Influence of Water Binders on the Activity and Thermal Inactivation of Lipase

ENRIQUE J. GUARDIA AND GERHARD J. HAAS

When water binders (sucrose, glycerol, or propylene glycol) were added to liquid olive oil emulsions at pH 9.0, the activity of hog pancreatic lipase was inhibited. At pH 7.0, however, addition of these water binders significantly accelerated lipolytic activity. Acceleration was also found when propylene glycol was used at pH 6.0. In contrast, a lipase of fungal origin was completely inhibited by propylene glycol at concentrations which yielded maximum activation of pancreatic lipase. Sucrose and glycerol significantly protected pancreatic lipase against thermal inactivation, while propylene glycol increased its thermolability. Pancreatic lipase was active at moisture levels as low as 0.06% under the conditions tested.

The removal of water from tissues arrests the growth of the associated microflora and inhibits the activity of enzymes. This susceptibility of microorganisms and enzymes to the lack of moisture appears to be related more directly to the physical and chemical availability of the water than to its absolute concentration (*2*, *19*). A useful index of the state of the water in foods is the ratio p/p_o, where p is the partial pressure of the water vapor above the food, and p_o is the partial pressure of saturated water vapor. This ratio is referred to as the Aw index (available water) and is also expressed as a percentage ($p/p_o \times 100$), which is the equilibrium relative humidity (ERH).

Several detailed reviews are available in the literature relating enzyme activity to water concentration and equilibrium relative humidity (*1*, *8–10*). McLaren (*13*, *14*) has suggested a system of kinetics based on the use of mole fractions, rather than moles per unit volume, for low moisture systems, or systems which are "structurally restricted."

In general the activity of the endogenous enzymes of foods decreases as the free water in the tissues is reduced. Most of these studies have been carried out on natural foods or dry model systems, and they have greatly advanced our understanding of the problem. However, one limitation to this type of system is the lack of homogeneous distribution of the water in the tissue. Acker (*1*), for example, believes that the interaction of enzyme and substrate in dry materials is largely dependent on free capillary water. Thus, the state of the water at these highly localized capillary sites would determine enzyme action, and measurements of the water activity of the whole tissue would not be representative of these conditions.

Several investigators, among them Blain (*3*), have studied the effects of water availability on enzyme activity using liquid model systems. The inhibition of several hydrolytic and oxidative enzymes has been demonstrated using these model systems, where water was made less available by adding water-binding compounds—i.e., glycerol, sucrose—to the substrate solution. However, there was no attempt to relate the results to the equilibrium relative humidity of the system. Liquid model systems permit the evaluation of the relative importance of other factors because they are not affected by the problems which are inherent to dry systems, such as lack of homogeneity.

Lipases catalyze the hydrolytic split of triglycerides, producing nonesterified fatty acids, di- and monoglycerides, and glycerol. Lipolytic activity can result in the development of "off" (soapy) flavors usually associated with the so-called hydrolytic rancidity. This type of enzymatic rancidity has long been recognized as a problem in dry foods, and it has been implicated in the development of undesirable flavors in many dry food products (*1*).

The effect of water activity on lipase action has been investigated in many foods. Lipase has been found active in oatmeals of 6 to 12% moisture content (*7*) and wheat flour having 6% moisture (*5*). Lovern (*11*) found an increase in free fatty acids in frozen fish muscle stored at −30° C. This increase is believed to be due to the action of endogenous enzymes. At −30° C. less than 10% liquid water (by weight) can be expected in fish muscle (*22*). The increase in fat acidity during the storage of dried beef (3.2% moisture) is believed due to lipase activity (*21*). These investigations suggest that lipase is capable of activity at very low moisture levels and presumably low relative humidity. To our knowledge, however, all studies on the effect of moisture on lipase have been conducted on dry systems.

Our study was undertaken to determine the effect of water concentration and water binders on the activity of hog pancreatic lipase, using liquid emulsion model systems. As means of comparison, two other lipases were used, a fungal lipase and a plant lipase. Unfortunately, under the conditions of our experiment, the plant (wheat germ) lipase was never active enough to yield meaningful results, because this enzyme is active only against esters of short carbon chain fatty acids and should be abeled an "esterase" rather than a lipase (*6*, *15*).

Sucrose was chosen as the water binder, since its presence in foods makes it a compound of choice. In addition, two other polyhydroxy compounds of known water-binding activity were used, propylene glycol and glycerol.

The threshold value for moisture necessary for lipase

Corporate Research Department, Technical Center, General Foods Corp., Tarrytown, N. Y.

activity in olive oil was also studied. For this, it was necessary to study lipase in enzyme-water-oil suspensions, in the absence of emulsifier. Finally, the effects of water binders on the thermal inactivation of the enzyme were evaluated.

Experimental

Enzyme. Pancreatic lipase (Miles Chemical Co., Clifton, N. J.), Lipase B (Rohm and Haas, Philadelphia, Pa.), and wheat germ lipase (Mann Research Laboratories, Inc.) were used. The first two enzyme preparations were not completely soluble in water, so 1 or 10% suspensions were made and centrifuged at 7000 G for 10 minutes. The clear supernatants were used as the enzyme solutions. One milliliter of the 1% centrifuged solution, containing 0.41 mg. of protein nitrogen (by Kjeldahl) of pancreatic lipase, was used at pH 7.0 and 9.0, and 1 ml. of a 10% centrifuged solution, containing 4.19 mg. of protein nitrogen (by Kjeldahl), at pH 6.0 (at this pH the enzyme was considerably less active). One milliliter of a 10% centrifuged solution of lipase B was used at pH 7.0. These enzyme solutions were not stable at refrigerator temperatures (4° C.), so when they had to be kept longer than 1 day, their activity had to be standardized daily. Wheat germ lipase was used at 0.1 to 1 per weight % of the oil present.

Emulsions. The emulsions were made by a modification of the method of Marchis-Mourem, Sarda, and Desnuelle (*12*). Commercially available "100% pure" Progresso olive oil (imported and packed by Uddo and Taormina Corp., Jersey City, N. J.) was used as a substrate. This oil had a free fatty acid value of 0.24% (as oleic acid) and a peroxide value of 6.2 mg. per kg. Five milliliters of this oil and 5 grams of ice were added to a suspension containing 4 grams of gum arabic in 40 ml.of water or an appropriate solution of sucrose (J. T. Baker reagent grade), glycerol (Mallinckrodt analytical grade), or propylene glycol (Matheson Coleman reagent grade). The mixture was emulsified by blending for 10 minutes in a high speed blender immersed in an ice-water bath. Thirty milliliters of the emulsion were pipetted into a 100-ml. beaker, and 0.3 ml. of a 20% solution of sodium taurocholate (City Chemical Corp., New York, N. Y.) were added. The emulsion was equilibrated to the proper temperature in a water bath, adjusted to the proper pH by adding a few drops of 1*N* NaOH, and at zero time 1 ml. of the appropriate enzyme solution was added.

Lipase Activity. The method of Marchis-Mourem, Sarda, and Desnuelle (*12*) was employed to determine lipase activity at pH 7.0 and 9.0. To avoid changes in the concentration of water binders during the reaction, the titrant (0.04*N* NaOH) was made up in solutions containing the water binder (sucrose, glycerol, or propylene glycol) in the same concentration as the emulsion being titrated. The final normality of the titrant was determined against standard HCl. A Beckman pH meter Model H2 was used in the potentiometric titration. All reactions were carried out for at least 30 minutes.

The activity of the enzyme was determined by extrapolating from the initial rate of the reaction. The lipase activity in the absence of the water binder was defined as 100%.

No attempt was made to identify any of the products of these reactions.

Milk Fat. As lipase action on milk fat has been of considerable recent research interest, the authors decided to use this substrate and study the effect of water binders in this system.

These experiments were carried out as follows: Fifteen milliliters of water, or 15 ml. of a sucrose solution, or 15 ml. of propylene glycol, were added to 15 ml. of a mixture of half cream, half milk (10% fat). Three milliliters of a 10% centrifuged solution of pancreatic lipase or, respectively, 150 mg. of wheat germ lipase were added to this mixture, and lipase activity was determined as before.

Low Moisture Activity. One-tenth of a gram of pancreatic lipase powder was added directly to 30 ml. of olive oil and agitated with a magnetic stirrer for 68 hours at 30° C. A control containing no enzyme was treated in an identical manner. Lipase activity was determined by taking the oil into one phase with chloroform, methanol, and water (*4*) and titrating with 0.04*N* alcoholic KOH to a phenolphthalein end point.

Activity in the Absence of Emulsifier. Twenty-seven milliliters of water, 60% (v./v.) propylene glycol in water, 40% (w./v.) sucrose in water, or 5% (v./v.) glycerol in water (adjusted to pH 7.0) were added to 3 ml. of olive oil. One milliliter of the enzyme solution was added and the mixture was kept in suspension by violent agitation with a magnetic stirrer. Activity was determined after 68 hours. The volume of alkali taken up by the control was reported as 100% and the others as a fraction or multiple of the control.

Karl-Fischer Titration. The titration described by Seaman, McComas, and Allen (*20*) was employed.

Equilibrium Relative Humidity. This parameter was determined using an electric hygrometer (American Instrument Co., Inc., Silver Spring, Md.). A 50-ml. sample of the emulsion was placed in a 250-ml. Erlenmeyer flask and agitated by means of a magnetic stirrer. The sensor was placed directly above the emulsion, and the whole system was sealed until it reached equilibrium. The equilibrium relative humidity was then determined.

Results

At pH 9.0 and 37° C. the activity of pancreatic lipase on olive oil decreased at increasing concentrations of glycerol, propylene glycol, or sucrose (Table I). The inhibition of the enzyme was not directly proportional to the concentration of the water binder, and in some cases inhibition was not significant until relatively high amounts of water binder were present in the emulsion (60% propylene glycol, 60% glycerol).

At pH 7.0 and 37° C. there was an initial increase in activity as the water binder concentrations increased, then the activity declined. The region of maximum activity was different for the three compounds (between

Table I. Activity of Pancreatic Lipase on Olive Oil in the Presence of Water Binders at 37° C.

	Activity, %		
Propylene glycol, % (v./v.)	pH 9.0	pH 7.0	pH 6.0
0	100	100	100
10	...	100	...
20	100	...	160
40	100	215	140
60	65	230	160
80	5	140	70
Glycerol, % (v./v.)			
0	100	100	100
5	100	130	75
20	110	60	65
40	100	...	30
50	...	25	...
60	55	...	1
80	40	20	...
Sucrose, % (w./v.)			
0	100	100	...
5	100	...	...
20	90	160	...
35	80	...	...
50	75	180	...
65	65	140	...
90	30	...	...

Table II. Equilibrium Relative Humidity[a] of Olive Oil/Gum Arabic Emulsions Containing Water Binders

	Equilibrium Relative Humidity		
	pH 6.0	pH 7.0	pH 9.0
Propylene glycol, % (v./v.)			
20	99	99	99
40	99	99	99
60	96	95	95
80	89	82	81
Glycerol, % (v./v.)			
20	99	99	99
40	93	99	99
60	72	83	73
80	45	49	46
Sucrose, % (w./v.)			
20	99	99	99
40	99	99	99
60	99	99	99
70	99	99	99

[a] Values are an average of two determinations.

0 and 20% for glycerol, 40 and 80% for propylene glycol, and 20 and 65% for sucrose). This increase in activity was apparent also at pH 6.0 and 37° C. when propylene glycol was used, but it did not occur with glycerol (Table I). If activity was determined by the total acidity released after 15 or 30 minutes rather than extrapolation, the relative activation by the binders was not as large, although the general qualitative effect was the same. This increase in lipase activity owing to the water binders tested does not seem to be related to the water content of the emulsion or its equilibrium relative humidity (Table II).

This increase in lipase activity, which was greatest in the presence of propylene glycol, could possibly be due to a change in the physical state of the fat in the emulsion —i.e., particle size. Figure 1 shows photomicrographs of the olive oil emulsions made in the presence and absence of propylene glycol at pH 7.0. The emulsion in 60% propylene glycol, which increased lipase activity to 230%, had a larger fat particle size than the control. Since the activity of lipases increases with surface area at the interface (*17, 18*), apparently emulsion size cannot explain this increase in activity.

The lipolytic activity of pancreatic lipase was also tested in mixtures of olive oil with solutions of water binders without gum arabic. The mixtures were kept violently agitated by a magnetic stirrer to avoid the separation of the two phases. The sample containing 60% propylene glycol showed considerably greater lipolysis than the control (Table III). Forty per cent sucrose showed no difference, and 5% glycerol slightly inhibited lipolysis under these conditions. Results indicate that the increase in lipolysis brought about by propylene glycol is not due to interactions with the emulsifier, since even in the absence of gum arabic there is greater lipase action upon addition of propylene glycol.

Fifty per cent propylene glycol increased the activity of pancreatic lipase in milk fat (half milk and half cream), while sucrose (40%) inhibited the enzyme activity in this substrate (Figure 2).

A fungal lipase (lipase B) was studied under identical conditions (except that temperature was increased to 45° C., since the rate at 37° C. was too slow). In contrast to pancreatic lipase, the activity of this enzyme decreased greatly as the concentration of propylene glycol increased, and was completely inhibited by 60% propylene glycol (Table IV), the concentration at which pancreatic lipase shows maximum activity (Table I).

Figure 3 shows the effect of sucrose, glycerol, and propylene glycol on the thermal inactivation of pancreatic lipase at 60° C. In the presence of sucrose (50% w./v.), or glycerol (70% v./v.), the enzyme followed a typical activity *vs.* time curve. In the absence of water binders the enzyme was inactivated after about 10 minutes. Propylene glycol offered no protection against thermal inactivation and, in fact, increased the susceptibility of the enzyme to heat (Figure 3). Thus, it is not likely that propylene glycol increases the activity of lipase by stabilizing the enzyme.

Table V shows that even at 0.06% moisture (45% ERH) lipase is active. The rate of reaction at this low moisture level could be increased about 25-fold by addition of 2% water (99% ERH).

To test whether surface inactivation of the enzyme in oil could account for the reduced activity at low moistures, 2% water was added to a sample of enzyme in oil containing 0.06% water, which had been incubated for 48 hours. Twenty hours after the addition of water, the total activity was approximately 20 times greater

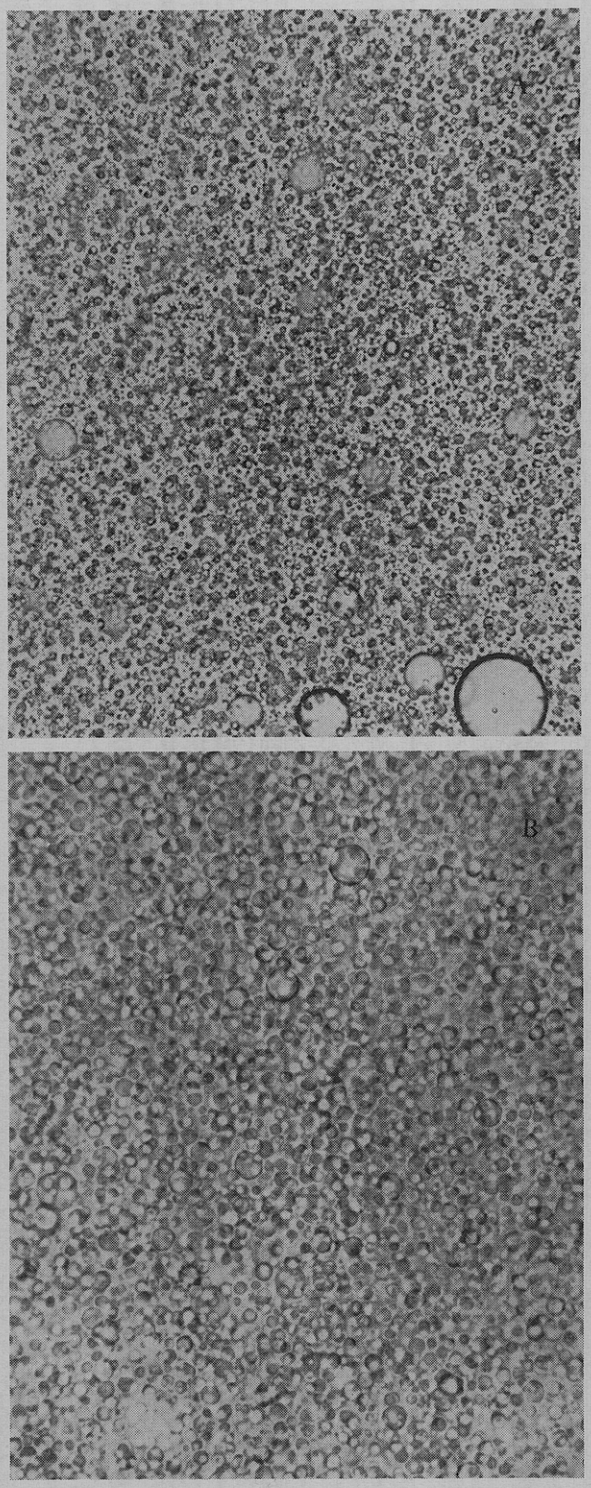

Figure 1. Gum arabic emulsions of olive oil in the presence and absence of propylene glycol

Top, *A*. Control, pH 7.0
Bottom, *B*. 60% Propylene glycol, pH 7.0

than that in the low moisture sample (total reaction time was 68 hours, Table V).

Discussion

During the preparation of this manuscript Purr (*16*) described the investigation of the activity of a lipase

Table III. Effect of Water Binders on the Activity of Pancreatic Lipase on Olive Oil in the Absence of Emulsifiers at 30° C., 68 Hours

	Activity, %[a]
Water (control)	100
60% v./v. propylene glycol	170
40% w./v. sucrose	100
5% v./v. glycerol	80

[a] Average of two titrations.

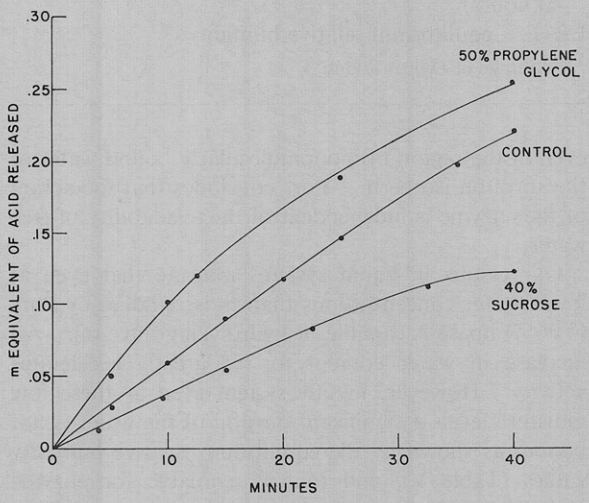

Figure 2. Effect of water binders on the activity of pancreatic lipase on milk fat

pH 7.0 and 37° C.

Table IV. Activity of Fungal Lipase in the Presence of Propylene Glycol pH 7.0, 45° C.

Lipase B (fungal lipase)	
Propylene glycol, % (v./v.)	Activity, %
0	100
10	65
40	2
60	0

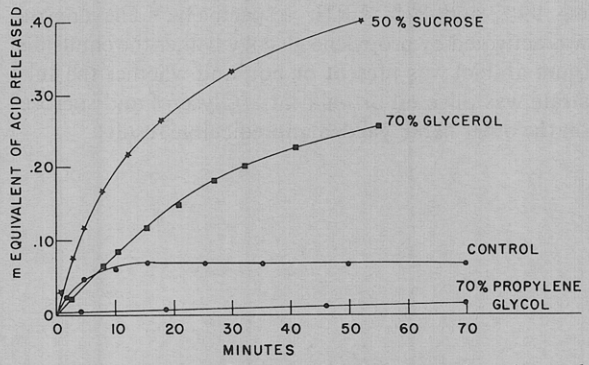

Figure 3. Effect of water binders on the thermal inactivation of pancreatic lipase

pH 7.0 and 60° C.

mixture (pancreatic lipase and lipase from *Aspergillus oryzae* and a lipase from an unknown source) in dry systems of variable water content and relative humidity. His results indicate that the enzyme activity proceeded

Table V. Activity of Pancreatic Lipase on Olive Oil at Low Moisture Levels 30° C. for 68 Hours

	0.04*N* KOH, Ml.[a]
Oil control (no enzyme)	1.80
Oil + 2% water (no enzyme)	1.95
Oil + enzyme (0.06% water-ERH 43%)	4.65
Oil + enzyme (2% water-ERH 99%)	102.00
Oil + enzyme (2% water added after 48 hours)	90.00

ERH = equilibrium relative humidity

[a] Average of two titrations.

even in the region of monomolecularly bound water of the sorption isotherm. Purr concludes that the action of the enzyme is independent of the availability of free water.

Our results in liquid systems indicate that even at lower water concentrations than those studied by Purr (0.06%) lipase is capable of hydrolyzing olive oil. An increase in water content to 2% greatly accelerates activity. However, in our system even at these low moisture levels a significant portion of the water is not bound, as shown by the equilibrium relative humidity values (Table V), and thus is available for enzyme activity.

The enhancement of lipase activity by the water binders appears to be specific for the pancreatic lipase. This interaction was greatly pH-dependent and was not found when the fungal lipase was used. It is unlikely that this enhancement is brought about by modifications in the state of the fat in the emulsion, since such diverse results were obtained with the two lipases, or that the effects of water binders on the activity of the enzyme are due to the physical state of the water in the system, since similar enhancement of activity could be obtained with two different compounds at different equilibrium relative humidities—i.e., 140% activity was obtained at pH 7 with 80% propylene glycol and 65% sucrose having 99% and 82% ERH, respectively. The enzyme was activated by propylene glycol whether the emulsifier (gum arabic) was present or not, and whether the substrate was olive oil or milk fat. Glycerol and sucrose, on the other hand, yielded unpredictable results.

Glycerol, one of the products of fat hydrolysis, in addition to enhancing lipase activity may also inhibit the reaction by mass action. The over-all manifestation of its effect would then be the sum of these two actions.

The nature of the different and specific effects of the water binders on the pancreatic lipase is not clear at this time. However, since water-binding compounds are present in many foods, their role as potential lipase activators should be realized. In addition, the effect of sucrose and glycerol in protecting the enzyme against thermal inactivation may be important in pasteurization and blanching processes designed to heat-denature the enzyme.

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Received for review October 13, 1966. Accepted March 6, 1967.