Stability of Porcine and Microbial Lipases to Conditions that Approximate the Proventriculus of Young Birds

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ABSTRACT In vitro experiments were conducted to characterize the activity and the stability of lipase from animal (crude porcine, CPL; lyophilized porcine, LPL), fungal (Rhizopus arrhizus, RAL; Aspergillus niger, ANL), and bacterial (two Pseudomonas spp., PL1, PL2; and Chromobacterium viscosum, CVL) sources when exposed to conditions associated with the glandular stomach. Activity was measured at pH 3 to 8, 40 C and then monitored in response to temperature (40 C), time of exposure (0 and 30 min), pH (3 and 7), and pepsin level (5, 50, and 500 U/mL). All lipases except ANL and CVL had maximum activity at pH 7 to 8. The optimal pH for ANL and CVL were 5 and 6 to 8, respectively. Exposure of lipases to 40 C and pH 7 for 30 min reduced the activity of all lipases except ANL. In contrast, 40 C increased ANL activity 2.5-fold. Although activity of all lipases was reduced by exposure to pH 3, it was nearly eliminated for CPL and LPL. Pepsin concentration had only minor effects on lipase activity and then only at high concentration. The results demonstrate that bacterial lipases (PL1, PL2, and CVL) and ANL are more stable under conditions that approximate the glandular stomach and may explain why dietary porcine lipase has been ineffective in preventing fat malabsorption in previous in vivo studies.

(Key words: porcine lipases, microbial lipases, enzyme activity, pepsin, acidic pH)


INTRODUCTION

Studies on the digestibility of fats by chickens (Carew et al., 1964) and turkeys (Sell et al., 1986; Leeson and Atteh, 1995) have shown that the utilization of saturated fats is lower in young than in mature birds. The reasons for poor fat digestibility are not resolved but may reflect the underdeveloped state of gastrointestinal functions at the time of hatch. Potential reasons are a rapid feed transit time (Vergara et al., 1989), and low levels of bile salt (Krogdahl and Sell, 1984) and pancreatic secretions (Noy and Sklan, 1995).

Relative to the adult, a lower digestibility of a given nutrient will occur in the young bird if the process of digestion and absorption is not complete during the period of gastrointestinal passage. Additives that are targeted toward improving the efficiency of the specific step or steps that limit the overall process of triglyceride digestion and absorption have the potential to increase fat digestibility in the young bird. In the young bird, bile salt secretion is lower and enterohepatic circulation of bile salts is higher than occurs in the adult (Jackson et al., 1971; Green and Kellogg, 1987). However, dietary supplementation with a low amount of bile salts (0.025%) has resulted in only a small change in fat utilization (Gomez and Polin, 1976). In comparison with the digesta of humans and rats (Watkins, 1975), relatively high concentrations of bile salts (2 vs 14 mM/L) have been found in the 2-d-old chick (Green and Kellogg, 1987). These observations suggest that bile salt insufficiency may not be the primary cause of poor fat utilization in the young birds (Gomez and Polin, 1974, 1976).

Numerous reports indicate that the concentration of digestive enzymes in poultry increase with age (Krogdahl and Sell, 1989; Pubols, 1991; Noy and Sklan, 1995). Duodenal activity of lipase in young chicks increases 20 times between 4 and 21 d of age (Noy and Sklan, 1995). In turkeys, the lipase activities of pancreas and intestinal contents were relatively low in the newly hatched bird, increased slowly to about 6 wk of age, and then increased rapidly to 8 wk of age (Krogdahl and Sell, 1984, 1989). The relatively low rate of pancreatic lipase

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secretion in young birds may be insufficient for substantial triglyceride hydrolysis and therefore could contribute to poor fat utilization.

Dietary supplementation with lipase has the potential to improve fat utilization in situations of pancreatic lipase insufficiency. In humans, pancreatic enzyme replacement therapy is used routinely to treat malabsorption (Saunders and Wormsley, 1975). However, dietary supplementation with pancreatic lipase results in only a partial improvement in fat digestibility (DiMagno et al., 1973). The use of dietary supplemented pancreatic lipase, with the exception of one paper (Polin et al., 1980), has not been reported in poultry studies and most results are based on pancreatic insufficiency disease in humans (DiMagno et al., 1977). Supplementation of crude porcine lipase (steapsin) in a corn-soybean diet containing 4% tallow was tested in young chicks but did not produce beneficial effects on fat utilization or bird performance (Polin et al., 1980). Possible reasons as to why supplemental pancreatic lipase is ineffective or less effective than anticipated include destruction of pancreatic enzymes in the acid environment of the stomach, the variability in the potency of commercial pancreatic lipase supplements, and loss of pancreatic enzymes during passage through the small intestine (DiMagno et al., 1977; Raimondo and DiMagno, 1994).

Dietary lipase can be protected from the adverse conditions of the gastric stomach by a number of methods. For example, gastric pH can be increased by the use of antacids or cimetidin, which inhibit gastric acid secretion. Lipase can be protected by the use of enteric coating procedures. Another possibility is to use enzyme sources that are stable under conditions of the stomach.

No information is available as to the relative stability of various native lipase sources under conditions of the avian proventriculus in young birds. The objective of this study was to evaluate the activity and stability of lipase sources after exposure to conditions that approximate passage through the proventriculus.

**MATERIALS AND METHODS**

A total of seven sources of lipase were used in this study. Crude porcine lipase (CPL, catalog no. L 3126), lyophilized porcine lipase (LPL, 50 kDa, catalog no. L 0382), and *Rhizopus arrhizus* lipase (RAL, 40 kDa, catalog no. L 4384), were obtained from Sigma. Aspergillus niger lipase (ANL, 35 kDa, experimental), and Pseudomonas spp. lipase (PL1, 30 kDa, experimental) were obtained from Finnfeeds International and *Chromobacterium viscosum* lipase (CVL, 73.5 kDa, catalog no. 5045), and *Pseudomonas* spp. lipase (PL2, catalog no. 6044) from Karlan Research Products Corp.

**Measurement of Lipase Activity**

Lipase activity was measured in 10 mL of reaction mixture containing 1 mM tris-HCl buffer, 2 mM CaCl$_2$, 150 mM NaCl (Borgstrom and Erlanson-Albertson, 1973). The tributyrin (ICN) concentration and the pH of the mixture were adjusted as required for the particular experiment. The reaction was initiated by addition of about 50 units of lipase activity and the rate of tributyrin hydrolysis monitored by continuously measuring the volume of a stock solution of NaOH added to the mixture to maintain pH over a 6-min period. Initial rates of tributyrin hydrolysis were calculated as the slope of the regression line for the linear portion of the reaction and expressed as micromoles fatty acids released per minute. Duodenal pH and the temperature of most birds are close to 7 and 40 C, respectively. These conditions, in the absence of any preincubation states, were chosen as the control. To determine the effect of temperature on lipase activity, the enzymes were exposed to 40 C for 30 min and then activity was measured at pH 7. Lipases were exposed to pH 3 for 30 min at 40 C and then activities were measured at pH 7 to see the effect of acidity on lipases. In order to study the effect of pepsin (catalog no. P 7125), lipases were exposed to varying concentrations of pepsin for 30 min at pH 3, 40 C; activities were then measured at pH 7. Each assay was replicated three times. Protein concentration of lipases was measured spectrophotometrically at 595 nm using Sigma procedure 610-A that employs Coomassie Brilliant Blue as a protein dye reagent.

Data were analyzed according to the General Linear Models (GLM) procedure of SAS (SAS Institute, 1985) as

![FIGURE 1. Activity of *Pseudomonas* spp. lipase (PL1) at 40 C, pH 7, using 2 mL tributyrin and 50 unit lipase. Y1 to Y3 are the regression equations of replicates of each treatment. The slope of the equations represents the lipase activity.](https://academic.oup.com/ps/article-abstract/77/11/1665/1541656/5126262)
a completely randomized design. When treatment effects were significant \( P < 0.05 \), Duncan’s multiple range test (Steel and Torrie, 1980) was used to compare means.

**RESULTS**

**Initial Rate of Tributyrin Hydrolysis**

Figure 1 shows the time course of 2 mL tributyrin hydrolysis at 40 C and pH 7. Under these conditions, the time course was linear for 4 min from initiation. In all subsequent experiments the initial rate of hydrolysis was defined as the slope of the linear portion of the time course of the reaction.

**Kinetics of PL and CPL at pH 7 and 8**

Figure 2 shows the effect of various concentrations of tributyrin on the initial rate of the reaction in the presence of PL1 and CPL at pH 7 and 8. Under these conditions the two lipases exhibited a Michaelis-Menten type of enzymatic activity. Increasing media pH from 7 to 8 had no effect on maximum velocity \( (V_{max}) \) but was associated with a slight decrease in the affinity of the substrate \( (K_m) \) for CPL. For both lipases near saturation of enzyme activity occurred at a substrate concentration of 2 mL. In all further experiments 2 mL of tributyrin was used to approximate the maximum activity of the lipases.

**Effect of pH on Lipase Activity**

Activity of lipases at 40 C and different pH in the absence of any pre-incubation period are presented in Figure 3. Enzyme activity tended to increase with higher pH. Little if any lipase activity was seen for all sources at pH 3 and 4. Maximum activity was seen in the neutral pH range (6 to 8) for all sources except ANL, which demonstrated maximum activity at pH 5. Unlike other sources with maximum activity at neutral pH, the activity of RAL was substantially lower at pH 8 than pH 7.

**Effect of Preincubation Conditions on Lipase Activities**

The results of preincubation of lipases under varying conditions are shown in Figure 4. Preincubation for 30 min at 40 C and pH 7, reduced the activity of all lipases except for ANL. Longer term exposure to 40 C resulted in a 2.5-fold increase in the activity of ANL measured at pH 7. Pre-incubation of lipases for 30 min at 40 C and in acidic

![Image of graphs showing enzyme activity and kinetics](link-to-image)
sonication, the apparent Km was drastically decreased. When the substrate was dispersed by sonication, the apparent Km was drastically decreased. Borgstrom and Erlanson-Albertson (1973) observed that when the substrate was dispersed by sonication, the apparent Km was drastically decreased.

7. Preincubation of LPL, CPL, RAL, and ANL with pepsin inhibited their activities when measured at pH 7. This result confirms the fact that porcine lipase is not acid resistant and inactivation of porcine or other mammalian pancreatic lipases before they reach the small intestine can contribute to the failure of oral enzyme replacement therapy for treatment of fat malabsorption (steatorrhea) in human studies (DiMagno et al., 1979). Exposure of most lipases to 40°C, for 30 min causing a slight but significant additional inhibition of CVL, PL1, and PL2 activities measured at pH 7. Preincubation of LPL, CPL, RAL, and ANL with pepsin had no effect on their activities.

**DISCUSSION**

The titrimetric methodology employed in this study provided an accurate measure of true initial rates of tributyrin hydrolysis. Thus, it was possible to test kinetic models for fit to initial rate data obtained with varying substrate concentrations. For each of the lipases tested, the data exhibited a Michaelis-Menten type of enzyme activity. Maximum velocities were obtained at substrate concentrations greater than 0.5 mL and thus in subsequent experiments initial rates of the reaction were measured with 2 mL of tributyrin incorporated in the mixture to insure that the reaction was measured at maximal velocity.

A measure of reaction rates at a high concentration of substrate provides a good indication of Vmax that is independent of the physical degree of dispersion of the solution. Borgstrom and Erlanson-Albertson (1973) observed that when the substrate was dispersed by sonication, the apparent Km was drastically decreased. They showed that an increasing amount of substrate concentration has the same effect as sonication of the substrate emulsion. Thus, using a high concentration of tributyrin allows the reaction to proceed at its maximal rate. This optimization assures a reliable titrimetric assay that follows the classical Michaelis-Menten kinetics.

The lipases tested varied in optimal pH, ranging from 5 to 8. Microbial lipases had higher activities at lower pH. The pH dependency for ANL obtained in our study is comparable with the results of Zentler-Munro et al. (1992), which demonstrated ANL activities in pH ranges of 2.5 to 5.5 with a pH optimum of 4.5. Our results with *Pseudomonas* lipase agree with Stead (1986), who reported that the pH and temperature optima were in the range of 7 to 9 and 30 to 50°C, respectively.

Domestic chickens have an internal body temperature of approximately 40°C (Whittow, 1986), and feed passes through the low pH of the proventriculus (Winger et al., 1962) in about 30 min (Noy and Sklan, 1995) before reaching the neutral pH of the small intestine (Duke, 1986). Exposure of most lipases to 40°C, for 30 min decreased their activities as expected; however, ANL showed an opposite response, increasing 2.5-fold in activity. This result was unexpected and requires further investigation.

Preincubation of CPL and LPL at pH 3 for 30 min (40°C) inhibited their activities when measured at pH 7. This result confirms the fact that porcine lipase is not acid resistant and inactivation of porcine or other mammalian pancreatic lipases before they reach the small intestine can contribute to the failure of oral enzyme replacement therapy for treatment of fat malabsorption (steatorrhea) in human studies (DiMagno et al., 1977). Antacids, inhibitors of gastric acid secretion (Graham, 1982) or enteric coated lipase (Roberts, 1989) have been used to protect pancreatic lipase from acid denaturation. However, these methods are likely not applicable in the poultry industry because of the grinding activity of the gizzard and the lower retention time of feed in the duodenum. Enzyme replacement therapy with acid-stable lipases may be more appropriate in birds. Microbial lipases were more resistant to acidic conditions and it is probable that these enzymes should work better than pancreatic lipases.

Preincubation with pepsin under acid conditions that approximate the stomach is known to have variable effects on enzyme activity dependent upon the enzyme in question. Heizer et al. (1965) showed that trypsin was inactivated by pepsin and acid whereas pancreatic lipase was inactivated by a pH of less than 4 in human subjects. DiMagno et al. (1977) also showed that trypsin and pancreatic lipase can be inactivated by pepsin and the acid of the stomach, respectively. Zentler-Munro et al. (1992) showed that ANL is pepsin- and trypsin-resistant and any inhibition below pH 4 is completely reversible. In our study, incorporation of pepsin in the pH 3 preincubation media had no further inhibitory effect on the acid-sensitive lipases, CPL, LPL, and ANL.
The activity of ANL and PL1 was not affected by pepsin addition to the acid preincubation media. At the highest concentration of pepsin the activity of PL2 and CVL was partially inhibited relative to the acid only preincubation treatment. Despite this loss, these sources still retained 16 to 27% of their maximal activity. Our findings are in agreement with Raimondo and DiMagno (1994) in that bacterial lipase lipolytic activity was shown to be more resistant to inactivation by acid than was the lipolytic activity of porcine lipase. As such, one would predict that bacterial lipases would retain more activity during passage to the small intestine than the lipolytic activity of either mammalian or fungal lipase.

In summary, not all lipases retain activity during incubation under conditions that mimic those of the chicken proventriculus. The activities of CPL, LPL, and RAL were very low after low pH exposure and therefore are not considered suitable candidates for use in the diet to prevent steatorrhea in young chickens. Despite some loss of activity, PL1, PL2, CVL, and ANL may be suitable sources of lipase for dietary supplementation. Further research is required to establish whether these enzymes can also be active in the conditions of the small intestine.

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