Studies on Free and Immobilized Lipases from *Mucor miehei* [1]

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An extracellular lipase produced by fermentation with a selected strain of *Mucor miehei* has been purified partially in two forms: A and B. The forms have a high degree of antigenic identity and have similar pH-activity profiles with tributyroylglycerol as the substrate with optima at pH 7. The differences are A, in contrast to B, requires activation at alkaline pH before analysis; A binds with concanavalin A more completely than B, the net charges are slightly different at pH 8; the isoelectric points are different. Our results indicate that the B lipase is formed by partial deglycosylation of the A lipase and that this influences the activity toward emulsions.

The two enzymes have been immobilized by adsorption. These preparations and the soluble forms were highly specific for primary ester of triacylglycerols (TG), usually hydrolyzed TG of 12:0, 14:0, 16:0 and 18:1 more rapidly than those of 4:0, 6:0, 8:0 and 10:0 in mixtures of monoacid TG (4:0 to 18:1), and were not stereospecific for TG. Immobilization altered the specificity of the preparations somewhat in that slightly more 14:0 and 16:0 were released.

Enzymatic modification of foods and other material has occurred either spontaneously or deliberately for generations. This has been done primarily for production of characteristic flavors. The recent availability of large quantities of enzyme preparations has increased activity in the search for industrial applications of enzymes and simultaneously for enzymes with desirable characteristics such as thermostability and certain specificities. For several reasons, including relative ease of production, many of these enzymes are obtained from microbial fermentations. These aspects, with emphasis on lipases, recently have been discussed by Kilara (1).

During our search for microbial lipases with industrial applications, we selected a strain of *M. miehei* for further study. This mold produces an active extracellular lipase (2). Results from preliminary experiments suggested that there were at least two different forms of the enzyme. In this paper, we describe the partial purification of two of these soluble forms. These were immobilized and the four preparations partially characterized. The purifications and immobilizations were done in Denmark and the specificity studies in Connecticut. Preliminary data on use in industrial applications and some characterization of the lipase have been presented (3,4). We believe that this is among the few reports (5,6) on the characteristics of immobilized lipases and is the first on the A and B forms of *M. miehei* lipases. A complete report of this investigation has been published (7).

[1]Presented at the symposium "The Biology, Biochemistry and Technology of Lipases" at the 78th annual meeting of the American Oil Chemists' Society held May 17-21, 1987, in New Orleans, Louisiana.

MATERIALS AND METHODS

Materials and Determinations. DE-cellulose 52 was purchased from Whatman Chemical Separation Ltd. (Kent, England). DEAE-Sepharose, Phenyl-Sepharose, concanavalin A (Con A)-Sepharose and Sepharose 4B were obtained from Pharmacia Fine Chemicals (Piscataway, New Jersey).

Lipase activity was measured with a pH-stat essentially as described by Brockman (8) except the 0.5% of polyvinylpyrrolidone was used as an emulsion stabilizer. Substrates were tributyrolglycerol (Novo method AF 95.1/3-GB) and olive oil (Novo AF 182.2/3-GB). These designations are given for those who may use the Novo methods, otherwise they are very similar to those described by Brockman (8). Units of activity are 1 lipase unit (LU), the amount of enzyme that liberates 1 μmol of titratable 4:0 per min under standard conditions, and 1 Novo lipase unit (NLU), the amount of enzyme that liberates 1 μmol of titratable fatty acid from olive oil per min under standard conditions. Note that these Novo methods require that the *Mucor* lipase be diluted in alkaline buffer (pH 10.5) before analysis. This buffer did not interfere with subsequent analyses. Protein determination was performed according to Lowry et al. (9) with bovine serum albumin as a standard. Reference 7 has a full description of these and the purification procedures.

Purification of Lipase A. The supernatant from the culture broth of a selected strain of *M. miehei*, with mycelia and low molecular weight substances removed, was used for production of a crude powder. The powder was treated as follows: (a) anion exchange chromatography on DE-cellulose 52, (b) concentration of the lipase fraction by ultrafiltration and lyophilization, (c) affinity chromatography on concanavalin A-Sepharose and (d) desalting, ultrafiltration and lyophilization of the pooled lipase fractions.

Purification of Lipase B. The sequence of purification was (a) anion exchange chromatography of the crude powder on DEAE-Sepharose, (b) hydrophobic interaction chromatography on Phenyl-Sepharose, and (c) concentration by ultrafiltration and lyophilization.

Monitoring of Purifications and Lipase Activity. These were performed as follows: (a) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described in Bio-Rad's manual (10) for the Laemmli system, using Bio-Rad vertical slab gel cell model 220. The separating gel was a gradient of 7%–20% polyacrylamide. Reference proteins were employed for molecular weight determination of low molecular weight proteins; (b) isoelectric focusing (IEF) was done on LKB's Multiphore apparatus using ready-made polyacrylamide gels as described in LKB's manual (11); (c) crossed immunoelectrophoresis (CIE) was performed as described by Hojby and Axelsen (12) using 0.02 M barbital buffer pH 8.0. The polyspecific antibodies were produced in rabbits against the crude enzyme powder; (d) tandem-CIE was per-

formed as described by Kroll (13), using 0.02 M barbital buffer pH 8.0; (e) crossed immunoaffinity electrophoresis (CIAE) was performed by a modification of the CIE methods; and (f) overlayer technique for detection of lipase activity was done as follows. Lipase activity in IEF and CIA/CAE was detected by immediately after the electrophoretic run, incubating the gel under approximately 1.5 mm overlayer gel (1% agarose) containing a 1% tributyroylglycerol emulsion. Incubation time varied from two to six hr at 45 C. Binding of antibodies did not interfere with activity. Lipase activity appeared as cleared zones. In the photographs made of these, the cleared zones appear as dark areas because a dark background was used during photography.

Preparation of immobilized lipase A and B. Each of the lipases A (from the Con-A-Sepharose chromatography) and B (from the phenyl-Sepharose chromatography) was immobilized on Duolite ES 562 resin (Rohm and Haas, Philadelphia, PA) according to patent application No. 0,140,542. The conditions used for the immobilization were pH 6.0 and two hr at 5 C. The intention was to immobilize 14 mg of Lipase A preparation and 18 mg of lipase B preparation on 1 gm carrier each. Direct specific activity determinations on these immobilized preparations were not possible. Indirect determinations were done.

Determination of specificities. (a) Position specificity was determined by thin layer chromatographic (TLC) separation and then visualization of extracts from digestions of trioleoylglycerol. In these, the relative amounts of 1 (3)- and 2-monooleoylglycerol and 1,2 (2,3)- and 1,3-dioleoylglycerols were ascertained by inspection and quantified by gas liquid chromatography (GLC) (14). Confirmation was obtained by digestion of the synthetic TG (1,3-dioleoyl-2-palmitoyl glycerol (18:1-16:0-18:1) and 1,3-dipalmitoyl-2-oleoylglycerol (16:0-18:1-16:0) and identification of the free fatty acids (14,15). These TG were synthesized as described by Jensen and Pitas (16); (b) fatty acid specificity was determined as described by Wang et al. (17). Equimolar mixtures of the monoacid TGs of 4:0, 6:0, 8:0, 10.0, 12:0, 14:0, 16:0 and 18:1 were hydrolyzed, and the residual TGs were recovered and separated by temperature programmed GLC; and, (c) for stereospecificity, the specific rotation of the entire extract of a trioleoylglycerol digestion was determined in hexane with a Perkin-Elmer polarimeter sensitive to 0.00001 degree. In the absence of unequal amounts of optically active 1,2 or 2,3 *sn*-diacylglycerols and with the adequate amounts of both, there should be no rotation, hence no stereospecificity (14,18).

RESULTS AND DISCUSSION

Conversion of the lipase from the A-form to the B-form

TABLE 1

Purification of Lipase A from *Mucor miehei*[a]

Purification steps	Yield, % based on LU-values[a]	Protein content %	Specific activity LU/mg preparation	LU/mg protein	NLU/mg[b] preparation	NLU/mg protein
Crude powder	100	42	157	374	100	238
De-cellulose 52 chromatography	77	67	1700	2537	1073	1601
Con A-Sepharose chromatography	57	66	3590	5439	2300	3485

[a]LU (Lipase Unit) is the amount of enzyme that liberates 1 μmol of 4:0 per min (see text).

[b]NLU (Novo Lipase Unit) is the amount of enzyme that liberates 1 μmol of fatty acid from olive oil per min (see text).

TABLE 2

Purification of Lipase B from *Mucor miehei*[a]

Purification steps	Yield, % based on LU-values[b]	Protein content %	Specific activity LU/mg preparation	LU/mg protein	NLU/mg[c] preparation	NLU/mg protein
Crude powder	100	42	157	374	100	238
DEAE-Sepharose chromatography	72	80	945	1181	600	750
Phenyl-Sepharose chromatography	32	100	3000	3000	1900	1900

[a]Lipase B is formed by partial deglycosylation of lipase A, Table 1 and text.

[b]See a, Table 1.

[c]See b, Table 1.

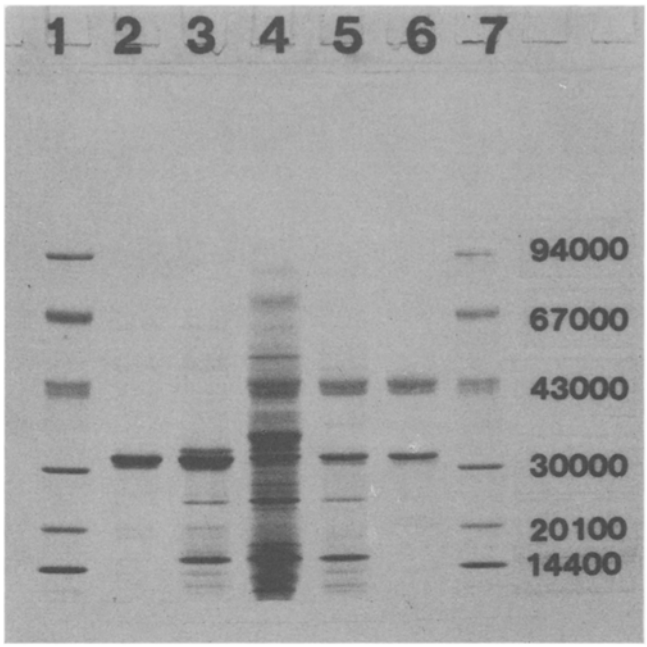

FIG. 1. SDS-PAGE of lipase preparations obtained from the various purification steps. 1) and 7), respectively, 18 μg of the total reference proteins. 2) 25 μg of the Con A-Sepharose purified lipase A preparation. 3) 63 μg of the DE-cellulose 52 purified lipase A preparation. 4) 375 μg of the crude powder. 5) 63 μg of the DEAE-Sepharose purified lipase A/B preparation. 6) 25 μg of the phenyl-sepharose purified lipase B preparation. Proteins were stained with Coomassie Brilliant Blue.

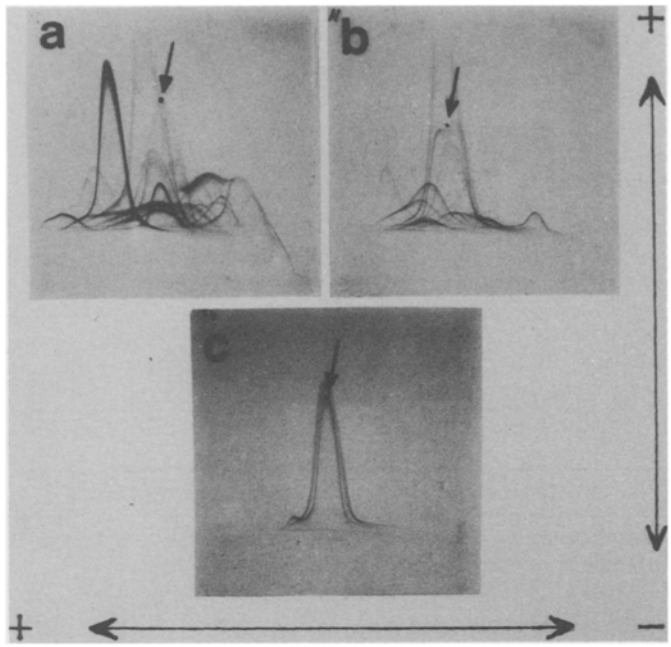

FIG. 3. Crossed immuno electrophoresis (CIE) of purification steps of lipase B. The antigens applied were a, 100 μg of crude powder; b, 20 μg of DEAE-Sepharose purified lipase (A/B) preparation; c, 4.4 μg of Phenyl-Sepharose purified lipase B preparation. 500 μl of the polyspecific antibodies were applied on each CIE. The CIE's were stained with Coomassie Brilliant Blue. The arrow indicates the lipase containing immunoprecipitate, detected as described in Figure 2.

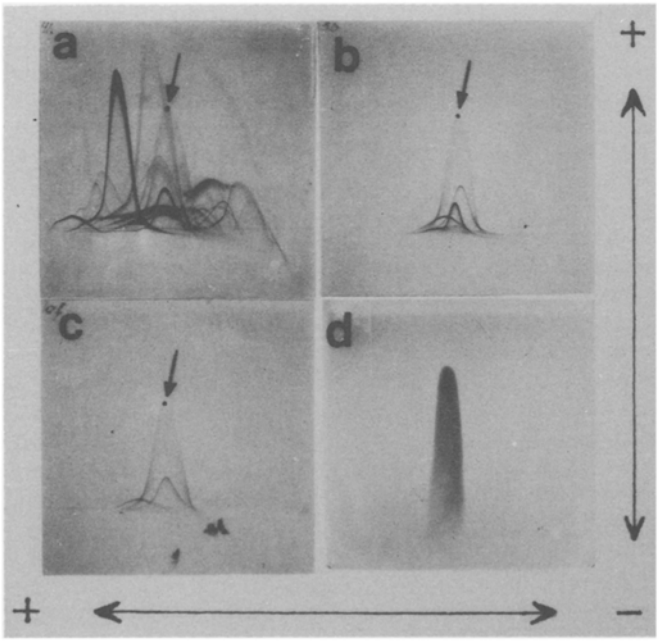

FIG. 2. Crossed immuno electrophoresis (CIE) of purification steps of lipase A. a, b and c are CIE stained with Coomassie Brilliant Blue. The arrow indicates the lipase containing immunoprecipitate, detected as described for d. d is the duplicate CIE of b but with tributyrolglycerol overlayer, where the lipase peak shows as a clearing zone. The antigens applied were a, 100 μg of crude powder; b, and d, respectively, 10 μg of DE-cellulose 52 purified lipase A preparation; c, 5 μg of Con-A-Sepharose purified lipase A preparation. 500 μl of the polyspecific antibodies were applied on each CIE.

was done by keeping the lipase fractions obtained from purification steps a and b at low pH during recovery and by treating the enzyme at low pH at an elevated temperature before step b. Lipase activity bands and peaks in IEF and CIE/CAE, respectively, were identified by performing duplicates of the electrophoretic runs. These duplicates were used for the substrate overlayers and compared with the Coomassie stained plates. The purifications were followed by activity measurements on both 4:0-4:0-4:0 and olive oil as well as by the electrophoretic methods SDS-PAGE and CIE. Tables 1 and 2 summarize the purification steps and yields of lipases A and B, respectively. Figures 1, 2 and 3 shows the successive purifications of lipase from the multiprotein mixture in the crude powder with removal of many nonlipase proteins in the starting material.

The differences in lipolytic and molecular behavior of the lipases were investigated by determining activities on water dilutions of the enzyme preparations as well as by CIAE. In Table 3, we present data on differences between lipases A and B in their activity ratios when they are diluted in glycine buffer (pH 10.5) and in water, respectively, before analysis. It is obvious that lipase B needs no activation at high pH, whereas lipase A does.

Effect of pH on activities of lipase A and B on tributyrolglycerol also was investigated using the appropriate analysis pH-values in Novo method AF 95.1/3-GB. Figure 4 shows similar pH-activity profiles of the two lipases, both having optima at pH 7.0. Only a minor difference is seen at pH 8.0, where lipase B has slightly lower activity than lipase A.

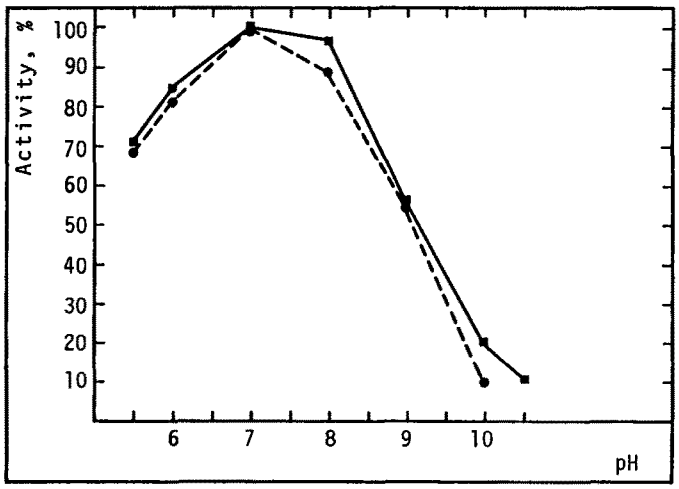

FIG. 4. Effect of pH on activity on tributyroylglycerol, 30 C, ph-stat method (8) using the appropriate analysis pH values. (■) Lipase A, Con-A-Sepharose purified preparation. (●) Lipase B, Phenyl-Sepharose purified preparation.

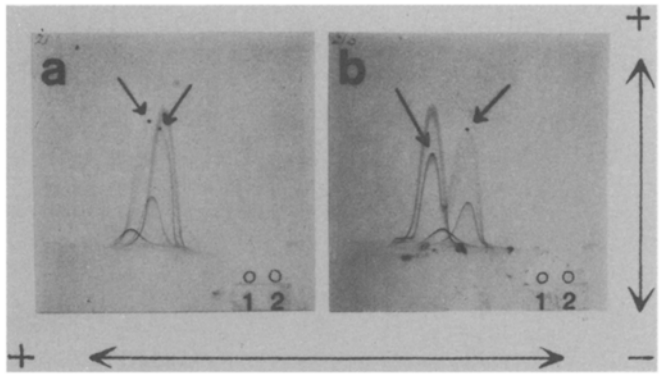

FIG. 5. Tandem-crossed immunoelectrophoresis, stained with Coomassie Brilliant Blue. Arrows indicate lipases, detected as described in Figure 2. (a) Well 1, 5 μg of Con-A-Sepharose purified lipase A preparation, (b) Well 2, 4.4 μgm of Phenyl-Sepharose purified lipase B preparation. Well 1 and Well 2, reverse order of (a).

Figure 5 (tandem-CIE) clearly shows a high degree of antigenic identity between lipases A and B, as defined (13). Their immunoprecipitates fuse into a double peak practically without any spurs. A difference in the morphology of the immunoprecipitates is seen, though. The reverse orders of the well positions of lipase A and B in the first dimension gel also visualize the greater anodic mobility of A-B.

TABLE 3

Activation Factors[a] of Lipase Preparations Based on LU-values[b]

Purification steps	Lipase preparations A	B
Crude powder	6.3	6.3
Anion exchange-chromatography	6.3	1.7
Affinity-chromatography	5.6	1.1

[a]Activation factor refers to ratio of activities of the enzyme when diluted in glycine buffer pH 10.5 and in water, respectively, before analysis.

[b]LU, see Table 1.

The results indicate that the B-form of the lipase is derived by partially deglycosylation of the A-form, and that this influences the activity toward emulsions. Lipase B is the activated form, but by the recovery yields in Tables 1 and 2 it seems to be less stable than lipase A. We postulate that this partial deglycosylation at low pH of the original lipase is due to enzymatic reactions.

All preparation were highly specific for the primary positions of 18:1-18:1-18:1 as indicated by the absence of 1 (3)-monoacylglycerols (MG) and 1,3-DG in boric acid-TLC plates of extracts from 10 min digestions. The profiles of digestion products were similar for all preparations; rapid appearance of fatty acids and 1,2 (2,3)-DG accompanied by disappearance of TG at four and eight min. Not much MG was produced at four min, but the amounts increased dramatically at eight min, reflecting the accumulation of 2-MG. These will convert to 1-MG by acyl migration and eventually be hydrolyzed, resulting in glycerol, which we did not determine. By eight min, 30%–50% of the TG was gone, and by 12 min the amounts were less than 10%.

Confirmation of specificity for the primary positions of TG was obtained by analyses of the fatty acids liberated by the lipolyses of 18:1-16:0-18:1 and 16:0-18:1-

TABLE 4

Composition of Fatty Acids Produced by Digestion of 18:1-16:0-18:1[a] and 16:0-18:1:16:0 with Several Preparations of *Mucor miehei* Lipase[b]

Substrate composition of fatty acids, mol %	Lipase preparation Soluble A	Immobilized A	Soluble B	Immobilized B
18:1-16:0-18:1				
16:0	17.7	17.9	20.3	17.2
18:1	82.3	82.1	79.7	82.8
16:0-18:1-16:0				
16:0	90.9	93.2	91.5	94.7
18:1	9.1	6.8	8.5	5.3

[a]Average of five determinations.

[b]18:1-16:0-18:1 is 1,3-dioleoyl-2-palmitoyl-glycerol.

TABLE 5

Digestion (%) of Equimolar Mixtures of Monoacid Triacylglycerols by Lipase Preparations from *Mucor miehei*

Length of digestion (min)	Monoacid triacylglceryol, % digested							
	4:0	6:0	8:0	10:0	12:0	14:0	16:0	18:1
				Soluble A				
1	35.6	38.2	43.4	40.1	47.4	47.1	45.3	50.0
2	42.4	46.5	52.8	48.9	54.9	53.8	50.7	53.7
3	54.2	61.1	65.4	61.0	65.7	64.7	62.7	62.2
5	61.0	66.7	73.6	70.3	76.6	75.6	76.6	74.5
				Soluble B				
1	27.6	33.4	40.7	40.7	44.3	45.8	39.4	38.0
2	32.1	49.2	51.4	57.7	63.1	63.4	62.4	56.7
3	74.6	73.1	78.8	78.8	81.8	51.5	80.7	78.9
5	87.5	86.5	81.4	89.3	92.8	92.1	91.7	90.9
				Immobilized A				
1	31.8	30.6	35.6	39.3	42.8	57.4	52.3	43.8
2	43.9	42.0	49.5	54.8	54.6	61.5	63.2	55.7
3	—	43.3	52.1	56.0	57.7	64.3	65.4	56.6
5	65.2	65.6	72.9	74.6	76.4	79.7	82.7	75.8
				Immobilized B				
1	25.4	17.5	18.3	14.2	21.2	18.7	21.2	10.4
2	36.6	28.5	26.9	20.9	22.9	37.5	36.0	29.8
3	39.7	39.7	47.0	44.2	52.9	57.5	55.9	57.8
5	42.9	45.3	51.2	47.2	57.7	61.9	59.2	59.9

16:0 (Table 4). In both cases, all preparations released much greater amounts (79.7–94.7M%) of the acids in the primary positions than 67M%, the quantity that would be produced by a nonspecific lipase (14). Interestingly, there was a slight preference for 16:0 as compared with 18:1 in the primary positions.

We analyzed the whole extracts of digestions of 18:1-18:1-18:1 with a sensitive polarimeter but found no deviations from zero specific rotation (14). We used this technique in our study on human lingual lipase (17), finding a rotation denoting the presence of *sn*-1,2-DG. It is possible that enantiomeric MG as well as DG could be responsible for rotation, but because we observed no rotation, further investigation was not needed.

In contrast to Wang et al, who found specificity by a breast milk lipoprotein lipase (17) and a bile salt-stimulated lipase (19) for the shorter fatty acids in mixtures of monoacid TG ranging from 4:0-18:0, we observed the opposite. The percent digestions of the 4:0, 6:0, 8:0 and 10:0 TG almost always were lower than those of 12:0, 14:0, 16:0 and 18:1 with all preparations except soluble B. These data are presented in Table 5. In contrast to soluble A, immobilized A released more 14:0 and 16:0 than the other acids, including 18:1. This also occurred with the B preparations, but to a lesser extent. It is possible that the enzymes showed some preference for 14:0 and 16:0. This is supported by the results from the digestions of 16:0-18:1-16:0 and 18:1-16:0-18:1, above and in Table 4.

In summary, there are distinct differences in some characteristics, including specificity for fatty acids among the several preparations. Neither deglycosylation of *M. Miehei* lipase A to produce B nor immobilization of either preparation altered other specificities studied.

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REFERENCES

1. Kilara, A., *Process Biochem. 20*:36 (1985).
2. Moskowitz, G.J., R. Cassaigne, I.R. West and L.I. Feldman, *J. Agric. Food Chem. 25*:1146 (1977).
3. Eigtved, P., T.T. Hansen and B. Huge-Jensen, in *Proceedings of the 13th Scandinavian Symposium on Lipids* (Lipid Forum), Scandinavian Forum Lipid Res. Technol., Goteborg, Sweden, 1985, pp. 135–148.
4. Nielsen, T., *Fette Seifen Anstrich 87*:15 (1985).
5. Lavayre J., and J. Baratti, *Biotechnol. Bioeng. 24*:1007 (1982).
6. Roland, J.F., R.J. Wargel, W.L. Alm, S.P. Kiang and F.M. Bliss, *Appl. Biochem. Biotechnol. 9*:15 (1984).
7. Huge-Jensen, B., D.R. Galluzzo and R.G. Jensen, *Lipids 22*:559 (1987).
8. Brockman, H.L., *Methods Enzymol. 71*:619 (1981).
9. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, *J. Biol. Chem. 193*:265 (1951).
10. Bio-Rad Model 220 and 221, *Vertical Slab Electrophoresis Cell Instruction Manual*, April 1977.
11. KB Manual 1804, Instructions, LKB Ampholine PAG Plates for Analytical Electrofocusing on Polyacryamide Gels.
12. Hojby, N., and N.H. Axelsen, *Scand. J. Immunol. 17, Suppl. 10*:125 (1983).

13. Kroll, J., Scand. *J. Immunol. 17, Suppl. 10*:135 (1983).
14. Jensen, R.G., F.A. DeJong and R.M. Clark, *Lipids 18*:239 (1983).
15. Jensen, R.G., G.S. Tardiff and R.M. Clark, *J. Pediatr. Gastroenterol. Nutr. 4*:580 (1985).
16. Jensen, R.G. and R.E. Pitas, *Adv. Lipid Res. 14*:213 (1976).
17. Wang, C-S., A. Kuksis and F. Manganaro, *Lipids 17*:278 (1982).
18. Jensen, R.G., F.A. DeJong, R.M. Clark, L.G. Palmgren, T.H. Lia and M. Hamosh, *Lipids 17*:570 (1982).
19. Wang, C-S., A. Kuksis, F. Manganaro, J.J. Myher, D. Downs and H.B. Bass, *J. Biol. Chem. 258*:9197 (1983).