Hydrolysis of Animal Fat and Vegetable Oil with *Mucor miehei* Esterase. Properties of the Enzyme

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Enzymatic action is largely responsible for the enhancement of flavor in a number of food products. The development of the characteristic flavor of Italian cheese is enhanced by the addition of various esterases to the milk, including the *Mucor miehei* (strain Cooney and Emerson) esterase. *M. miehei* esterase will readily attack a number of natural fats such as vegetable oils, beef tallow, and lard oil and a number of synthetic substrates including sorbitol esters of fatty acids. Fatty acid profiles produced by the hydrolysis of soy oil and beef tallow at pH 8.0 with either *M. miehei* esterase or pancreatic lipase are similar. *M. miehei* esterase shows a pH dependent substrate specificity on synthetic triglycerides. The enzyme is more specific for low molecular weight fatty acid containing triglycerides at pH 5.3 than at pH 8.0. At 50 °C, the enzyme has a pH optimum of 9.0 on beef tallow and a temperature optimum of 45 °C when evaluated on olive oil. At 25 °C, *M. miehei* esterase is stable over the pH range of 4.0–10.0 and is also stable up to 45 °C at pH 8.0.

Desirable flavor modifications in food products can be obtained through the controlled lipolysis of fats. The most thoroughly studied application of controlled lipolysis in the food industry is the development of Italian cheese flavor through the use of animal derived pregastric esterases (Arnold et al., 1975). The characteristic flavors of certain Italian cheeses are due in large part to the hydrolysis products of milk triglycerides produced by pregastric esterases derived from calf, kid, or lamb (Harper, 1959; Long and Harper, 1956). Similar flavors can be developed in Fontina and Romano cheese using *Mucor miehei* (strain Cooney and Emerson) esterase in place of the animal pregastric esterases (Peppler et al., 1976; Huang and Dooley, 1976).

Flavor concentrates for salad dressings, snacks, and dips have been developed using microbial lipases to modify milk fat. Blue cheese flavor concentrates have been produced by incubation of milk fat containing substrates with *Penicillium roqueforti* spores or whole cell pastes. These processes have been reviewed by Kinsella and Hwang (1976). Kosikowski has reported that Cheddar cheese flavor can be enhanced by the addition of several fungal lipases in continuous cheese processes (Arnold et al., 1975).

Studies have been undertaken on the role of lipolysis for flavor development in foods containing animal or vegetable fats. The charactertistic aroma of dry or fermented sausages can be attributed to the enzymatic lipid modifications occurring during curing (Shahani et al., 1976). Pet foods consisting of a combination of grains and animal by-products treated with pancreatic lipase preparations have enhanced palatibility (Marshall, 1976). Acceptable flavors have been produced by treatment of pork, beef, or chicken fat with a combination of pregastric esterase and a lipase (Nelson, 1972).

The potential role for *Mucor miehei* esterase in flavor modification of foods containing animal fat or vegetable oil prompted a study of the properties of the enzyme.

MATERIALS AND METHODS

Isolation of *Mucor miehei* Esterase. Esterase and microbial rennet were produced by *Mucor miehei* in the same fermentation. Esterase was obtained, essentially free of rennet, by filtration of the fermentation medium at pH 5.0. The esterase retained in the filter cake was recovered by suspension of the cake in five volumes of water at pH 10.5. The suspension was then filtered, the filtrate was adjusted to pH 6.0 with 1.0 N HCl and spray dried with a NaCl carrier (Moskowitz et al., 1975). The activity of the preparations used varied from 270 to 950 LA units/g.

Reagents. Pancreatic Lipase 250 was purchased from Miles Laboratories, Inc., Elkhart, Ind. Roccal was obtained from Hilton Davis Co., Cincinnati, Ohio. Tallow (a combination of beef and pork tallow) was a gift from Ralston Purina Co., St. Louis, Mo. and lard oil was obtained from Mayco Oil & Chemical Co., Neville Island, Pa. Vegetable oils were obtained as standard supermarket items. The fatty acid standards butyric through stearic were purchased from Polyscience Corp., Niles, Ill. The unsaturated fatty acids were purchased from Sigma Chemical Co., St. Louis, Mo.

ANALYTICAL PROCEDURES

Enzyme Assays. Enzymatic activity was determined titrimetrically. Assays were run at either pH 6.5 or 8.0 using two basic systems that were further modified as required. Enzyme activity (LA units) was defined on the basis of the rate of hydrolysis of an olive oil substrate at pH 6.5. In all experiments, a reaction mixture containing enzyme that had been heat inactivated at 100 °C for 5 min served as the control. In all the kinetic studies, the reactions were linear for up to 1 h under the conditions of substrate and enzyme concentration used.

pH 6.5 Reaction Mixture. A stock of emulsified substrate was prepared by homogenizing 6.0 g of Amerchol L101 (American Cholesterol Products Co., Edison, N.J.), 72 g of olive oil and 222 g of a 10% gum arabic solution in a Waring Blendor for 5 min. The pH was adjusted to 6.5 with 0.5 N NaOH and the emulsion blended for an additional 7 min.

The reaction mixture containing 8.0 mL of homogenized substrate and 2.0 mL of enzyme was incubated with stirring at 30 °C for 5 min. The reaction was stopped by the addition of 40 mL of 95% ethanol and the quantity of free fatty acids released determined by titration to pH 9.5 with 0.02 N NaOH. One LA unit is defined as the amount of enzyme required to produce 1 microequivalent of acid/min under the test conditions.

pH 8.0 Reaction Mixture. Substrate was prepared by dissolving 2.2 g of gum arabic in 21 mL of 0.1 M Tris–HCl buffer, pH 8.0. Thymol (8.3 mg) and 3.8 g of the indicated substrate were added and the mixture homogenized in a Waring Blendor for 5 min to produce an emulsion. The reaction was started by the addition of 1.7 LA units of

Wallerstein Co., Division of Baxter Travenol Laboratories, Inc., Morton Grove, Illinois 60053 (G.J.M., I.R.W., T.S., L.I.F.) and Societé Rapidase, Seclin, France (R.C.).

enzyme in 5.0 mL of water, and the reaction mixture incubated at 37 °C for 40 min. A 5.0-mL aliquot of the reaction mixture was removed and added to 20 mL of 95% ethanol to stop the reaction. Enzyme activity was determined as described previously.

Modified Reaction Mixtures. The effects of salts and inhibitors were studied using modifications of both the LA and pH 8.0 assay. The reaction mixtures were similar to the LA or pH 8.0 assay except that 2.5 mL of the indicated salt or inhibitor was added as a pH adjusted solution prior to the addition of 2.0 LA units of enzyme in 2.5 mL of water.

pH and Temperature Optima Studies. For studies on the pH optimum of the enzyme, the homogenized reaction mixture contained, in a final volume of 25 mL, 2.2 g of gum arabic, 8.3 mg of thymol, and 3.8 g of olive oil. The reaction mixture was adjusted to the appropriate pH with either 0.5 N NaOH or 0.5 N HCl prior to the addition of 2.0 LA units of enzyme in 5.0 mL of water. The pH was maintained potentiometrically throughout the incubation period with 0.02 N NaOH and a pH stat (Radiometer, Copenhagen, Denmark). At the end of the incubation period a 5.0-mL aliquot was removed and titrated as previously described.

For studies on the temperature optimum of the enzyme, the pH 8.0 reaction mixture containing 2.0 LA units of enzyme was used. The reaction mixture was incubated at the indicated temperatures for 40 min. At the end of the incubation period a 5.0-mL aliquot was removed and titrated as previously described.

Hydrolysis of Animal Fats and Vegetable Oils. Product Determination. Studies on the degree of hydrolysis of various animal fats and vegetable oils were conducted under the following conditions. The reaction mixture containing 15.8 g of gum arabic, 0.17 g of thymol, 0.66 mL of Roccal preservative, 0.66 g of sodium taurocholate, and 15.8 g of substrate was adjusted to pH 8.0 with 0.2 N NaOH. The reaction mixture (final volume 150 mL) was then homogenized for 5 min in a Waring Blendor to produce an emulsion. The *M. miehei* esterase enzyme was added as a slurry (5.95 g containing 5670 LA units of enzyme in a total volume of 20 mL of water) and the reaction mixture was incubated at 50 °C for 5 h, at which time the reaction had reached equilibrium. The pH was maintained potentiometrically throughout the incubation period with 0.02 N NaOH. A 5.0-mL aliquot of the reaction mixture was removed periodically and 20 mL of 95% ethanol added to stop the reaction. The quantity of fatty acids released was determined by titration of the aliquot to pH 9.5 with 0.02 N NaOH. The degree of hydrolysis was calculated from the total milliequivalents of base used to maintain pH and to titrate the final 5-h sample. A reaction mixture containing heat inactivated enzyme was used as a control. Reaction mixtures containing pancreatic lipase were similar except that 0.77 g of enzyme containing an equal number of LA units in a final volume of 20 mL of water was used.

A 25-mL aliquot of the reaction mixture was adjusted to pH 3 with 2 N HCl and 50 mL of deionized water plus 15 mL of ethanol were added. The free fatty acids were extracted four times with 50-mL portions of 25/75 petroleum ether–diethyl ether. The extracts were combined and evaporated to 5 mL. The concentrated fatty acids were methylated according to the procedure of Metcalfe and Schmitz (1961) and injected directly into the gas chromatograph.

Gas–Liquid Chromatography. Product Determination. Analysis were performed using a Hewlett-Packard 5830A gas chromatograph equipped with dual-flame ionization detectors. A 6 ft × $^1/_8$ in. i.d. stainless steel column was packed with 10% DEGS-PS on 80–100 mesh chromosorb W, HP, prepared according to Metcalfe (1960). The injector and detector temperatures were 250 and 300 °C, respectively, and the oven temperature programmed from 45 to 200 °C at 6 °C/min with a 45-min hold at 200 °C. The helium carrier gas flow was 18 mL/min. Samples were identified and quantitated by comparison of their retention times with known standards using an attached Hewlett-Packard 18850A computing processor. A 1.0-μL sample was used for injection.

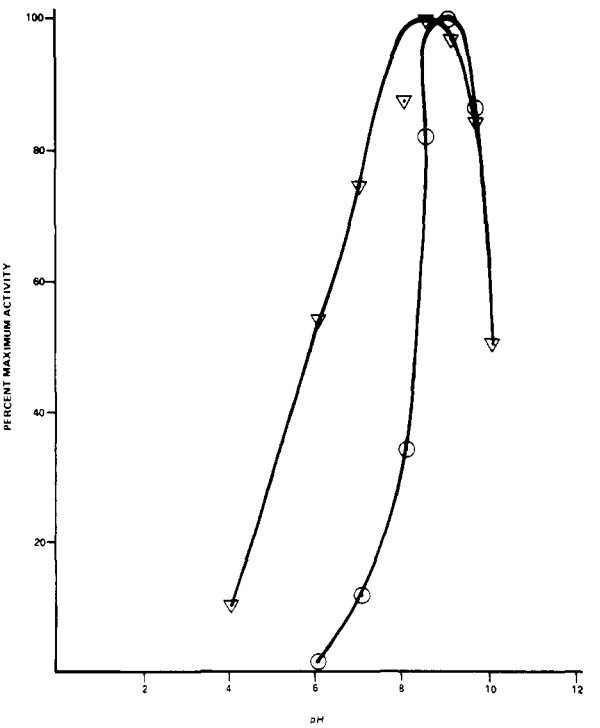

Figure 1. Effect of pH on the activity of *Mucor miehei* esterase: (△) activity at 37 °C on olive oil; (○) activity at 50 °C on beef tallow.

RESULTS AND DISCUSSION

pH and Temperature Optima. As indicated in Figure 1, *M. miehei* esterase has a pH optimum of 8.5 when measured at 37 °C with an olive oil substrate. In contrast, the optimum pH range obtained for the *Mucor* lipase described by Somkuti on olive oil was 5.0–5.5 (Somkuti et al., 1969). At 50 °C on beef tallow, the pH rise for the *M. miehei* enzyme is steeper, the pH optimum is sharper and shifted slightly to a higher pH of 9.0 (Figure 1). This slight shift in pH optimum and change in shape of the curve may be a function of the temperature of incubation and the substrate used. Olive oil is a liquid at room temperature while higher temperatures are required to melt animal fats. Animal fats contain a higher percentage of saturated fatty acids than olive oil and most other vegetable oils (Morrison and Boyd, 1973).

As indicated in Figure 2, enzyme activity increased with increasing temperature up to 45 °C. Above the 45 °C temperature optimum, enzyme activity rapidly decreased.

Effect of pH and Temperature on Enzyme Stability. An enzyme solution (70 mg/mL) was adjusted to the appropriate pH with 0.1 N NaOH or 0.1 N HCl and allowed to stand at room temperature for 4 h. The pH was then adjusted to 6.5 with either 0.1 N HCl or 0.1 N NaOH and the residual activity determined via the LA procedure. As indicated in Figure 3, the enzyme was stable over the

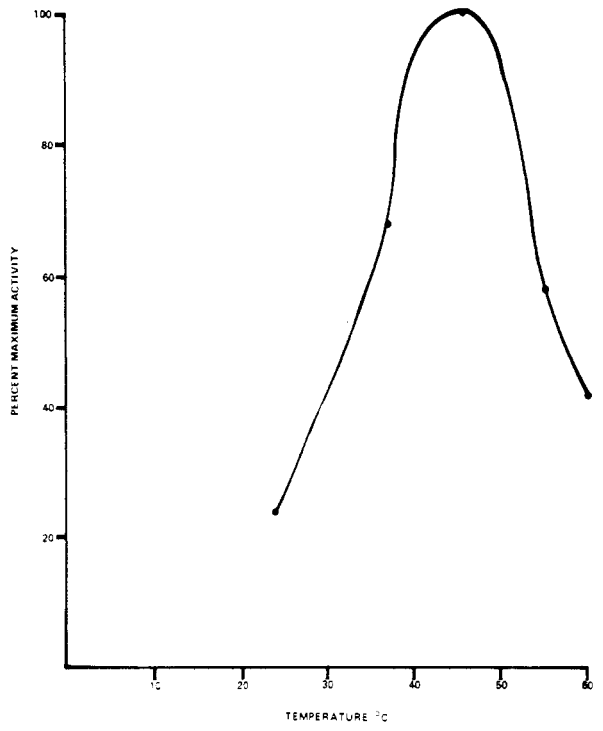

Figure 2. Effect of temperature on the activity of *Mucor miehei* esterase with olive oil substrate at pH 8.0.

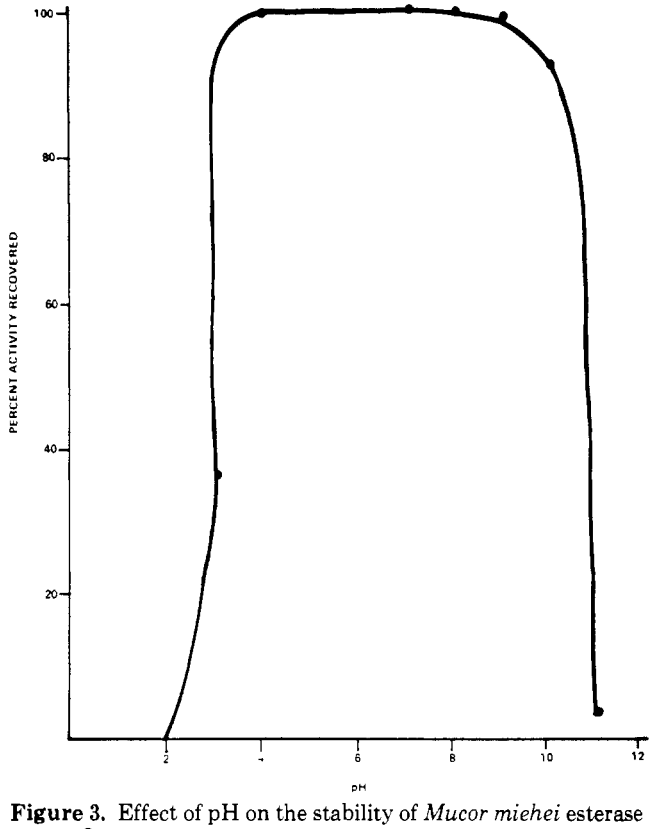

Figure 3. Effect of pH on the stability of *Mucor miehei* esterase at 25 °C.

broad pH range of 4.0–9.0. At pH 10.0, the enzyme retained 93% of the original activity after 4 h at room temperature. The enzyme was unstable at pH's below 4.0.

For studies on the temperature stability of the enzyme, a solution of esterase (33 mg/mL) was adjusted to pH 8.0 with 0.1 N NaOH and aliquots were removed and incubated at various temperatures for 4 h. Residual activity was then determined via the LA procedure. As indicated in Figure 4, the enzyme stability decreased with increasing

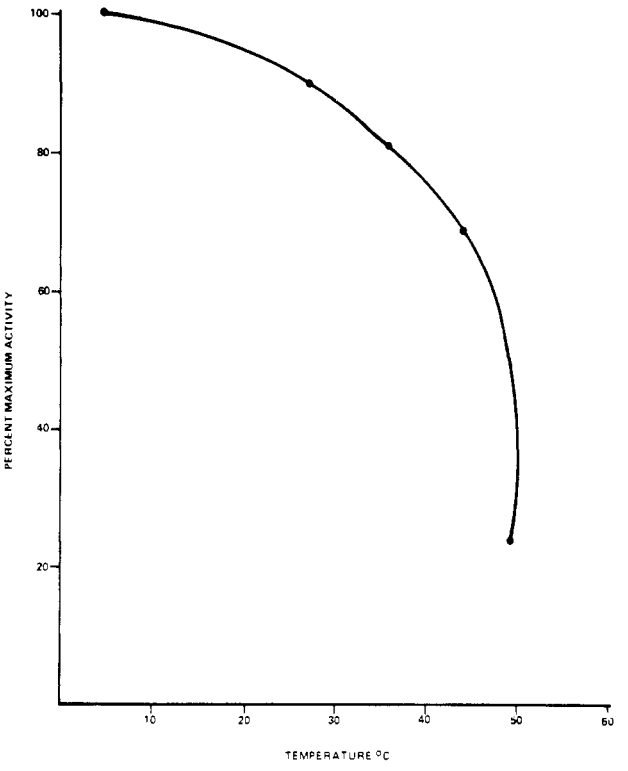

Figure 4. Effect of temperature on the stability of *Mucor miehei* esterase at pH 8.0.

Table I. Effect of Salts on *Mucor miehei* Esterase Activity with Olive Oil Substrate

Metal salt	Concn, M	% activity obtained pH 6.5	pH 8.0
NaCl	0.4	86	94
KCl	0.1	92	100
$CaCl_2$	0.05	100	100
$MgCl_2$	0.005	99	103
$MnCl_2$	0.005	96	66
$CoCl_2$	0.005	97	80
$ZnCl_2$	0.01	28	23
$FeSO_4(NH_4)_2SO_4$	0.001	72	85
$Pb(NO_3)_2$	0.001	81	105
$AgNO_3$	0.01	83	103
$HgCl_2$	0.001	94	87
$CuSO_4$	0.001	70	112
$SnCl_2$	0.001	84	95

temperature. The enzyme was most stable at 5 °C but lost activity even at room temperature in dilute solution. Above 45 °C at pH 8.0, the enzyme activity was rapidly lost.

Effect of Salts and Inhibitors on *M. miehei* Esterase. Table I shows the effect of various salts on enzyme activity with olive oil as the substrate. Except for $ZnCl_2$, none of the salts tested including lead, silver, and mercury salts showed a significant effect on enzyme activity at either pH 6.5 or 8.0. $FeSO_4(NH_4)_2SO_4$, $MnCl_2$, and $CuSO_4$ had a slight inhibitory effect on the enzyme. $MnCl_2$ was a more effective inhibitor at pH 8.0 than at pH 6.5 while the opposite effect was obtained with $CuSO_4$ and $FeSO_4(NH_4)_2SO_4$.

As with the *Mucor* lipase described by Somkuti (Somkuti et al., 1969), the enzyme is not inhibited to any great degree by the chelating agent EDTA nor by a variety of sulfhydryl inhibitors such as PCMB, iodoacetamide, and iodoacetic acid. Thus neither free sulfhydryl groups or metallic cofactors are required for activity. Sodium taurocholate stimulates the reaction about twofold at pH

Table II. Effect of Sulfhydryl Inhibitors EDTA and Sodium Taurocholate on *Mucor miehei* Esterase Activity with Olive Oil Substrate at pH 8.0

Reagent	Concentration	% activity obtained	
		pH 6.5	pH 8.0
EDTA[a]	0.01 M	90	97
PCMB[b]	0.005 M	97	96
Iodoacetamide	0.005 M	88	106
Iodoacetic acid	0.005 M	85	97
Sodium taurocholate	0.4 %	475	184

[a] Ethylenediaminetetraacetate. [b] *p*-Chloromercuribenzoate.

Table III. Relative Rate of Hydrolysis of Natural Lipids with *Mucor miehei* Esterase at pH 8.0

Substrate	% activity of olive oil
Tributyrin	121
Milk fat	113
Olive oil	100
Soy oil	100
Peanut oil	100
Cottonseed oil	97
Corn oil	89
Sesame oil	86
Sunflower oil	84
Lard oil	83
Beef tallow	77
Castor oil	45

8.0 and approximately fivefold at pH 6.5 (Table II).

Reaction Rate on Soluble vs. Emulsified Substrates. Desnuelle and Savary (1963) differentiate between esterases and lipases based on their relative rates of reaction on water soluble as opposed to emulsified, water insoluble substrate. Esterases were more active on soluble than insoluble, emulsified substrates.

Although triacetin was the most water soluble synthetic triglyceride, it was a poor substrate for the enzyme. The effect of the enzyme on soluble and emulsified substrate was therefore tested using tributyrin. The rate of reaction of the enzyme on emulsified tributyrin was studied in the pH 8.0 reaction mixture containing 2.7 LA units of enzyme. The system was modified for studies on the soluble substrate. In order to render the tributyrin soluble, the reaction mixture contained one-third the level of tributyrin indicated in the previous assays and 8% ethanol. Previous studies had indicated that neither modification significantly effected the enzyme activity. The reaction mixture for soluble substrate studies contained 0.063 M Tris–HCl buffer (pH 8.0), 8.3 mg of thymol, 1.3 g of tributyrin, 2.5 mL of 95% ethanol and enzyme (2.7 LA units in 2.5 mL of water) in a final volume of 30 mL. Incubation and assay were performed as previously described.

The relative rate of hydrolysis of soluble substrate was 1.21 times that of the emulsified insoluble substrate. On this basis, the *Mucor miehei* enzyme can be classified as an esterase.

Substrate Specificity. *M. miehei* esterase hydrolyzed a number of natural fats and oils as indicated in Table III. Of the substrates tested, milk fat, olive, soy, peanut, and cottonseed oils were the most readily attacked. Corn, sesame, and sunflower oils were hydrolyzed at about 80–90% of the rate on olive oil. Lard oil and beef tallow were hydrolyzed at a slightly lower rate. Castor oil was hydrolyzed at about half the rate of olive oil.

When the enzymatic hydrolysis had gone to completion (reached equilibrium), at pH 8.0 and 50 °C, 90% of the fatty acid esters in soy and corn oil, 70% of the fatty acid esters in beef tallow and lard oil, and 57% of the fatty acid esters, in butter fat had been hydrolyzed (Table IV). Although the enzyme rapidly hydrolyzed butter fat as measured by the initial rate of reaction (Table III), the degree of hydrolysis at equilibrium, did not approach that obtained with the other substrates tested. No explanation for this was apparent.

Table IV. Percent Hydrolysis of Natural Lipids at pH 8.0 and 50 °C at Equilibrium

Substrate	% hydrolysis
Soy oil	89.5
Corn oil	89.1
Beef tallow	69.9
Lard oil	67.3
Milk fat	56.7

Table V. Free Fatty Acids Produced by *Mucor miehei* Esterase and Pancreatic Lipase on Soy Oil and Beef Tallow at pH 8.0 and 50 °C

Fatty acid	Mole % at equilibrium	
	M. miehei	Pancreatic lipase
	Soy oil	Soy oil
Palmitic	9.2	11.1
Stearic	4.0	4.7
Oleic	20.6	20.9
Linoleic	60.1	54.5
Linolenic	6.0	8.8
	Beef tallow	Beef tallow
Myristic	3.1	3.0
Palmitic	23.8	23.7
Stearic	22.9	22.9
Oleic	43.8	43.7
Linoleic	6.1	6.0
Eicosenoic	0.4	0.8

Table VI. Relative Rate of Hydrolysis of Synthetic Substrates by *Mucor miehei* Esterase at pH 8.0

Substrate	Relative rate of hydrolysis
Tributyrin	100
Tween 20	123
Tween 40	63
Tween 60	54
Tween 80	148

The free fatty acids produced from soy oil and beef tallow at equilibrium at pH 8.0 were similar to those produced by pancreatic lipase under the same conditions (Table V). In both cases the profiles reflected the fatty acid distribution found in the natural fat. The beef tallow sample consisted of a mixture of beef and pork fat as evidenced by the 6% content of linoleic acid (Morrison and Boyd, 1973).

The enzyme readily hydrolyzed the Tween series which consist of sorbitol esters of fatty acids (Table VI). The enzyme, therefore, is not specific for the alcohol portion of the ester. *M. miehei* esterase most readily attacked the lauric and oleic acid containing sorbitol esters (Tween 20 and Tween 80, respectively) while those containing palmitic (Tween 40) and stearic (Tween 60) acids were less readily attacked.

The relative rates of hydrolysis of a series of synthetic triglycerides containing fatty acids of varying chain length were studied at pH 5.3 (the approximate pH of Italian cheese ripening) and pH 8.0. The triglyceride series triacetin through tridecanoin were studied in the pH 8.0 reaction mixture described in the analytical procedures section. The triglyceride series containing lauric through stearic acid and a tributyrin control were studied in a similar system using 0.43 g of polyvinyl alcohol to stabilize

Table VII. Relative Rate of Hydrolysis of *Mucor miehei* Esterase on Synthetic Triglycerides of Different Chain Length

Triglyceride	% activity of tributyrin at pH 5.3	pH 8.0
Triacetin	7.7	8.7
Tripropionin	42	30.6
Tributyrin	100	100
Trihexanoin	80	79.8
Trioctanoin	96.5	175.5
Tridecanoin	32.6	86.5
Trilaurin	35.2	137.6
Trimyristin	24.5	111
Tripalmitin	18	333
Tristearin	1.3	100

the emulsion in place of gum arabic. The controls contained heat inactivated enzyme. The reaction mixture at pH 5.3 was similar except that it was buffered with Na acetate buffer, pH 5.3, and 2.7 LA units of enzyme were used.

As indicated in Table VII, at pH 5.3 and with the exception of triacetin, the enzyme preferentially attacked low molecular weight fatty acid esters as measured by the rate of hydrolysis after 40 min. Tributyrin and trioctanoin were most readily hydrolyzed at pH 5.3. At pH 8.0, tributyrin and trioctanoin were also attacked, but in contrast to the data at pH 5.3, the high molecular weight fatty acids were readily hydrolyzed. At pH 8.0, tripalmitin was reproducibly hydrolyzed at three times the rate of tributyrin while at pH 5.3 the relative rate of hydrolysis of tripalmitin was only 18% that of tributyrin. This apparent specificity for palmitic acid was not observed in the product analysis (Table V) probably because 70–90% of the substrate had been hydrolyzed.

Both *M. miehei* esterase and the lipase described by Somkuti (Somkuti and Babel, 1968) attacked synthetic triglycerides of varying chain length as well as vegetable oils and butter fat. The *M. miehei* enzyme at pH 5.3 showed optimum activity on tributyrin and trioctanoin while the other Mucor enzyme was most active on trioctanoin and tridecanoin (Somkuti and Babel, 1968).

The apparent specificity of *M. miehei* esterase for the fatty acid portion of the triglyceride was altered with change in pH. At pH 5.3, the enzyme showed a specificity for low molecular weight fatty acid esters. This observation could explain the successful use of the enzyme in the manufacture of Italian cheeses. At pH 8.0, this specificity was altered. The enzyme readily hydrolyzed high molecular weight fatty acid esters of both saturated and unsaturated fatty acids. The free fatty acid profile produced by hydrolysis of animal fats and vegetable oils at pH 8.0 was similar to the product profile produced by pancreatic lipase. This property of the enzyme could have significant effects on the flavor modifications of foods containing milk fat.

The ability of the enzyme to hydrolyze a number of natural fats and oils and the similarity of the products produced by *M. miehei* esterase to those produced by pancreatic lipase at pH 8.0 may prove to be of considerable value in the processing of foods containing animal fats or vegetable oils.

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