Influence of Surfactants on an Extracellular Lipase from *Pythium ultimum*

Z. Mozaffar, J.D. Weete* and R. Dute
Department of Botany and Microbiology, Alabama Agricultural Experiment Station, Auburn University, Alabama 36849

The activity of an extracellular lipase from the fungus *Pythium ultimum* was studied as a function of emulsion properties, and the amount and type of surfactants used in preparing the emulsions. The highest emulsified globule surface area as a function of surfactant concentration and emulsion stability were in the order taurocholic acid > Triton X-100 > 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). Lipase activity was dependent on both the type and the concentration of surfactant, with taurocholic acid and CHAPS giving tenfold stimulation over the control at 0.1 and 0.8 mM, respectively. The evidence suggests that the influence of surfactant is related more to interaction with the enzyme at the oil–water interface than to providing greater interfacial surface area.

KEY WORDS: CHAPS, lipase, *Mucor miehei*, *Pythium ultimum*, surfactants, taurocholic acid, Triton X-100.

Lipases (EC 3.1.1.3) are exceedingly versatile enzymes that mainly catalyze the hydrolysis of triacylglycerols. They are active at the oil–water interface of emulsified substrates. Emulsions are inherently unstable, their degree of stability is influenced by the nature and concentration of surfactant used (1). Emulsifiers may function by one of several mechanisms, such as lowering the interfacial tension between phases, surrounding the emulsion droplet with a charged thin film and providing a physical barrier to the approach of other droplets, thus preventing coalescence (2). Gum arabic, polyvinyl alcohol, Triton X-100, bovine serum albumin (BSA), gelatin, ovalbumin and lecithin are examples of surfactants that have been used to stabilize triacylglycerol emulsions for lipase activity. These substances are present at the oil–water interface and, therefore, may alter the activity of lipases (3). Although there are several studies on the effects of bile salts on mammalian lipases (3–6), relatively little is known about the relationship between the emulsifier and lipase activity, particularly for extracellular microbial lipases.

We have previously described the purification and partial characterization of an extracellular lipase from the primitive plant-pathogenic fungus *Pythium ultimum* (7). Because the specific activity of this lipase was relatively low, the study reported here was conducted with the primary aim of increasing its activity and gaining a better understanding of the relationship between the lipase activity and surfactant at the oil–water interface.

MATERIALS AND METHODS

Materials. Trizma (Tris) maleate, taurocholic acid, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), polyoxyethylene-sorbitan monooleate (Tween 80) and olive oil were purchased from the Sigma Chemical Co. (St. Louis, MO). Triton X-100 was obtained from Boehringer Mannheim (Mannheim, Germany). Lipase from *Mucor miehei* (Piccantase™ A) was supplied by Gist-Brocades (King of Prussia, PA). All reagents were of the highest purity available.

Fungal growth and lipase production. Pythium ultimum strain #144 was acquired and grown on a synthetic medium as described previously (8). The fungus was cultivated for 6 d at 18°C in 250-mL Erlenmeyer flasks containing 100 mL medium and 1 mL soybean oil in a Lab-line rotary incubator-shaker (Labline Instruments, Inc., Melrose Park, PA) at 120 rpm. Inoculations were made with 5 mL blended mycelium from a 6-day-old culture grown as described above. Mycelia were separated from the medium by suction filtration in a Buchner funnel. Proteins were precipitated from the culture medium with ammonium sulfate (85% saturation), dissolved in 50 mM sodium phosphate buffer at pH 7.5 containing 10% glycerol (Buffer A), and dialyzed against the same buffer as described previously (7).

Lipase from M. miehei. Lipase from *M. miehei* (15 g) was reconstituted in Buffer A (30 mL) and dialyzed overnight against the same buffer prior to use.

Emulsion preparation. The emulsions were prepared by sonicating 2.5 mL of aqueous solutions of taurocholic acid, CHAPS or Triton X-100 with 2.5 mL olive oil, yielding final surfactant concentrations of 0, 1, 2, 4, 6 and 8 mM. The emulsions were prepared at 4°C for 30 s in a Ross micro-mixer emulsifier (Charles Ross & Son Co., NY, NY). Emulsions were standardized by preparing them in tubes of the same geometry (borosilicate glass, 13 × 100 mm; Fisher Scientific, Malvech, PA) by using 20% of total power at 50 watts as the energy for emulsification.

Emulsion activity index (EAI). The EAI of emulsions containing different surfactants was determined according to the method of Pearce and Kinsella (9). A sample of the emulsion (20 μL) was pipetted from the bottom of the sample and added to 10 mL of 10 mM sodium phosphate buffer containing 0.1% sodium dodecylsulfate (SDS), pH 7.5. The absorbance was then determined at 500 nm. The EAI was expressed as surface area in m^2/g surfactant:

$$EAI = 2(2.303A/l)/\phi C \quad [1]$$

where l is the light path in cm, ϕ is the dispersed phase fraction, A is the absorbance at 500 nm and C is the emulsifier concentration (mg/mL).

Emulsion stability (ES). The ES was determined by centrifuging 1 mL of the emulsion in a microcentrifuge (Fisher Scientific Model 235C) at 13,600 × *g* for 15 min (25°C) and by determining the weight of emulsion remaining relative to the total weight prior to centrifugation (10). Emulsions were stored at 25°C, and the ES was determined at 0, 24 and 48 h after preparation.

Assay of lipase activity. Lipase activity was measured by a modification of the method described by Ota *et al.* (11). Composition of the reaction mixture was 2 mL 0.2 M Tris-maleate-NaOH buffer (pH 8.0), 6 mL deionized water, 1 mL olive oil emulsion (prepared as described above) and 1 mL enzyme preparation containing 0.18 mg protein. When *M. miehei* lipase was used, 1 mL of the

*To whom correspondence should be addressed at Nuclear Science Center, Auburn University, AL 36849.

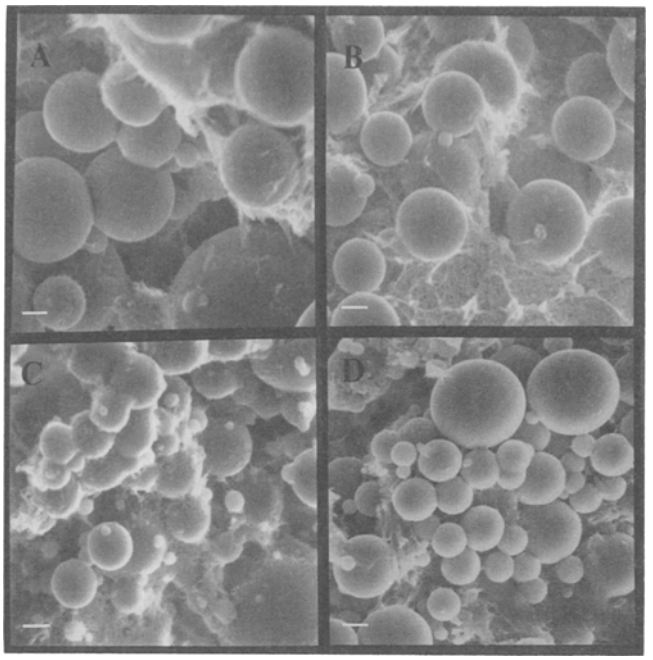

FIG. 1. Scanning electron microscopy micrographs of olive oil/water (1:1, vol/vol) emulsions prepared with no surfactant (control) (A and B), taurocholic acid at 1 mM (C) and 8 mM (D). Magnification: 1920 ×, except for B: (960 ×). The scale bar equals 2 μm for A, C and D, and 5 μm for B.

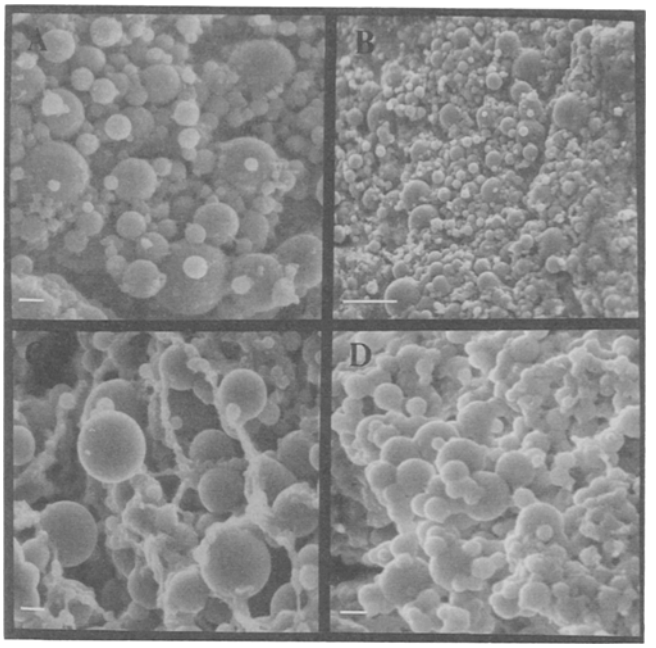

FIG. 2. Scanning electron microscopy micrographs of olive oil/water (1:1, vol/vol) emulsions prepared with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate at 1 mM (A) and 8 mM (B) and with Triton X-100 at 1 mM (C) and 8 mM (D). Magnification: 1920 ×. The scale bar equals 2 μm.

enzyme preparation contained 0.02 mg protein. When Tween-80 was the substrate, the reaction mixture consisted of 2 mL 0.2 M Tris-maleate-NaOH buffer (pH 8.0), 1 mL deionized water, 1 mL surfactant solution (taurocholic acid, CHAPS or Triton X-100) at 0, 1, 2, 4, 6 or 8 mM, 5 mL 20% Tween-80 and 1 mL enzyme preparation containing 0.36 mg protein. For *M. miehei* lipase, 1 mL enzyme preparation contained 0.02 mg protein. The reaction mixtures were incubated at 30°C with constant reciprocal shaking (45 strokes/min). In experiments involving nonemulsified substrates (Tween-80), the reaction mixtures were agitated only slightly during incubation. After 60 min incubation, 20 mL of acetone/ethanol (1:1, vol/vol) were added to stop the reaction, and the free fatty acid content was determined by titration with 0.05 N aqueous NaOH. The reaction mixture without enzyme (blank) was also incubated as described above, and the enzyme was added to the blank prior to titration with NaOH as before. The apparent free fatty acid content of the blank was subtracted from that of the reaction mixture. One unit of lipase activity was defined as the amount of enzyme required to liberate 1 μmole of fatty acid per min under the specified conditions.

Scanning electron microscopy (SEM). The preparation of samples for examination by SEM was carried out by a slight modification of the method described by Liboff *et al.* (12). Freshly prepared emulsions (3 parts by vol) were mixed gently with warm (35–40°C) 4% agar (1 part by vol) by using a wooden applicator, and allowed to set at 4°C. The gel was cut into 1-mm^3 pieces. The gelled emulsion was lifted with a spatula to cause free fractures and to expose fresh surfaces for examination. The fractured gelled emulsion was kept in 4% glutaraldehyde in 0.1 M phosphate buffer for 2 h at 4°C, and the samples were post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.0) for 16 h at 4°C. The specimens were briefly rinsed with the phosphate buffer, dehydrated in a graded series of ethanol and then dried in a Denton vacuum critical point drier (Cherry Hill, NJ). Samples were mounted on aluminum stubs and coated with gold/palladium in a Conductavac IV sputter coater (SeeVac Inc, Pittsburgh, PA) and examined with a Zeiss digital scanning electron microscope (DSM 940; Oberkochen, Baden Württemberg, Germany) interfaced with a Thomas Optical Image Analysis System, which was equipped with the software Optimas (Bioscan Inc., Edmonds, WA) for image analysis. The micrographs in Figures 1 and 2 are representative of the fields of globules used for measurements. Between 100 and 350 individual globules for each sample were measured for average-diameter calculations. In the case where the emulsion globules aggregated (Fig. 1D), globules were measured individually rather than as aggregates.

Protein determination. Protein was determined at 595 nm with Bio-Rad Protein reagent according to the Bradford method (13) with BSA as the standard.

RESULTS

Emulsion properties. The emulsifiers used in this study were taurocholic acid (anionic), CHAPS (zwitterionic) and Triton X-100 (nonionic). The EAI is a measure of surface area of the dispersed (discontinuous) phase of the emulsion as a function of surfactant concentration, and has been used by Haque and Kinsella (14) to compare the

emulsifying properties of proteins. This measure of surface area was used in this study to determine which surfactant (taurocholic acid, CHAPS or Triton X-100) gave the highest EAI, and to understand the relationship between EAI and activity of the *Pythium* lipase. The emulsification activity of the three surfactants differed markedly for the olive oil/water emulsion. Taurocholic acid was the best emulsifier of the three at 1 mM, with EAI values of over eight and four times greater than those from CHAPS and Triton X-100, respectively (Table 1). For each surfactant, the EAI decreased substantially with increasing concentration up to 8 mM. For example, the EAI decreased by 82% from 1 to 8 mM taurocholic acid, whereas the EAI values of emulsions containing Triton X-100 and CHAPS decreased 36 and 75%, respectively.

Emulsions prepared with taurocholic acid were also more stable to centrifugation and over time than those prepared with the other two surfactants, particularly at the lower concentrations. The stability of emulsions prepared with taurocholic acid and Triton X-100 was inversely related to the EAI (surface area) of the dispersed phase, but directly related to emulsifier concentration (Table 1). Emulsions prepared with CHAPS were completely unstable to centrifugation at all concentrations tested.

SEM. The emulsions were examined by SEM to determine variability of emulsion particle (globule) diameter as a function of surfactant type and amount. Although the control emulsion (no surfactant) was relatively unstable with time, the particle diameters ranged from 0.5 to 4.5 μm with a mean of 1.8 ± 1.1 μm (Figs. 1A and 1B). The range and mean of particle diameters for emulsions prepared with 1 mM taurocholic acid were similar to the control (Fig. 1C). Although mean particle diameters for the emulsion prepared with 8 mM taurocholic acid was similar to the value with 1 mM and the control, their range was smaller—0.5–3.5 μm (Fig. 1D). Also, unlike that with lower concentration (1 mM), the emulsion prepared with 8 mM of taurocholic acid caused aggregation between the globules without apparent coalescence (Fig. 1D). Emulsions prepared with 4 mM taurocholic acid showed an intermediate level of aggregation (data not shown). There was no aggregation between globules with CHAPS and Triton X-100 at any of the concentrations tested.

The particle diameters for emulsions prepared with CHAPS at 1 mM ranged from 0.5 to 10.5 μm, and from 0.5 to 8.5 μm at 8 mM (Figs. 2A and 2B). The average diameters were 4.0 ± 2.5 and 4.7 ± 2.4 μm for 1 and 8 mM, respectively. For emulsions prepared with Triton X-100, the particle diameters ranged from 0.5 to 6.5 μm at 1 mM and from 0.5 to 4.5 μm at 8 mM (Figs. 2C and 2D). For Triton, the average diameter decreased from 3.2 ± 2.3 to 2.0 ± 0.9 μm with increasing surfactant concentration.

Pythium *lipase activity.* Lipase activity varied both as a function of surfactant type and concentration. In emulsions containing taurocholic acid, the highest lipase activity was observed with emulsions prepared with 1 mM surfactant (0.1 mM after dilution for lipase assay), but it decreased linearly with increasing surfactant concentration. On the other hand, lipase activity in emulsions prepared with CHAPS increased exponentially (Table 2). Lipase activity in emulsions prepared with 1 mM taurocholic acid was essentially the same as that for emulsions prepared with 8.0 mM CHAPS. Lipase activity was lower in emulsions containing Triton X-100 than those prepared with the other two surfactants, but the changes as a function of concentration followed a pattern similar to that for emulsions containing CHAPS. Activities increased with increasing surfactant concentration for emulsions

TABLE 1

Effects of Surfactants on Emulsion Surface Area and Stability

Surfactant	Surfactant concentration (mM)	EAI[a] (m^2/g)	ES (%)[b] 0 h	24 h	48 h
Taurocholic	1	1179 ± 70[c]	51 ± 1[c]	37 ± 1[c]	33 ± 1[c]
	2	692 ± 21[d]	55 ± 1[d]	50 ± 1[d]	46 ± 1[d]
	4	389 ± 15[e]	56 ± 1[d]	52 ± 2[d]	49 ± 1[e]
	6	274 ± 9[f]	57 ± 1[d]	52 ± 2[d]	52 ± 1[f]
	8	209 ± 6[g]	56 ± 1[d]	55 ± 0[d]	52 ± 0[f]
CHAPS	1	141 ± 17[c]	—	—	—
	2	100 ± 6[d]	—	—	—
	4	65 ± 3[e]	—	—	—
	6	47 ± 2[f]	—	—	—
	8	36 ± 1[g]	—	—	—
Triton X-100	1	268 ± 16[c]	16 ± 3[c]	6 ± 1[c]	3 ± 0[c]
	2	279 ± 10[c]	36 ± 6[d]	20 ± 1[d]	14 ± 1[d]
	4	237 ± 12[d]	48 ± 2[e]	39 ± 3[e]	35 ± 1[e]
	6	204 ± 6[e]	50 ± 1[e]	48 ± 2[f]	44 ± 1[f]
	8	172 ± 7[f]	52 ± 1[e]	49 ± 1[f]	49 ± 1[g]

[a]EAI, Emulsion activity index. Each value is a mean ± SD of three determinations. In columns for each surfactant, means with the same letter are not significantly different. Significance was calculated by Duncan's Multiple Range Test ($\alpha = 0.05$ level). Other details are given in the Materials and Methods section.

[b]ES, Emulsion stability. Values represent the percentage of emulsion remaining after centrifugation as described in the Materials and Methods section. CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfate.

TABLE 2

Effect of Surfactants on the Activity of an Extracellular Lipase from *Pythium ultimum*

Surfactant[a] concentration (mM)	Lipase activity (μmol/min/mg protein)[b]		
	Taurocholic acid	CHAPS	Triton X-100
0	0.7 ± 0.0[c]	0.7 ± 0.0[c]	0.7 ± 0.0[c]
0.1	7.3 ± 0.4[d]	0.7 ± 0.0[c]	0.8 ± 0.2[c,d]
0.2	6.3 ± 0.4[e]	0.9 ± 0.2[c,d]	1.0 ± 0.0[d]
0.4	5.2 ± 0.4[f]	1.1 ± 0.3[d]	1.4 ± 0.1[e]
0.6	4.2 ± 0.4[g]	3.4 ± 0.2[e]	2.0 ± 0.2[f]
0.8	3.5 ± 0.4[h]	7.1 ± 0.4[f]	3.7 ± 0.2[g]

[a]Lipase activity was measured in the emulsions containing the three surfactants as described in Table 1. The factor of 10 difference in surfactant concentration is due to the 1 in 10 dilution of the emulsion for the enzyme assay.
[b]Each value is a mean ± SD of three determinations. In columns for each surfactant, means with the same letter are not significantly different. Significance was calculated by Duncan's Multiple Range Test ($\alpha = 0.05$ level). Other details are given in the Materials and Methods section. Abbreviation as in Table 1.

prepared with CHAPS and Triton X-100. Taurocholic acid, CHAPS and Triton X-100 stimulated *Pythium* lipase activity by factors of 10.4, 10.1 and 5.3, respectively, over the controls when the optimal surfactant concentrations were compared.

Although activity was relatively low, these surfactants had essentially no effect on the activity of the *Pythium* lipase under nonemulsifying conditions when Tween-80 was the substrate (data not shown).

Mucor *lipase activity.* Activity of the lipase from *Mucor* was also measured as functions of the nature and concentration of surfactant as described above for the *Pythium* lipase. Each of the surfactants stimulated activity of the *Mucor* lipase with activities reaching up to 50–52 μmol/min/mg protein for taurocholic acid and CHAPS, and 43 μmol/min/mg for Triton X-100 (Table 3), which are four- to fivefold higher than for the controls without surfactant.

TABLE 3

Effect of Surfactants on the Activity of a Lipase from *Mucor miehei*

Surfactant[a] concentration (mM)	Lipase activity (μmol/min/mg protein)[b]		
	Taurocholic acid	CHAPS	Triton X-100
0	10.5 ± 0.0[c]	10.5 ± 0.0[c]	10.5 ± 0.0[c]
0.1	43.8 ± 3.0[d]	18.4 ± 2.6[d]	19.3 ± 3.0[d]
0.2	50.8 ± 3.1[e]	49.1 ± 3.1[e]	43.8 ± 3.0[e]
0.4	50.8 ± 3.1[e]	50.8 ± 3.1[e]	38.6 ± 3.1[f]
0.6	52.6 ± 5.3[e]	29.8 ± 3.0[f]	22.8 ± 3.1[d]
0.8	50.8 ± 3.1[e]	21.0 ± 5.3[d]	12.3 ± 3.1[c]

[a]See Tables 1 and 2 for explanation of surfactant concentration and abbreviations.
[b]Each value is a mean ± SD of three determinations. In each column, means with the same letter are not significantly different. Significance was calculated by Duncan's Multiple Range Test ($\alpha = 0.05$ level). Other details are given in the Materials and Methods section.

Lipase activity in the emulsions containing taurocholic acid reached a threshold in emulsions prepared with 2.0 mM without significant change at higher concentrations (Table 3). Activity in emulsions prepared with CHAPS and Triton X-100 responded similarly in that an optimum surfactant concentration was found for emulsions prepared with 2–4 mM for the former surfactant and with 2.0 mM for the latter. Activity decreased at higher concentrations of these surfactants. Activity appeared to be less related to surface area of the emulsified substrate or nature of the surfactant than to the amount present.

DISCUSSION

The properties of emulsions prepared as functions of the nature and concentration of surfactants (emulsifier) were compared and then evaluated with respect to the activity of an extracellular lipase from the filamentous fungus *P. ultimum*. The magnitude of the effect at any of the respective concentrations used, from the highest surface area to the lowest, was in the order taurocholic acid > Triton X-100 > CHAPS. Taurocholic acid and CHAPS had similar effects on the surface area (EAI) of emulsified olive oil globules, *i.e.,* surface area decreased with increasing surfactant concentration. With Triton X-100, EAI was unchanged up to 2 mM concentration; however, it decreased thereafter with increasing concentration. However, according to the SEM examination and optical imaging analysis, the average globule diameters did not differ substantially between emulsions prepared with 1 or 8 mM taurocholic acid or CHAPS, but the average particle size diameter for emulsions prepared with the latter surfactant was about 2.5 times larger than that for the former. Therefore, the total interfacial surface area for the emulsions prepared with taurocholic acid would be expected to be greater than that for those prepared with CHAPS.

On the other hand, lipase activity responded differently in emulsions prepared with taurocholic acid as compared to those prepared with CHAPS and Triton X-100, *i.e.,* activity decreased with increasing taurocholic acid concentration whereas it increased in emulsions containing the other two surfactants. This, combined with the above results on globule size, strongly suggests that stimulation of the *Pythium* extracellular lipase by surfactants is more a function of the nature of the surfactant *per se* than of the surface area of the oil–water interface, where the catalytic activity occurs. However, the fact that these surfactants do not affect lipase activity, under nonemulsifying conditions supports the requirement for the surfactants to create a favorable environment at the oil–water interface for lipase activity. Our results with taurocholic acid differ from those of Naka and Nakamura (15), who found that deoxycholate at 1–10 mM concentration inhibited the activities of lipases from various microbial sources. For *Chromobacterium viscosum* and ex-*Candida* lipase, it was observed that lipase activity in the presence of surfactants depended on the source of the enzyme (16). *Chromobacterium viscosum* lipase was competitively inhibited by SDS and cetyltrimethylammonium bromide (CTAB), and noncompetitively by aerosol-OT (AOT). On the other hand, activity of the ex-*Candida* lipase was unaffected by SDS, and markedly reduced by AOT and CTAB (16).

The results of this study are consistent with those of Brockerhoff and Jensen (3), who concluded that the binding of bile salts to the lipase was essential for subsequent orientation of the enzyme at the oil–water interface. In this study, taurocholic acid appeared to play a dual role, *i.e.,* it served as an emulsifier as well as an activator of the lipase. The *Pythium* lipase responded similarly to the bile salt as pancreatic lipase (4), *i.e.*, it stimulated activity at low concentrations and reduced activity at higher concentrations. In a previous study, we showed that taurocholic acid stimulated activity of the *Pythium* lipase 2.7-fold when included in an emulsion assay mixture where it did not serve as the primary emulsifier (7). Momsen and Brockman (4,5) concluded that low concentrations of the bile salt increased stability of the enzyme, and that higher concentrations prevented absorption of the enzyme to the oil–water interface. In this study, the emulsified substrate was diluted 1:10 for the lipase assay, and, therefore, the surfactant would be expected to occur primarily at the oil–water interface and not in excess.

Unlike taurocholic acid, the two other surfactants used in this study stimulated activity of the *Pythium* lipase progressively with increasing concentration. Similar observations have been made for Triton X-100 with other fungal extracellular lipases, except for a lipase from *Rhizopus arrhizus*, which increased in activity with 1% (15.5 mM) surfactant (17).

It is interesting that the specific activity of *Pythium* lipase in the emulsion prepared with 1 mM taurocholic acid was about the same as that in the emulsion prepared with 8 mM CHAPS, which suggests that the two surfactants may stimulate activity of the *Pythium* lipase by different mechanisms. Perhaps this is related to the relatively large (270 kD) structure of this lipase (7), which, except for a recently reported lipase from *C. parapsilosis* (18), is unusual for fungal lipases. Nevertheless, both surfactants, one anionic and the other zwitterionic, may create a similar environment for the enzyme at 1 and 8 mM, respectively, perhaps by improving the interaction of the enzyme with the interface and/or by facilitating access of the substrate to the active site of the enzyme.

Effects of the surfactants on the activity of an extracellular lipase from *M. miehei* were compared to those of the *Pythium* lipase because the former shows both esterase as well as hydrolase activities (19,20). The *Mucor* lipase showed 16 times greater activity toward Tween-80 in a nonemulsified system than the *Pythium* lipase (unpublished data). The *Mucor* lipase was about seven times more active than the *Pythium* lipase in an emulsion system when specific activity is expressed on a milligram protein basis. However, when expressed on a mole basis, the specific activity of the *Pythium* lipase is 1.97 μmol/min/mol, and that of the *Mucor* lipase is 1.55 μmol/min/mol (21).

The *Mucor* lipase responded quite differently to the surfactants than the *Pythium* lipase. Emulsions prepared with each surfactant at 2.0 mM stimulated activity over the controls by four- to fivefold, suggesting that specific interaction of the surfactant with the enzyme is less important for activity than it is for the lipase from *Pythium*.

ACKNOWLEDGMENTS

This work was supported by AAES project Ala 00773 (JDW). It is AAES publication no. 6-933562. We thank Franck Dayan and Ananda Mahadaven for their assistance in data processing. We also thank Gist-Brocades (King of Prussia, PA) for kindly providing *Mucor miehei* lipase (Piccantase™ A).

REFERENCES

1. Sharma, M.K., and D.O. Shah, in *Macro and Microemulsions: Theory and Applications*, edited by D.O. Shah, American Chemical Society, Washington, D.C., 1985, pp. 4–5.
2. Klemaszewski, J.L., Z. Haque and J.E. Kinsella, *J. Food Sci. 54*:440 (1989).
3. Brockerhoff, H., and R.G. Jensen, *Lipolytic Enzymes*, Academic Press Inc., New York, 1974.
4. Momsen, W.E., and H.L. Brockman, *J. Biol. Chem. 251*:378 (1976).
5. Momsen, W.E., and H.L. Brockman, *Ibid. 251*:384 (1976).
6. Alvarez, F.J., and V.J. Stella, *Pharmaceut. Res. 6*:449 (1989).
7. Mozaffar, Z., and J.D. Weete, *Lipids 28*:377 (1993).
8. Gandhi, S.R., and J.D. Weete, *J. Gen. Microbiol. 137*:1825 (1991).
9. Pearce, K.N., and J.E. Kinsella, *J. Agric. Food Chem. 26*:716 (1978).
10. Mozaffar, Z., and Z. Haque, *Food Hydrocolloids 5*:573 (1992).
11. Ota, Y., K. Gomi, S. Kato, T. Sugiura and Y. Minoda, *Agric. Biol. Chem. 46*:2885 (1982).
12. Liboff, M., H.D. Goff, Z. Haque, W.F. Jordon and J.E. Kinsella, *Food Microstructure 7*:67 (1988).
13. Bradford, M.M., *Anal. Biochem. 72*:248 (1976).
14. Haque, Z., and J.E. Kinsella, *J. Food Sci. 54*:1241 (1989).
15. Naka, Y., and T. Nakamura, *Biosci. Biotech. Biochem. 56*:1066 (1992).
16. Schomaecker, R., B.H. Robinson and P.D.I. Fletcher, *J. Chem. Soc. Faraday Trans. I 84*:4203 (1988).
17. Veeraragavan, K., *Anal. Biochem. 186*:301 (1990).
18. Riaublanc, A., R. Ratomahenina, P. Galzy and M. Nicolas, *J. Am. Oil Chem. Soc. 70*:497 (1993).
19. Sonnet, P.E., *Ibid. 65*:900 (1988).
20. Huge-Jensen, B., D.R. Galluzzo and R.G. Jensen, *Ibid. 65*:905 (1988).
21. Brady, L., A.M. Brzozowski, Z.S. Derewenda, E. Dodson, G. Dodson, S. Tolley, J.P. Turkenburg, L. Christiansen, B. Huge-Jensen, L. Norskor, L. Thim and I.U. Menge, *Nature 343*:767 (1990).

[Received July 13, 1993; accepted October 27, 1993]