Composition and Activity of Commercial Triacylglycerol Acylhydrolase Preparations

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ABSTRACT: Commercial lipase preparations were surveyed to determine gross composition, amounts of nonprotein impurities, and esterolytic and lipolytic activities. Most of the 34 commercial lipase preparations contained more than 80% nonproteinaceous material, with salt and carbohydrate being the most abundant materials. The tributyrin hydrolase activity of these commercial lipase preparations was determined and expressed as lipase/esterase forestomach units (LFU). Tributyrin hydrolase activity ranged from negligible (5.3 LFU/g) to very high (>1,000,000 LFU/g). *Aspergillus* and *Penicillium* preparations were low in tributyrin hydrolase activity. *Candida rugosa* preparations were intermediate in activity. Preparations of porcine pancreas, *Rhizomucor, Pseudomonas,* and *Rhizopus* lipases exhibited a broad range of levels of activity. No relation between protein content and tributyrin hydrolase activity was observed. Isoelectric focusing of the proteins present in the preparations demonstrated the presence of between 2 and 27 isophoretically discrete bands in the isoelectric range of 3 to 9. Although there were many similarities of distribution of protein isoelectric points within genera and species, the preparations generally displayed unique patterns of isophoretically discrete protein bands. Lipase zymography demonstrated the presence of 0 to 7 isophoretically discrete lipase activities in each preparation, spanning the entire range of isoelectric points surveyed.

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KEY WORDS: Commercial lipase preparations, esterases, isoelectric focusing, lipases, lipase screening, pI, side activities, triacylglycerol hydrolase, tributyrin hydrolysis activity, zymography.

The application of triacylglycerol acylhydrolases (lipases, EC 3.1.1.3) has become a mature field of research (1,2). Lipases are routinely employed to produce high-purity structured fats with a wide variety of nutritional and functional properties (3–5). Although a broad spectrum of microbial cultures is available for fermentation and harvest of lipolytic activities, commercial lipase preparations (CLP) have been employed in many investigations. They are convenient, readily available, allow work to be verified and extended by other investigators, and simplify calculations of process economics for potential full-scale commercial development.

CLP are often complex mixtures of proteins, fats, carbohydrates, cell debris, nucleic acids and salts remaining from fermentation of the host organism and downstream processing of the enzyme-containing product stream (6). Complete purification of the desired lipolytic activity is costly, and loss of activity and stability may result (7). In spite of the vast amount of research carried out on CLP, the composition of impurities and nonlipase activities (side activities) in CLP are largely unknown. Both positive and negative effects may be exerted by impurities or side activities in lipase-catalyzed reactions. Proteinaceous material can cause up to an eightfold activity enhancement in lipases from milk spoilage organisms (8). Stabilization and activation by proteinaceous material occurs in ester synthesis and transesterification reactions catalyzed by immobilized lipases (9). On the other hand, nonproteinaceous material can interfere with the control of water activity or present mass-transfer barriers in microaqueous reactions. The activity of CLP can be sharply diminished by the presence of even small amounts of glucose (10). Salts can exert strong effects on the pH, ionic strength, and water activity of a microaqueous environment, affecting lipolytic activity. In the production of natural flavors, the presence of large amounts of salt may render a flavor mixture created by lipase or esterase action too salty.

In addition to nonproteinaceous material, CLP may contain more than one form of lipase, possibly with different selectivities. The presence of multiple lipase activities with different selectivities has been shown to exert strong effects on reaction products (6). Although it is generally known that the majority of fungal lipases, especially from the *Rhizomucor* and *Rhizopus* genera, contain more than one lipolytic activity, the presence of multiple forms of lipolytic activity in most CLP is poorly understood (11). CLP from *C. rugosa* have been demonstrated to contain at least two lipase/esterase isoenzymes; the organism is known to contain seven different lipase-encoding genes (6,7).

In order to employ CLP in modifications of complex substrates, such as flavor development in cheese, knowledge of the presence and quantities of nonlipase materials in CLP is needed. Tributyrin hydrolysis activity is a vital determinant of the cost effectiveness of lipases used in dairy flavor production, even though tributyrin is not a true lipase substrate. Knowledge of the presence and isoelectric point (pI) of both nonlipase proteins and proteins with lipolytic activity can be useful to understand the effects of pH on flavor production. In addition, since any nonlipase protein present could express some other kind of catalytic activity, knowledge of the presence of nonlipase proteins is useful to explain modification of

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substrates not acted on by lipase when incubated together with CLP. At the very least, such comparisons can indicate the degree of batch-to-batch variation in a CLP and allow the identification of CLP that are substantially similar in content.

MATERIALS AND METHODS

CLP were gifts from their respective vendors: Amano Enzyme U.S.A. Co., Ltd. (Lombard, IL); Bio-Cat, Inc. (Troy, VA); Biocatalysts, Ltd. (Pontypridd, United Kingdom; preparations were supplied by Kaltron Inc., Bensonville, IL); other were from Danisco Ingredients (New Century, KS); DSM/Gist-Brocades (Menomonee Falls, WI); Enzyme Development Corp. (New York, NY); Lallemand Inc. (Montréal, Canada); Novo Nordisk (Franklinton, NC); Quest International (Hoffman Estates, IL); Rhodia, Inc. (Madison, WI); and Valley Research, Inc. (South Bend, IN). Preparation names and vendors of CLP are listed and classified according to their biological sources in Table 1. Although the former genus denotation *Mucor* has been replaced with *Rhizomucor* in fungal taxonomy, several enzyme manufacturers have maintained the use of the former genus name for simplicity. In this report, the naming convention currently employed by each manufacturer is observed (Table 1).

Kjeldahl nitrogen, moisture, fat, salt, and ash contents of CLP were determined by standard AOAC methods (12) conducted by an ISO 9002 certified analytical laboratory. Protein contents were calculated from Kjeldahl nitrogen values using standard conversion factors (13). Tributyrin hydrolase activity was determined according to the Food Chemicals Codex

TABLE 1
Biological Sources, Commercial Suppliers, Trade Names, and Abbreviations of Commercial Lipase Preparations Used in This Study

Genus	Species	Supplier	Preparation name	Enzyme no.
Sus	*scrofa*	Biocatalysts Ltd.	Lipase Porcine Pancreatin	PP1
	scrofa	Valley Research, Inc.	Pancreatic Lipase 250	PP2
	scrofa	Biocatalysts Ltd.	Lipomod 224P	PP3
	scrofa	Biocatalysts Ltd.	Lipomod 299P	PP4
Aspergillus	*niger*	Amano Enzyme U.S.A. Co.	Amano Lipase A	AN5
	niger	Enzyme Development Corp.	Microbial Lipase 50	AN6
	niger	Valley Research, Inc.	Lipase 8000	AN7
	oryzae	Danisco Ingredients	Grindamyl 16 (concentrate)	AO8
	oryzae	Danisco Ingredients	Grindamyl 16 (dilute)	AO9
Candida	*rugosa*	Amano Enzyme U.S.A. Co.	Amano Lipase AY	CR10
	rugosa	Bio-Cat, Inc.	Cylindracea	CR11
	rugosa	Biocatalysts Ltd.	Lipomod 34P	CR12
Mucor	*javanicus*	Amano Enzyme U.S.A. Co.	Amano Lipase M	MJ13
	javanicus	DSM/Gist-Brocades	Piccantase M10	MJ14
(Rhizomucor)	*miehei*	Novo Nordisk	Palatase M 1000	MM15
	miehei	Enzyme Development Corp.	Esterase Lipase	MM16
	miehei	DSM/Gist-Brocades	Piccantase A (concentrate)	MM17
	miehei	Rhodia, Inc.	Marlase 20X	MM18
	miehei	Novo Nordisk	Palatase 20,000	MM19
	miehei	Biocatalysts Ltd.	Lipomod 187P	MM20
Penicillium	*camembertii*	Amano Enzyme U.S.A. Co.	Amano Lipase G	PC21
	roqueforti	Biocatalysts Ltd.	Lipomod 338P	PR22
	roqueforti	Amano Enzyme U.S.A. Co.	Lipase R Amano G	PR23
Pseudomonas	*cepacia*	Amano Enzyme U.S.A. Co.	Lipoprotein lipase 200S	PsC24
	cepacia	Amano Enzyme U.S.A. Co.	Lipase PS	PsC25
	fluorescens	Amano Enzyme U.S.A. Co.	Lipase AK Amano 20	PF26
Rhizopus	*niveus*	Amano Enzyme U.S.A. Co.	Newlase F	RN27
	oryzae	Amano Enzyme U.S.A. Co.	Amano Lipase F-AP	RO28
	oryzae	Bio-Cat, Inc.	Javanicus	RO29
	oryzae	Bio-Cat, Inc.	Javanicus	RO30
	oryzae	Quest International	Biolipase P	RO31
	oryzae	DSM/Gist-Brocades	Fermizyme L80000	RO32
	oryzae	Lallemand Inc.	Lipase S80000	RO33
	oryzae	Enzyme Development Corp.	Lipase ROL	RO34

[a]Some manufacturers have retained the use of the label *Mucor* for the genus now classified as *Rhizomucor,* and their designation is used here. Preparations from genetically modified organisms have retained the genus and species designation of the organism from which the lipase genes were derived.

(FCC) lipase/esterase (forestomach) assay and quantified in lipase/esterase forestomach units (LFU/g) (14). One LFU is the activity that releases butyric acid at the rate of 1.25 μmol/min at pH 6.2 and 42°C under the conditions of the assay.

Isoelectric focusing (IEF) of CLP was carried out on a PhastSystem (Pharmacia LKB, Piscataway, NJ) according to the manufacturer's recommendations (Separation Technique File No. 100). Solutions of CLP were applied to IEF 3–9 Phastgels (Amersham Pharmacia Biotech, Piscataway, NJ) using an eight-lane sample application comb with a 0.5 μL application load. To prevent streaking of gels and wavy protein bands, CLP with a salt concentration greater than 5% were desalted using Micron centrifugal filters with a molecular weight cutoff of 10,000 (Amicon, Inc., Beverly, MA). A series of three centrifugation (14,000 × *g*, 30 min) and reconstitution steps was carried out at room temperature. CLP high in carbohydrate diluents were purified by dialysis using a Slide-A-Lyzer 10K dialysis cassette (Pierce, Rockford, IL) followed by concentration using Micron centrifugal filters in the same manner as for CLP high in salt. CLP solutions containing insoluble solids were centrifuged at 14,000 × *g* for 5 min at room temperature prior to IEF of the supernatant.

IEF was carried out on two gels simultaneously, each with identical applications of CLP; one gel was stained to detect protein bands, and the other gel was used for detection of lipolytic activity by zymography. IEF and zymography were carried out in duplicate, after testing some preparations up to eight times to ensure reproducibility. Protein bands were detected by silver stain using a PhastGel Silver Kit according to the manufacturer's instructions (Amersham Pharmacia Biotech). The isoelectric points of the protein bands of electrofocused CLP were estimated by comparison of their mobility to those of standard proteins (Broad pI Calibration Kit, Amersham Pharmacia Biotech). Assignment of pI to bands was facilitated by densitometry of digital images of gels. Gels were illuminated from beneath on a light table and photographed with a digital camera (Sony DKC-5000; Meyer Instruments, Houston, TX) equipped with a 25-mm F/2.8 lens (Century Precision Optics, North Hollywood, CA). Digital images were scanned using Quantiscan software version 1.5 (Biosoft, Ferguson, MO) to produce images similar to chromatograms, with a peak corresponding to each gel band. Isoelectric points were determined by comparing the unique abscissal coordinate of each band with the abscissal coordinate of reference proteins run on both ends of the same gel.

Lipolytic activity on isoelectrically focused gels was detected by zymography. The second gel of a gel pair was incubated on a solid lipase detection medium containing anhydrous butteroil (Land O'Lakes, St. Paul, MN) and rhodamine B (Sigma Chemical Co., St. Louis, MO) in agar. The pH of the gel was 6.7, which corresponds to the pH typical of bovine milk prior to acid development in cheesemaking. The gel was incubated at room temperature for 5–60 min and the lipase detection medium was examined under ultraviolet (UV) light for pink bands (15). The pI of activity bands were assigned by comparison of their mobility to the proteins in the corresponding silver-stained gel.

RESULTS AND DISCUSSION

Composition of CLP. The composition of most CLP was dominated by nonprotein material, which ranged in content from 48% to greater than 99% (Table 2). The predominant nonprotein materials in CLP were carbohydrates, sodium chloride, and noncombustible material that reduced to ash in our analyses. The activity of CLP is often standardized with inert diluents, which allows the manufacturer to guarantee a given activity from fermentation products that typically vary from batch to batch. The two most common diluents for powdered commercial enzymes reported by enzyme manufacturers are maltodextrin and sodium chloride. Ammonium sulfate, diatomaceous earth, lactose, nonfat milk powder, sodium caseinate, sodium benzoate, and starch are also used. Liquid enzymes are usually standardized with glycerol, sorbitol, and water.

High carbohydrate contents (greater than 50 wt%) were found in 22 CLP (PP2, PP3, PP4, AN7, AO9, CR10, CR11, CR12, MJ13, MJ14, MM20, PC21, PR22, PR23, PF26, RN27, RO29, RO30, RO31, RO32, RO33, and RO34) (Table 2). These high levels may be detrimental to lipolytic activity in nonaqueous systems, guiding the choice of enzymes for such applications (10,16). The sodium chloride content of 5 CLP (AN6, MM16, MM17, PF26, and RO33) was higher than would be expected from a typical fermentation broth. High sodium chloride content can account for high contents of noncombustible material, but in 9 CLP (AN5, CR12, MJ13, MJ14, MM17, MM20, PR23, PsC25, and RN27) the amount of noncombustible material exceeded the salt content by a broad margin. The use of other salts in precipitation steps or the addition of solid diluents such as diatomaceous earth could account for this phenomenon. Salts can exert strong effects on the pH and ionic strength of a microaqueous environment, affecting lipolytic activity. They can also interfere with efforts to control water activity with salt hydrates or saturated salt solutions (17). Moisture contents were generally low in powdered CLP (below 5 wt%). Maintaining low moisture content in CLP during storage is vital to retention of lipolytic activity. Fat contents of microbial CLP were generally very low (below 0.3 wt%). Slightly higher fat content were present in some CLP from porcine pancreas (PP1–PP3), which are prepared by removing fat from pancreas tissue with cold acetone.

Most preparations of the same genus and species from different manufacturers were of dissimilar protein content (Table 2). Porcine pancreas CLP spanned the broadest range of protein contents of all genera in this study (2.1–51.8 wt%), but were generally high in protein content. All but one *Aspergillus* sp. CLP was low in protein. *Aspergillus niger* CLP AN5 and AN6 were much higher in protein than the CLP from *A. oryzae*. The protein content of *Candida rugosa* CLP ranged from 2.1 to 17.6 wt%. The *Rhizomucor* CLP were dissimilar along species lines. The two *R. javanicus* preparations

TABLE 2
Composition and Activity of Commercial Lipase Preparations

Enzyme no.	Carbohydrate (wt%)	NaCl (wt%)	Ash (wt%)	Moisture (wt%)	Fat (wt%)	Protein (wt%)	Activity[b] (LFU/g CLP)
PP1	35.5	0.6	3.8	7.0	1.9	51.8	7,617
PP2	63.6	0.5	1.9	4.8	1.1	24.0	3,938
PP3	55.7	0.9	3.1	4.8	0.9	29.5	2,783
PP4	94.4	0.4	0.2	3.3	0.1	2.1	253
AN5	68.0	0.1	6.0	5.1	0.1	20.8	17
AN6	19.7	65.1	69.2	2.4	0.2	8.5	7.9
AN7	92.6	0.3	0.5	5.1	0.1	1.7	1,067
AO8	1.7	0.8	0.6	96.5	ND	1.3	37
AO9	86.5	0.3	0.4	12.1	ND	1.0	5.3
CR10	91.9	ND	1.1	4.3	0.1	2.7	4,000
CR11	91.5	ND	1.3	4.9	0.2	2.1	3,429
CR12	57.6	0.4	17.6	6.9	0.3	17.6	2,586
MJ13	62.5	0.5	7.9	3.4	0.2	20.0	2,560
MJ14	71.4	0.2	7.7	2.8	ND	18.1	2,909
MM15	22.3	0.1	0.2	76.0	0.0	1.4	656
MM16	0.5	98.1	99.3	ND	0.1	0.1	163
MM17	23.7	22.7	41.1	9.5	2.4	23.4	20,782
MM18	48.9	0.2	0.2	49.8	ND	0.7	8,533
MM19	48.4	0.1	0.2	49.3	0.1	0.6	6,120
MM20	79.0	0.1	5.8	5.6	0.2	9.4	517
PC21	86.5	0.1	0.7	5.8	0.1	6.9	103
PR22	83.5	0.3	3.4	5.6	0.2	6.1	213
PR23	72.2	0.1	6.1	7.2	0.1	14.5	1,326
PsC24	NT	NT	NT	NT	NT	50.0	1,047,273
PsC25	11.4	0.3	81.7	1.5	0.2	4.9	107
PF26	53.0	28.9	29.7	3.9	0.6	13.0	16,457
RN27	62.2	0.3	7.5	4.9	0.2	19.6	853
RO28	43.7	1.4	4.3	4.7	0.3	35.7	22,588
RO29	52.6	0.2	4.6	4.2	0.2	38.5	16,537
RO30	54.1	ND	3.5	5.2	ND	37.1	32,000
RO31	90.9	0.1	ND	8.6	0.3	0.2	727
RO32	65.1	13.5	15.8	4.8	0.4	14.0	20,000
RO33	54.5	20.6	25.1	4.1	0.6	15.7	10,667
RO34	91.7	0.4	0.2	3.5	0.1	4.6	5,486

[a]ND, Not detected, below detection limit of assay; NT, not tested due to insufficient sample amount.
[b]Compositions were determined by standard AOAC methods (Ref. 12). Activity is expressed as lipase/esterase forestomach units per gram (LFU/g). One unit is the amount of activity which releases 1.25 μmol/min of butyric acid from tributyrin at 42°C and pH 6.2 under the conditions of the assay (Ref. 14).

were almost identical in protein content. Several CLP from *R. miehei* (MM15, MM16, MM18, and MM19) were very low in protein content. *Rhizomucor miehei* lipases were originally contaminants of microbial rennet production for cheesemaking and were removed from the fermentation broth by acid precipitation. Because they were easily available to the dairy industry to accelerate flavor development in cheesemaking, these lipases were historically standardized by the manufacturer to correspond in activity to animal pregastric esterases, requiring significant dilution and low protein content (18,19). CLP from *Penicillium* (PC21, PR22, and PR22) and *Pseudomonas* (PsC25 and PF26) species were low in protein (below 20 wt%) except for the one purified CLP in this study (PsC24). Among the CLP from *Rhizopus* species, a broad range (0.2–38.5 wt%) of protein contents was observed. Several *Rhizopus* species CLP (RN27, RO28, RO29, and RO30) were in the high range of protein content in this study, consistent with their production by surface fermentation. CLP produced by surface fermentation often include proteins from the matrix on which the fungus is grown. Two CLP, RO28 and RO29, were from two different batch fermentations of the same culture; the protein content of these CLP was similar between surface fermentation batches.

Tributyrin hydrolase activities of CLP. Since lipolytic activities can be determined in many ways, investigators must take pains to select a method appropriate for their needs

(20,21). The hydrolytic activity of most triacylglycerol acylhydrolases is dependent on the choice of substrate (22). In our work, the activity of CLP was determined by automatic titration of tributyrin hydrolysis according to the FCC lipase/esterase forestomach assay. This method for quantification of activity of CLP was chosen because of the importance of tributyrin hydrolase activity in dairy flavor development. The solubility of tributyrin in 0.15 M NaCl is about 0.4 mM, rendering it available as a soluble substrate for esterases; the assay mixture also contained an excess of insoluble tributyrin (23). However, tributyrin is poorly suited for determination of true, interface-dependent, lipolytic activity, so we report tributyrin hydrolase activity only.

Tributyrin hydrolase activity was calculated by weight of CLP (Table 2). The lowest tributyrin hydrolase activity, 5.3 LFU/g, was found in AO9. The highest activity, >1,000,000 LFU/g, was found in PsC24, which is a purified CLP. Porcine pancreas CLP activity levels fell in the low range. Their physiological role is to aid in digestion of triglycerides containing long-chain fatty acids; low activity against tributyrin is not surprising. The tributyrin hydrolase activities of *Aspergillus* sp. CLP fell into the low range, which may be a consequence of their preference for long-chain fatty acids. The tributyrin hydrolase activities of the two *A. niger* CLP were among the lowest detected. *Candida rugosa* lipases were very similar in their activity levels. The *Rhizomucor* CLP were dissimilar along species lines. *Rhizomucor javanicus* preparations were almost identical in activity. The activity of *R. miehei* CLP covered a very broad range; the CLP highest in activity (MM17) is marketed as a concentrate. As their species names (*camembertii* and *roqueforti)* indicate, *Penicillium* species enzymes are important in cheese ripening. However, all three *Penicillium* CLP were low in activity against tributyrin. The manufacturer reports that PC21 (*P. camembertii*) CLP has a strong preference for mono- and diglycerides, exhibiting almost no activity against triglycerides. The purified *Pseudomonas* CLP (PsC24) was very high in activity, but PsC25, another *P. cepacia* preparation from the same manufacturer, was very low in tributyrin hydrolase activity. CLP PsF26, from *P. fluorescens,* was among the most active CLP tested. *Rhizopus niveus* CLP was relatively high in protein but low in activity, possibly due to its selectivity toward medium- and long-chain fatty acids. Only among the *Rhizopus oryzae* CLP did tributyrin hydrolase activity roughly correspond to the protein content. RO28 and RO29 were from two different surface fermentation batches of the same culture. Although the protein content of these CLP was similar between batches, the tributyrin hydrolase activities were not.

Isoelectric profiles of proteins and lipolytic activities in CLP. Most CLP are the products of submerged or surface fermentation of bacteria or fungi, including yeasts. These fermentations are directed toward synthesis of a target enzyme by the enzyme manufacturers. Growth of the fermenting organism necessitates the expression of a broad range of nonlipase proteins, which can be visualized on electrophoresis gels. The presence of as many as 50 proteins was demonstrated in a previous survey of 60 CLP (22). Most CLP contained 10 to 50 bands; only two contained a single protein band. IEF and lipase zymography were carried out on some of the CLP; most of those in which activity was detected showed two to four active bands. Zymography has been applied to detect a wide variety of enzyme activities (24,25). Tributyrin and butteroil have been successfully employed in zymography to detect lipase activities (26). The addition of lipases to milk used for cheesemaking is widely practiced in the cheesemaking industry to increase and accelerate flavor development in cheese. The pH of our zymography medium was 6.7, which is the pH of normal milk, to elucidate lipolytic activities that could be operating in CLP added to cheesemilk.

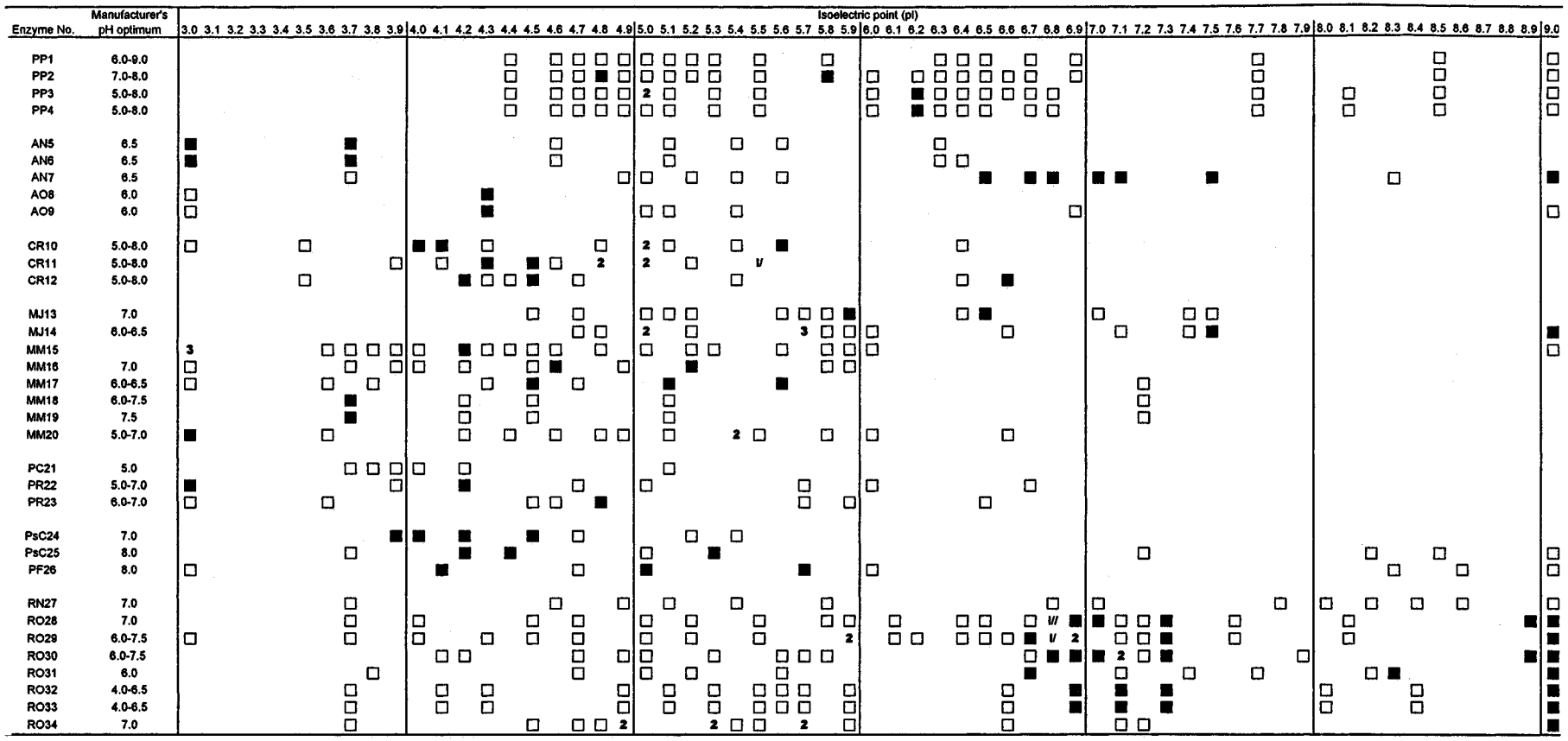
Although lipolytic enzymes with different primary structure are expected to have different apparent pI, the presence of multiple lipolytic activities with discrete pI or molecular weights can result from many biochemical events. Glycosylation, the presence of lipopolysaccharides, and partial proteolysis of lipase molecules with identical primary sequences also affect enzyme molecular weight and can alter the pI of enzyme activities (6,27). Peptides and phospholipids can bind to lipase molecules, generating differences in pI and selectivity (11). Lipase secretion and folding is sometimes mediated by molecular chaperones, which can remain bound to the active enzyme in these CLP (27). More significant than these observable dissimilarities is the effects that could arise in reaction mixtures using these preparations. While the source of heterogeneity of the pI in each CLP is not yet understood, it has been demonstrated that differences in pI can correspond to differences in lipolytic activity against a broad range of substrates (11).

Each porcine pancreas CLP contained about 20 isophoretically discrete protein bands (Table 3) with apparent pI spanning the range from 4.4 to ≥9.0. Porcine pancreas CLP are often simply solvent-defatted pancreas tissue. Thus, all the proteins present in the pancreas are incorporated into the lipase powder. The pI of protein bands in different porcine pancreatic CLP were very similar in spite of the broad range of protein content, but the pI of the lipolytic activities were not the same. No activity was detected in PP1. PP2 contained two active bands in the acid region (pH 4.8 and 5.8). Two pancreatic CLP (PP3 and PP4) contained a single active band at pH 6.2, about in the middle of the manufacturer's recommended pH optimum range. In our study, the lipolytic activity detected by Desnuelle, with pI 4.25, was not found (28).

Aspergillus niger CLP (AN5 and AN6) contained only six or seven isoelectrically discrete protein bands that were almost identical in distribution of pI. Both contained only proteins with pI in the acid region with two lipolytically active bands in the extreme acid end of the detectable region, at ≤3.0 and 3.7. This similarity was not observed in *A. oryzae* CLP. AN7 contained proteins with 12 discrete pI spanning a broad range of pI. Seven of these proteins exhibited lipolytic activity, five being tightly grouped in the pI range of 6.5 to 7.1. One other lipolytic activity focused at pI 7.5 and the remain-

TABLE 3

Apparent Isoelectric Points of Protein Bands and Lipolytic Activity Present in Commercial Lipase Preparations, and Manufacturers Recommended pH Optima for Lipolytic Activity[a]



[a]□, 1 inactive protein at pI; ■, 1 active protein at pI; **2**, 2 inactive proteins at pI; **3**, 3 inactive proteins at pI; **1/**, 1 active 1 inactive protein at pI; **1//**, 1 active 2 inactive proteins at pI. All protein bands detected by protein silver stain are indicated, as are those exhibiting lipolytic activity at pH 6.7.

ing active band migrated to the alkali end of the gel (pI ≥ 9.0). AO8 is a lipase concentrate from which AO9 is prepared for commercial sale. The concentrate (AO8) contained only two bands, both in the acidic region, at ≤3.0 and 4.3, indicating that an acid precipitation purification step may have been executed. According to the manufacturer, the dilute form (AO9) was standardized with a protein-rich material without enzymatic activity to allow for more consistent dosage and distribution in the reaction matrix.

The CLP of *C. rugosa* (CR10, CR11, and CR12) contained 9 to 12 protein bands, all with apparent pI values less than 6.6; the pI of most of the proteins clustered in the 4.0 to 5.6 region. Each of the *Candida* CLP contained three discrete bands of lipolytic activity. Each preparation had one active band within the pH optimum range (5.0 to 8.0) and two active bands at pI values from 4.0 to 4.5. CLP of *C. rugosa* have been used in a broad range of asymmetric catalysis reactions, and several discrete lipases with differing substrate selectivities have been characterized (7). Many of these reactions have been carried out with a lipase preparation from Sigma Chemical Co., which contains a lipolytic activity at pI 4.2 (29).

The CLP from the genus *Rhizomucor* contained proteins with pI spanning the entire gel (≤3.0 to ≥9.0). The *javanicus* species CLP (MJ13 and MJ14) were similar in distribution of protein pI. Extremely acidic proteins were lacking and the pI range spanned 4.5 to ≥9.0, with most of the proteins clumped in the pI range 4.5 to 6.0. Each *Rhizomucor javanicus* CLP contained two active bands. The pI values of lipase activities in MJ13 were at 5.9 and 6.5; CLP MJ14 had two active bands with pI at 7.5 and ≥9.0. Remarkably, the stated pH optimum for the former lipase is higher than the pI of the active bands, while the pH optimum of the latter lipase is lower than the pI of the active bands.

Rhizomucor miehei CLP (MM15–MM20) were originally an unwanted by-product of the production of fungal protease preparations, and the lipase was an impurity removed from the fermentation broth by acid precipitation. Not surprisingly, the pI of most of the proteins of *Rhizomucor miehei* CLP in this study fell in the acid region. One exception was MM15 (Palatase M 1000 from Novo Nordisk), which was a special preparation of the former Lipozyme 10,000L liquid CLP provided by the manufacturers for this study. This CLP contained a large number of protein bands (22), most of which were in the acidic region. However, one protein band was found at pI ≥9.0. The lipolytic activities of all *R. miehei* CLP surveyed in this work were in the acid region (≤3.0 to 5.6), and most (MM15, MM18, MM19, and MM20) contained only a single active band. In our study, no lipolytic activity was detected by zymography of the standard commercial preparation of MM16 (results not shown). A special concentrate provided by the supplier, however, enabled the detection of two lipase activities at pI 4.6 and 5.2. Three active bands were detected in MM17 (Piccantase A). Marlase 20X (MM18) from Rhodia and Palatase 20,000 from Novo Nordisk (MM19) were identical in the number and pI of protein bands and lipase activities (5). Our assignment of pI to the active band present in MM19 was 3.7; other investigators have determined the pI to be 3.9 (11). The isoelectric point of MM20 lipase is reported by the manufacturer to be 4.1; in our study no activity was observed at this pI, but a single band was detected at ≤3.0.

Penicillium CLP contained proteins only in the acidic and neutral region (≤3.0 to 6.7). The *P. camembertii* CLP (PC21) contained six proteins that were in the tightest range in this study (pI 3.7 to 5.1). However, no lipolytic activity was detected. The manufacturer reports that this enzyme is not a true triacylglycerol hydrolase and does not hydrolyze triglycerides at any appreciable rate, which rendered the lipolytic activity undetectable in our matrix. Although each of the *P. roqueforti* lipases contained proteins with eight discrete pI, they were not identical. PR22 contained two active lipase bands, at ≤3.0 and 4.2, while PR 23 contained one active band at 4.8. These enzymes are often implicated in blue cheese flavor development at pH ~5.2.

CLP from *Pseudomonas* species contained seven to nine proteins that spanned the entire pH range tested, each with three or four discrete active bands in the acid region, and contained the fewest number of proteinaceous impurities in spite of the broad range of protein contents (Table 2). PsC24 was a purified preparation of very high activity. All of the proteins detected in PsC24 had isoelectric points of 3.9 to 5.4, with four active lipase bands tightly clustered at 3.9, 4.1, 4.2, and 4.5 (Table 3). The pI of protein bands in a different *P. cepacia* lipase from the same supplier (PsC25) ranged from 3.6 to ≥9.0. The only *P. fluorescens* CLP tested contained proteins that spanned the entire IEF gel (≤3.0 to ≥9.0), and the three active bands were spread over an unusually broad range (4.1, 5.0, and 5.7). The manufacturer's reported pH optimum is 8, which is rather high for a lipase, and far removed (2.3–3.9 pH units) from the pI of activity detected in this work.

Rhizopus species have been shown to express several forms of lipase (30). In at least one preparation the binding of phospholipids to lipase induced a change in pI and changed the hydrolytic activity against several substrates (11). *Rhizopus* CLP are typically produced by surface fermentations, and residual proteins and peptides from the fermentation substrate are often incorporated into the final product. This can account for the large number of protein bands (12 to 27) present in *Rhizopus* CLP found in this study.

No active bands were detected in the *Rhizopus niveus* CLP (RN27). *Rhizopus oryzae* CLP tested in this study contained many lipolytic activity bands. These were tightly clustered between 6.7 and 7.3; however, each CLP also had an active band that focused to ≥9.0. The two *R. oryzae* CLP from Bio-Cat, Inc. (RO29 and RO30) were from different fermentation batches of the same strain. The number (26 and 20, respectively) and apparent pI of their protein bands differed. More importantly, the differences in number (4 and 6, respectively) and pI of lipase activities indicate the occurrence of batch-to-batch differences between CLP produced by surface fermentations and the need for testing every batch of enzyme received. *Rhizopus oryzae* CLP from DSM/Gist-Brocades and Lallemand were identical in distribution of pI of proteins and

lipolytic activity bands; the manufacturer's recommended pH range for these enzymes falls into the most acidic conditions of any of the CLP reported here (4.0 to 6.5). A *Rhizopus* lipase containing four major protein bands in the pI range 4.7–5.3 and one band at pI 8.8 has been reported (24); the latter band was identified as the active lipase by zymography. In our hands, almost all *Rhizopus oryzae* CLP demonstrated lipolytic activity at a pI of >9.0. However, they also contained lipolytically active proteins with pI values near neutrality. Interestingly, the *Aspergillus* CLP AN7 was almost identical to the *Rhizopus oryzae* CLP in number and distribution of active lipase bands.

The causes and practical implications of the presence of several isophoretically discrete forms of lipolytic activity in most CLP are poorly understood. Lipase activities of different pI could respond differently to pH changes in a reaction medium. If these different activities exhibit differences in selectivity, activity, or operational stability, large impacts on yield, purity, quality, organoleptic properties, and process economics will result. The identity and activity of the many nonlipase bands present in most CLP is also a fertile field for investigation, both to eliminate side activities for use in complex substrates and to take advantage of hitherto unnoticed side activities for special applications.

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