoretic Analyses of the Enzymatic Fractions. The enzymatic fractions FI, FIIb, FIII, and FIVa were subjected to native polyacrylamide gel electrophoresis (PAGE), using minigels (5 × 4 cm) in which the concentrations of total monomers (T) and cross-linkers (C) were equal to 12.5 and 3%, respectively. The electrophoretic separation was performed, by the Phast System electrophoresis unit (Pharmacia), for 40 min at a constant current of 10 mA, using PhastGel native buffer strips (Pharmacia); the buffer strips consisted of 0.88 M L-alanine, 0.25 M Tris, pH 8.8, in 2% agarose isoelectric focusing buffer. After separation of the proteins, the gels were transferred to the development section of the PhastSystem unit and stained with silver nitrate according to the method described by Pharmacia (1992). The molecular mass values of the purified enzymatic fractions (FIII and FIVa) were estimated from a calibration standard curve established from the use of a mixture of molecular weight protein markers (Pharmacia) including α-lactalbumin (M_r = 14.4 kDa), soybean trypsin inhibitor (M_r = 20.1 kDa), carbonic anhydrase (M_r = 30.0 kDa), ovalbumin (M_r = 43.0 kDa), bovine serum albumin (M_r = 67.0 kDa), and phosphorylase *b* (M_r = 94.0 kDa).

The enzymatic fractions FI, FIIb, FIII, and FIVa were also subjected to sodium dodecyl sulfate (SDS)−PAGE (Laemmli, 1970), which was performed in a similar manner as described above for the native PAGE; however, the standards and samples were treated with SDS (2.5%) and mercaptoethanol (5%) at 100 °C (10 min), and PhastGel SDS buffer strips (Pharmacia), consisting of 0.20 M tricine, 0.20 M Tris (pH 8.1), and 0.55% SDS in 3% agarose isoelectric focusing buffer, were used.

Protein Determination. The enzymatic fractions were assayed for protein content according to a modification of the Lowry method (Hartree, 1972). Bovine serum albumin (Sigma Chemical Co., St. Louis, MO) was used as a standard for calibration.

Enzyme Assay. The lipase assay was performed using a butter oil emulsion as substrate according to a modification of the procedure described by Watanabe et al. (1977). The butter fat emulsion was prepared by suspending 0.5 mL of butter oil into 5 mL of 2% Arabic gum in sodium phosphate buffer solution (0.1 M, pH 7.0 for the commercial lipase extract and pH 7.7 for the purified fractions); the mixture was homogenized (Polytron PT 3000, Beckman Canada, Mississauga, ON) for 1 min at 18000 rpm. After the addition of 100 μL of 1.1 M $CaCl_2$, the reaction mixture was preincubated at 37 °C for 1 min. The enzymatic reaction was initiated by the addition of 100 μL of enzyme suspension (7 mg of protein) and incubated for 5 min at 37 °C, using an ultrasonic water bath (Branson Ultrasonic Corp., Danbury, CT). The enzymatic reaction was halted by the addition of 20 mL of a acetone/ethanol mixture (1:1, v/v), and the liberated free fatty acids were titrated with a 0.05 N solution of NaOH. One unit of lipase was defined as the amount of enzyme that liberated 1 μmol of free fatty acid per minute.

RESULTS AND DISCUSSION

Enzyme Purification. Table 1 shows that the partial purification of the commercial crude lipase N (Amano) extract (FI) by ammonium sulfate precipitation

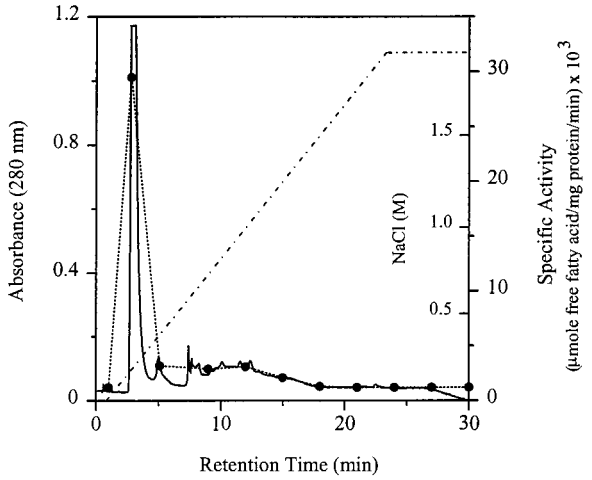

Figure 1. Elution profile of the purified enzymatic extract (FIII) by preparative ion-exchange chromatography on a Mono Q HR 10/10 column, using the FPLC system. The protein sample was eluted by a sodium phosphate buffer solution (0.01 M, pH 7.7) with a gradient of 2 M NaCl at a flow rate of 1 mL/min: absorbance at 280 nm (—), specific activity (●···●), and NaCl gradient (- — -).

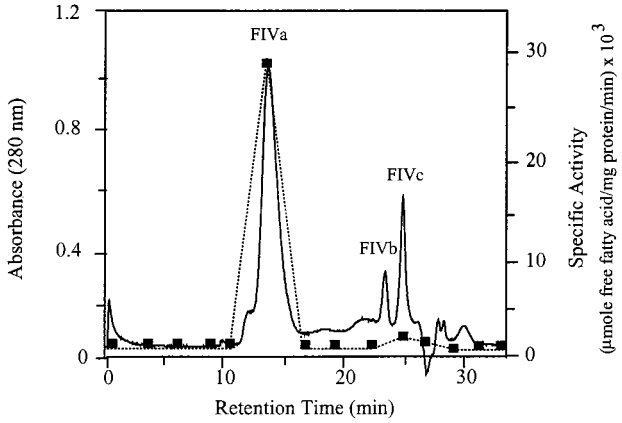

Figure 2. Elution profile of the purified enzymatic fraction (FIV) by preparative size exclusion chromatography on a Superdex 75 column, using the FPLC system. The protein sample was eluted by sodium phosphate buffer solution (0.01 M, pH 7.7) at a flow rate of 0.7 mL/min: absorbance at 280 nm (—) and specific activity (■···■).

at 40−60% of saturation (FIIb) resulted in a 2-fold increase in the specific activity, with a recovery of 10.1%.

The results (Figure 1) also indicate that the purification of FIIb by ion-exchange chromatography resulted in one major active enzymatic fraction (FIII); however, this purification step (Table 1) showed only a slight increase in specific activity from 27.6×10^3 to 29.9×10^3 units/mg. Kohno et al. (1993, 1994) reported that the purification of an exo-lipase, from the culture medium of *R. niveus*, on a DEAE-Toyoperol column followed by a CM-Toyopearl column, resulted in one major enzymatic fraction possessing a specific activity of 4.9×10^3 units/mg, a purification fold of 54.4, and a recovery of 31.1%.

The results (Figure 2) indicate that the purification of FIII by size exclusion chromatography on a Superdex 75 10/30 column resulted in one major (FIVa) and two minor (FIVb and FIVc) protein fractions with relative percentage total protein contents of 82.0, 7.5, and 10.5%, respectively (Table 1). The results (Table 1) also show that the specific activity of FIVa (36.1×10^3 units/mg) was much higher than those for FIVb and FIVc (1.5×10^3 and 1.2×10^3 units/mg, respectively). In addition, the results (Table 1) demonstrate that the purified enzymatic fraction FIVa showed the highest recovery of 4.77%. These findings (Table 1) are in agreement with those reported by Aisaka and Terada (1980), who indicated that the partial purification of lipase from *Rhizopus javanicus* on Sephadex G-100 resulted in three protein fractions exhibiting elution profiles similar to that obtained in our laboratory for the purified lipase fractions of *R. niveus* (Figure 2; Table 1). However, Wisdom et al. (1987) reported that the purification of lipases from *Rhizopus arrhizus* and *Aspergillus* sp. on Sephadex G-100 resulted in one enzymatic fraction possessing a specific activity of 7×10^3 units/mg of protein, a 14-fold increase in purification and an overall yield of 20%. The discrepancies between our findings and those reported in the literature could be related to the differences in microbial sources (Iwai and Tsujisaka, 1984).

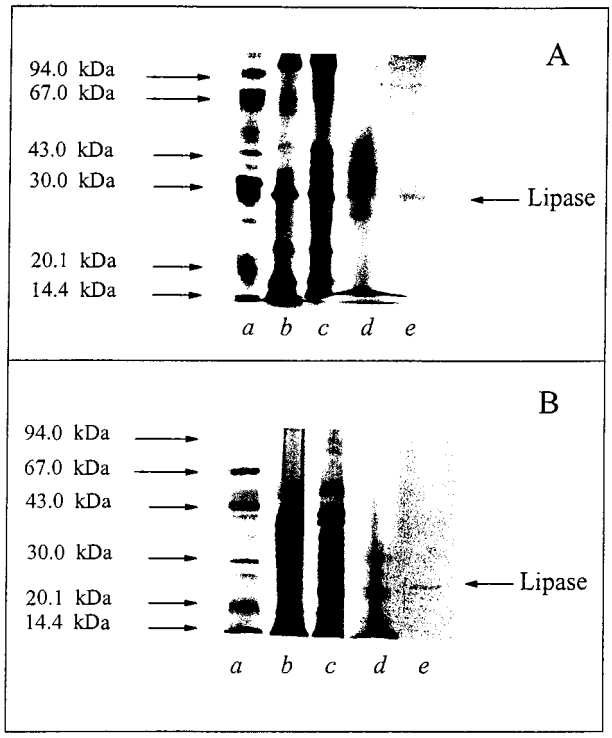

Figure 3. Electrophoregrams of (A) native PAGE and (B) SDS−PAGE: (lanes *a*) mixture of protein standards, including α-lactalbumin (M_r = 14.4 kDa), soybean trypsin inhibitor (M_r = 20.1 kDa), carbonic anhydrase (M_r = 30.0 kDa), ovalbumin (M_r = 43.0 kDa), bovine serum albumin (M_r = 67.0 kDa), and phosphorylase *b* (M_r = 94.0 kDa); (lanes *b*) commercial crude lipase extract FI; (lanes *c*) partially purified lipase fraction FIIb; (lanes *d*) purified lipase fraction FIII obtained by ion-exchange chromatography; (lanes *e*) purified lipase fraction FIVa obtained by size exclusion chromatography.

Electrophoretic Analyses. The native PAGE (Figure 3A) electrophoregram shows the presence of six major protein bands in the commercial crude lipase extract (FI). The results (Figure 3A) also show that the relative concentrations of protein in the range of 20−40 kDa are higher for fraction FIIb compared to that of the commercial crude lipase extract FI. These results demonstrate the efficiency of the ammonium sulfate precipitation step in the partial purification of the commercial lipase.

The native PAGE (Figure 3A) electrophoregram also demonstrates that the purified protein fraction obtained

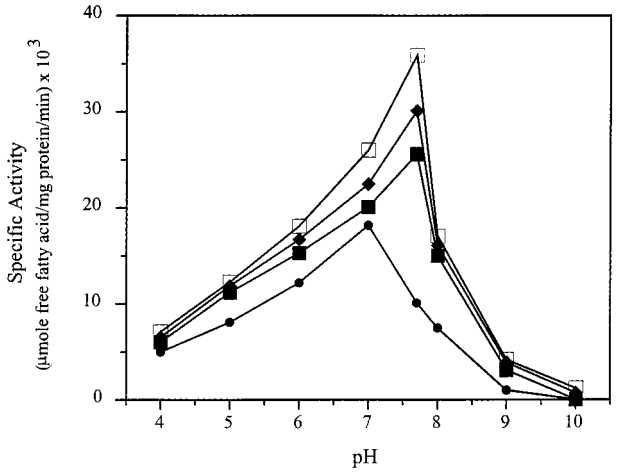

Figure 4. Effect of pH on the lipase activity: commercial crude lipase N extract FI (●); partially purified lipase fraction FIIb (■); purified lipase fraction FIII obtained by ion-exchange chromatography (◆); purified lipase fraction FIVa obtained by size exclusion chromatography (□).

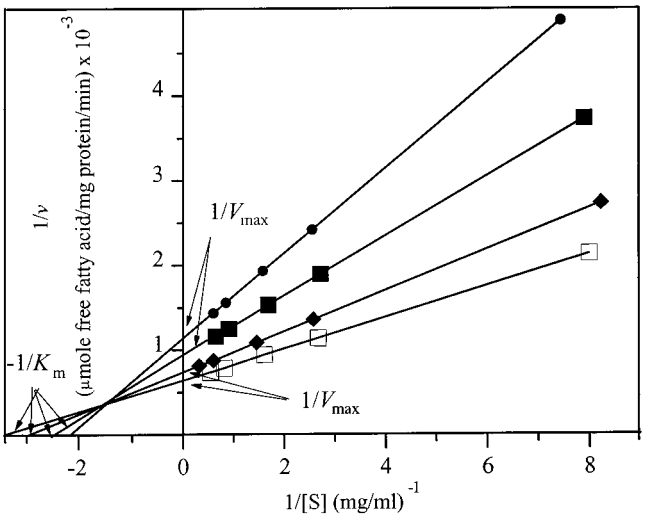

Figure 5. Lineweaver−Burk plots (1/*v* versus 1/[S]) of lipase activity: commercial crude lipase N extract FI (●); partially purified lipase fraction FIIb (■); purified lipase fraction FIII obtained by ion-exchange chromatography (◆); purified lipase fraction FIVa obtained by size exclusion chromatography (□).

by ion-exchange chromatography (FIII) showed the presence of three protein bands. The results (Figure 3) clearly confirm the homogeneity of the final purified enzymatic fraction (FIVa), obtained by size exclusion chromatography, as indicated by the presence of one protein band on both native and SDS−PAGE electropherograms. The overall results (Figure 3) also demonstrate the efficiency of the purification procedure developed in our laboratory, which allowed the purification of the commercial microbial lipase N (Amano) to homogeneity. Vasak (1991) reported that electrophoretic analysis could be used as a tool for the determination of the degree of purity of a given protein. Native PAGE analyses are generally used as an indication for the presence of oligomeric protein molecules, whereas those of SDS−PAGE are used as an indication of the homogeneity of monomeric protein molecules (Garfin, 1990); these results therefore suggest that the purified enzymatic fraction (FIVa) is a monomeric protein molecule.

Regarding the determination of the molecular weight of the purified enzymatic fraction (FIVa), the results indicated that in both native PAGE and SDS−PAGE analyses, the purified lipase fractions FIII and FIVa demonstrated the same relative molecular mass (26 ± 3 kDa), which was lower than that (34 kDa) reported for the purified lipase, also obtained from *R. niveus* by Kugimia et al. (1992). In addition, Kohno et al. (1992) reported that a native lipase from *R. niveus* had a molecular mass of 41 kDa. The differences in these findings could be due to the effect of pH and buffer concentration on the mobility of protein fractions (Laue and Rhodes, 1990) in the electrophoresis gels or to the pretreatment and purity of the enzyme preparations (Khoo and Steinberg, 1981).

Effect of pH on Lipase Activity. The results (Figure 4) demonstrate that the optimum pH for the commercial crude lipase extract (FI) was 7.0, whereas that for the partially purified fraction (FIIb) was 7.7. In addition, the purified enzymatic fractions (FIII) and (FIVa) demonstrated similar pH optima as that obtained for the partially purified enzyme (FIIb). The literature indicated a wide range of optimum pH values ranging from 5.6 for the lipase activity of *A. niger* (Fukumoto et al., 1962) to 8.0 for that of *Humicola lunuginosa* (Liu et al., 1973). Kohno et al. (1994) reported that the lipase from *R. niveus* showed optimal activity at pH 6.0. Moreover, lipases from *Penicillium crustusum* (Oi et al., 1967) and from *Mucor* (Nagaoka and Yamada, 1973) showed maximal activity at pH 9.0. However, Iwai et al. (1975) reported that lipases A and B from *Penicillium cyclopium* differed in terms of molecular weight, optimum pH, and pH stability. These slight variations in pH values may be due to the effects of factors such as incubation time, temperature, nature and concentration of buffer, ionic strength of the medium, and purity of the enzyme preparations (Whitaker, 1972).

Kinetic Studies. The kinetic studies of the enzyme fractions, obtained from Lineweaver−Burk plots (Lineweaver and Burk, 1934) of 1/*v* versus 1/[S], demonstrate (Figure 5) that the commercial crude lipase extract (FI), the partially purified enzyme (FIIb), the ion-exchange chromatography purified fraction (FIII), and the size exclusion chromatography purified faction (FIVa) showed K_m values of 0.46, 0.40, 0.34, and 0.29 mg of butter fat/mL, respectively. These results suggest that the binding affinity between the substrate and the enzyme increased steadily with the purification process (Fletcher et al., 1985).

The results (Figure 5) also show that the V_{max} values for FI, FIIb, FIII, and FIVa were 0.83×10^3, 0.98×10^3, 1.30×10^3, and 1.61×10^3 μmol (mg of protein)$^{-1}$ min^{-1}, respectively. These findings indicate that the major effect of the purification process was a large increase in the substrate turnover number (V_{max}) value for the purified enzymatic fraction FIVa, thereby indicating that more products were formed during the interesterification reaction.

Lee and Lee (1989) reported that the K_m and V_{max} values for the purified lipase of *Lactobacillus casei* subsp. *pseudoplantrum* LE2 were 1.20 mM and 90 μmol (mg of protein)$^{-1}$ min^{-1}, respectively; these authors (Lee and Lee, 1990) also reported that the K_m and V_{max} values for the purified lipase of *Lactobacillus casei* subsp. *casei* LLG were 0.57 mM and 76 μmol (mg of protein)$^{-1}$ min^{-1}, respectively.

Conclusion. The results gathered in this study indicated that the partial purification and the successive ion-exchange and size exclusion chromatographies of the

commercial crude lipase extract resulted in a well-purified monomeric enzyme fraction. The purification process also resulted in enzymatic fractions possessing a higher affinity for the substrate, as indicated by the lower K_m values. In addition, the purified lipase fractions demonstrated higher activity as shown by the increase in their V_{max} values. These findings could be used as a prelude for the development of biotechnological applications for the use of the purified lipase in the inter- and transesterification of fatty acids in lipids and fats.

ABBREVIATIONS USED

FPLC, Fast Protein liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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