FELINE PANCREATIC LIPASE: PURIFICATION AND VALIDATION OF A CLINICALLY SIGNIFICANT RADIOIMMUNOASSAY FOR THE DIAGNOSIS OF FELINE PANCREATITIS

A Thesis

by

BENJAMIN GREGG WILSON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2003

Major Subject: Veterinary Medical Sciences

FELINE PANCREATIC LIPASE: PURIFICATION AND VALIDATION OF A CLINICALLY SIGNIFICANT RADIOIMMUNOASSAY FOR THE DIAGNOSIS OF FELINE PANCREATITIS

A Thesis

by

BENJAMIN GREGG WILSON

Submitted to Texas A&M University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Approved as to style and content by:

Jörg M. Steiner (Chair of Committee)	David A. Williams (Member)
Luc R. Berghman (Member)	Sandee M. Hartsfield (Head of Department)

December 2003

Major Subject: Veterinary Medical Sciences

ABSTRACT

Feline Pancreatic Lipase: Purification and Validation of a Clinically Significant Radioimmunoassay for the Diagnosis of Feline Pancreatitis. (December 2003)

Benjamin Gregg Wilson, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Jörg M. Steiner

Serum lipase activity has traditionally been used for diagnosis of pancreatitis in human beings and dogs. However, serum lipase activity is not specific for exocrine pancreatic function and many cell types other than pancreatic acinar cells also synthesize lipases. Recently, an immunoassay for the measurement of canine pancreatic lipase immunoreactivity has been developed and validated. This assay has shown to be specific for exocrine pancreatic function and sensitive for the diagnosis of canine pancreatitis. The objectives of this project were to purify feline pancreatic lipase (fPL), have antibodies against fPL (anti-fPL antibodies) produced, and develop a radioimmunoassay (RIA) for the diagnosis of feline pancreatitis.

Pancreatic lipase was purified from feline pancreatic tissue by delipidation, anion-exchange chromatography, size-exclusion chromatography, and cation-exchange chromatography. Antiserum against fPL was raised in rabbits. Tracer was produced by iodination (^{125}I) of fPL using the chloramine T method. An RIA was established and validated by determination of sensitivity, dilutional parallelism, spiking recovery, intra-

assay variability, and inter-assay variability. A control range for fPLI in cat serum was established from 30 clinically healthy cats using the central 95th percentile.

The sensitivity of the fPLI assay was 1.2 µg/L. Observed to expected ratios for serial dilutions ranged from 58.0 to 164.3% for 4 different serum samples at dilutions of 1 in 2, 1 in 4, and 1 in 8. Observed to expected ratios for spiking recovery ranged from 76.0 to 156.5% for 4 different serum samples and 6 different spiking concentrations. Coefficients of variation for intra-assay variability for 4 different serum samples were 10.1, 4.5, 2.2, and 3.9%. Coefficients of variation for inter-assay variability for 4 different serum samples were 24.4, 15.8, 16.6, and 21.3%. The control range for serum fPLI concentration was established as 1.2 to 3.8 µg/L.

All of the objectives outlined above were successfully met, leading to the development of an RIA for the measurement of fPLI in cat serum. The RIA for fPLI described here is sufficiently accurate and precise, but has a limited linearity and reproducibility in the lower and higher end of the working range.

DEDICATION

To my loving mother, family and friends

ACKNOWLEDGMENTS

If it were not for Dr. Steiner, I would not be writing this thesis, much less these acknowledgments. Thank you for all the opportunities that you have brought my way. You took me on as an undergraduate student looking for an independent research project and allowed me to work together with you on a purification project. You also accepted me into a Master of Science degree program. I would also like to thank you for giving me the opportunity to learn to trust my instincts and myself when it comes to making decisions. I am also thankful for the publication and presentation opportunities you have granted. I have learned many skills while working in the Gastrointestinal Laboratory and made many life-changing decisions; thank you for the time and experience you have given me. All the late nights and weekends at the lab were grueling, but in the end, it was worth it.

I would also like to thank Dr. Williams for all of his advice and suggestions on the numerous projects I worked on and for being a member of my committee. I appreciate the time you have spent reading and proofing the publications, posters, and this thesis. Thank you for all the opportunities your lab has offered.

Furthermore, I would like to thank Dr. Berghman. You are a great professor and you really push your students. I not only received many hands-on laboratory techniques in your course but also learned a great deal about writing papers for scientific journals. Hopefully, it will all pay off in the following pages. I also want to thank you for being a

part of my committee and relinquishing a bit of your time to look over my thesis. Your time is greatly appreciated.

I also want to thank Dr. Nikos Zavros and Professor T. Rallis from the Aristotle University of Thessaloniki, Greece for allowing the use of their samples. The samples used for comparison of serum fPLI and fTLI concentrations in cats with experimentally induced pancreatitis originated from their laboratory.

I cannot forget to thank all of my co-workers at the lab. From other grad students, researchers, technicians, and student workers, all of you have helped me at one time or another. Thank you all for your assistance and for making the basement a better place. Long live the basement rats.

To all who may read this thesis: I thank you for your interest and wish you all the best.

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

LIPASES

Lipases were first discovered in 1856 by Claude Bernard when he studied the role of the pancreas in fat digestion.[1] Since then many different lipases have been identified in and isolated from bacteria, fungi, plants, and animals.[2-7] Lipases are characterized as enzymes capable of hydrolyzing water-insoluble substrates, such as triglycerides, into more polar lipolysis products.[1] It has been shown that lipases share a common amino acid sequence motif, G-X-S-X-G (glycine-variable-serine-variable-glycine). This amino acid sequence motif is also shared with esterases, some related proteins, and serine proteases.[1,8,9] While being hydrophilic in serine proteases, this amino acid sequence has been shown to be hydrophobic in nature in lipases. This indicates that the two sequences are phylogenetically unrelated.[10] Lipases and serine proteases also share another common property found within the active site of these enzymes.[11] They both contain three amino acids, serine, aspartic or glutamic acid, and histidine, commonly known as the catalytic triad.[11] The amino acid sequence motif for the active site of human pancreatic lipase is Ser152, Asp176, and His263.[11]

Many of the lipases that have been identified to date are phylogenetically related. The pancreatic lipase gene family is large and has been subdivided into nine subfamilies:

This thesis follows the style and format of the *American Journal of Veterinary Research*.

yolk proteins, lipoprotein lipase, hepatic lipase, classical pancreatic lipase, pancreatic lipase-related protein 1 (PLRP1), pancreatic lipase-related protein 2 (PLRP2), a group that contains a phosphatidylserine-specific phospholipase A1 from rat platelets and a protein expressed in human melanoma cell lines known as NMD, vespid phospholipases A1, and lastly endothelial lipase.[1,12-15] This project will focus solely on the digestive lipase known as classical pancreatic lipase.

Classical pancreatic lipase (also known as pancreatic lipase, PL) is a glycoprotein that has an estimated molecular mass of 50,000 Da in human beings.[16] Classical pancreatic lipase is synthesized in the pancreas, more specifically by pancreatic acinar cells. Pancreatic lipase is secreted as an active enzyme into the duct system of the exocrine pancreas from where it is transported into the duodenum.[17] Although the activity of PL towards water-soluble substrates is low in solution, the enzyme's activity is significantly increased at the interface between water and aggregated water-insoluble triglycerides.[15] This characteristic of increased lipase activity at an oil-water interface is known as interfacial activation.[15] Bile acids, even at physiological concentrations, have the ability to inhibit triglyceride hydrolysis by covering the oil-water interface. However, in the presence of colipase, which interacts with PL in a molecular ratio of 1:1 and also facilitates the adsorption of lipase at the bile acid covered interface, this inhibition is reversible.[15,16] Many detergents also exhibit an inhibitory action on PL activity.[18] The three-dimensional structure for many pancreatic lipases has previously been deduced, though the structure of human PL was the first described.[19] From all species where the structure has been determined, PL is folded into two domains, with the N-terminal

domain being larger than the C-terminal domain.[20] The smaller C-terminal domain plays a role in the binding of lipase to colipase and is very important for the interfacial binding of PL.[20-26] The catalytic triad or active site is located within the larger N-terminal domain, which also contains a glycosylation and a potential heparin-binding site.[20] In human beings, the PL active site contains the three essential amino acid residues Ser153, His264, and Asp177.[27] A mutation of any of these essential amino acid residues will result in a protein that does not display lipolytic activity.[27] However, a site-directed mutation of Asp177 to Glu177 leads to PL with lipolytic activity, but with an increased sensitivity to proteases.[28]

Two major digestive lipases have been reported in most mammalian species, PL and preduodenal lipase.[29] Pancreatic lipase has proven to be the most important lipase for digestion of dietary fats.[30,31] This water-soluble digestive enzyme is capable of hydrolyzing apolar dietary triglycerides into more polar lipolysis products, like monoglycerides and free fatty acids, that can be absorbed by the intestinal mucosa.[32] Upon completion of a meal, when foodstuffs reach the small intestine, specific receptors are activated that lead to the release of cholecystokinin which in turn activates a cyclic AMP pathway that stimulates the secretion of PL from the pancreas into the small intestine.[33,34] The activity of PL is limited to the small intestine due to the proteolysis by chymotrypsin.[25,35] Acid denaturation can also play a role in the loss of lipolytic activity as the enzyme progresses through the gastrointestinal tract.[36] The degradation and denaturation processes may be slowed by the presence of nutrients in the intestine.[36]

LIPASE ASSAYS

A myriad of different methods to evaluate catalytic lipase activity have been developed. One downside to having multiple methods for analysis is that the inter-assay variability between the assays of differing methods is large and a reference method has yet to be agreed upon.[37] The substrate used in the catalytic lipase activity assays is yet another factor of variability between methods. Many assays employ the use of oil based substrates such as olive oil or triolein suspensions while other assays depend on synthetic substrates.[38] As mentioned previously, lipase shows its maximal hydrolytic activity at a water-oil interface, thus the use of a water-soluble synthetic substrate could prove to be problematic in that it may yield a lower reading of catalytic activity when in fact there may be large amounts of lipase present.[38]

CLINICAL APPLICATION OF LIPASE ASSAYS

The measurement of PL activity has been used as a tool for the diagnosis of exocrine pancreatic disorders in human beings for many years.[39-41] However, enzymatic assays are not capable of distinguishing between lipase activity of different cellular origins and serum lipase activity does not exclusively originate from the pancreas. This is supported by research that shows lipase activity in serum after a total pancreatectomy in dogs.[42,43]

Research has shown that human beings with exocrine pancreatic insufficiency have a decreased serum lipase activity, while increased serum lipase activities have been observed in patients with acute pancreatitis.[44-46] One study conducted in dogs without

any lesions of the exocrine pancreas as determined by light microscopy showed serum lipase activities that were significantly elevated.[39] These dogs were diagnosed with renal diseases, hepatic lesions, and other lesions of the heart, gastrointestinal tract, and multiple other organs.[39] Other factors that can lead to an increased serum lipase activity in dogs, although they are small increases, are heat stress and muscular exercise.[47] Thus, increased serum lipase activity is not highly specific for pancreatitis.[45,48]

Pancreatitis used to be diagnosed infrequently in cats. However, recent studies have shown that pancreatitis occurs frequently in cats and have led to an increased level of suspicion by veterinary clinicians.[49] However, definitive diagnosis of feline pancreatitis remains elusive. Clinical signs in cats with pancreatitis are non-specific and key clinical signs seen in human beings and dogs with pancreatitis, such as vomiting and abdominal pain, are only reported infrequently in cats.[50] Also, findings on blood work are non-specific and serum amylase and serum lipase activities are of no clinical value in the cat. Abdominal radiography can show findings that are compatible with pancreatitis but do not allow for a definitive diagnosis. When stringent criteria are applied abdominal ultrasound is highly specific for feline pancreatitis but its sensitivity is only 11-35%.[51] Measurement of serum feline trypsin-like immunoreactivity (fTLI) is also highly specific for pancreatitis but its sensitivity of 30-60% is also less than optimal.[51-54] Thus, a minimally-invasive diagnostic test that is both highly sensitive and specific for feline pancreatitis is needed.

OBJECTIVE

The objective of this project was to evaluate the hypothesis that the measurement of serum feline pancreatic lipase immunoreactivity (fPLI) is useful for the diagnosis of exocrine pancreatic disorders in the cat. To test this hypothesis, feline pancreatic lipase was purified from pancreatic tissue, then antiserum against this protein was raised, and finally a radioimmunoassay for the measurement of fPLI in serum was developed and validated. To thoroughly analyze the hypothesis, a collection of serum samples from clinically healthy cats and cats with exocrine pancreatic disorders was compiled to compare serum fPLI concentrations between these two groups of cats.

CHAPTER II

PURIFICATION OF FELINE PANCREATIC LIPASE*

INTRODUCTION

Classical pancreatic lipase (also known as pancreatic lipase, PL) is a glycoprotein that has an estimated molecular mass of 50,000 Da in human beings.[16] Pancreatic lipase is synthesized in the pancreas, more specifically by pancreatic acinar cells. Pancreatic lipase is secreted as an active enzyme into the duct system of the exocrine pancreas from where it is transported into the duodenum.[17] Although the activity of PL towards water-soluble substrates is low in solution, the enzyme's activity is increased at the interface between water and aggregated water-insoluble triglycerides.[15] This characteristic of increased lipase activity at an oil-water interface is known as interfacial activation.[15] Bile acids, even at physiological concentrations, inhibit triglyceride hydrolysis by covering the oil-water interface. However, in the presence of colipase, which interacts with PL in a molecular ratio of 1:1 and also facilitates the binding of lipase at the bile acid covered interface, this inhibition is reversible.[15,16] Colipase is a small protein that is also secreted by pancreatic acinar cells and has an approximate molecular mass of 10 kDa.[55-57] Many detergents also have an inhibitory action on PL activity.[18] The three-dimensional structure for many pancreatic lipases has previously been deduced, though

---

*Parts of this chapter are reprinted from *Comparative Biochemistry and Physiology Part B*, Vol. 134, Jörg M. Steiner, Benjamin G. Wilson, David A. Williams, Purification and partial characterization of feline classical pancreatic lipase, Pages 151-159, © 2002 with permission from Elsevier Science Inc.

the structure of human PL was the first one described.[19] All pancreatic lipases for which the structure has been determined have exhibited a structure folded into two domains, with the N-terminal domain being larger than the C-terminal domain.[20] The smaller C-terminal domain plays a role in the binding of lipase to colipase and is very important for the interfacial binding of PL.[20-26] The catalytic triad or active site is located within the larger N-terminal domain which also contains a glycosylation and a potential heparin-binding site.[20] In human beings, the PL active site is comprised of the three essential amino acid residues Ser153, His264, and Asp177 which are also found in many other lipases as well as in serine proteases.[27] If there is a mutation of any of these essential amino acid residues the protein does not display lipolytic activity.[27] However, if the protein undergoes a site-directed mutation of Asp177 to Glu177 lipolytic activity will be present at the expense of a heightened sensitivity to proteases.[28]

The measurement of serum lipase activity has been used as a tool for the diagnosis of pancreatitis in human beings for many years.[39-41] Most studies in human beings utilize the measurement of lipase activity rather than that of a mass concentration of PL present. Catalytic assays may not be capable of distinguishing between lipase activity of different origins and lipase activity measured in serum does not exclusively originate from the pancreas. This is supported by research in dogs that shows that significant lipase activity remains in serum after total pancreatectomy.[42,43]

Recent studies have shown that pancreatitis occurs frequently in cats, which has led to an increased level of suspicion by veterinary clinicians.[49] However, definitive diagnosis of feline pancreatitis remains elusive. Clinical signs in cats with pancreatitis

are non-specific and key clinical signs seen in human beings and dogs with pancreatitis, such as vomiting and abdominal pain, are only reported infrequently in cats.[50] Also, findings on blood work are non-specific and serum amylase and serum lipase activities are of no clinical value in the cat. Abdominal radiography can show patterns that are compatible with pancreatitis but do not allow for a definitive diagnosis. When stringent criteria are applied, abdominal ultrasound is highly specific for feline pancreatitis but its sensitivity is only 11-35%.[51] Measurement of serum feline trypsin-like immunoreactivity (fTLI) is also highly specific for pancreatitis but its sensitivity of 30-60% is also less than optimal.[51-54] Thus, a minimally-invasive diagnostic test that is both highly sensitive and specific for feline pancreatitis is needed. The purification of feline PL, as described in this section, is a prelude to the development, validation, and clinical evaluation of a radioimmunoassay for the measurement of feline pancreatic lipase immunoreactivity in serum from cats suspected of having exocrine pancreatic disorders.

MATERIALS AND METHODS

Materials - All commonly used research materials and supplies were purchased from VWR Scientific, West Chester, PA. Chemicals and reagents used were from Sigma Chemicals, St. Louis, MO. The liquid chromatography system (Äkta® purifier) and all columns used for the purification of feline PL were purchased through Amersham Pharmacia Biotech, Piscataway, NJ. For the measurement of lipolytic activity a pH stat titration system was purchased from Brinkmann Instruments, Westbury, NY. The electrophoresis system used for SDS-PAGE was from Novex, San

Diego, CA. A NuPAGE 10% Bis-Tris Novex high-performance, pre-cast gel (1.0 mm X 10 well) from Invitrogen™ life technologies was used for estimation of molecular mass.

Lipase assay - Throughout the purification it was necessary to measure the amount of lipolytic activity present in each fraction collected. Lipolytic activity was measured with a pH stat assay using tributyrin as the substrate in the presence of feline pancreatic colipase. For this assay an acidic colipase preparation was prepared by dissolving 1 g of delipidated feline pancreatic extract in 50 ml of 20 mM Tris-HCl, 3.3 mM CaCl2, 2 mM benzamidine, 5 mM 3-phenyl propionate, 1 mM phenylmethylsulfonyl fluoride (PMSF) added in isopropyl alcohol, pH 8.0 and mixed at 4°C for 2 hours. This mixture was then centrifuged at approximately 12,000 g and 4°C for 30 min and the precipitate was discarded. The supernatant was analyzed for lipolytic activity and the pH was adjusted to pH 2.0 using 6 N HCl. The solution was centrifuged again under the same conditions as above and lipolytic activity of the supernatant was measured once again and the solution was adjusted to a pH of 5.0 using 2 N NaOH. Again the solution was centrifuged under the above-mentioned conditions and upon completion the supernatant was aliquotted, labeled, and frozen at -20°C. For the measurement of lipolytic activity a volume of 0.5 ml tributyrin was added to 14.5 ml of 0.28 mM Tris-HCl, 1.4 mM CaCl2, 4.0 mM taurodeoxycholate, 150 mM NaCl, pH 8.0 in a reaction vessel kept at 37°C. Fifty microliters of the acidic feline colipase preparation was also added to the vessel and the pH was adjusted to 8.0 with the use of 100 mM NaOH. Once the pH in the vessel was at 8.0, 10 µl of the sample were added and the pH was kept at 8.0 by the controlled addition of 100 mM NaOH. After a waiting

period of 1 min, the amount of NaOH added was measured for 3 min and lipolytic activity was expressed in international units with 1 unit representing the amount of lipolytic activity releasing 1 mmol of titratable fatty acid per minute under above-mentioned conditions.

Preparation of pancreatic extract - Pancreata were collected from cats sacrificed for unrelated projects and kept frozen at -20°C until they were used for extraction. For the pancreatic extraction, the pancreatic tissue was thawed at room temperature and the gross fat was removed with a razor blade. The partially delipidated pancreatic tissue was then cut into small pieces, mixed thoroughly with a tissue grinder (Polytron, Brinkmann Instruments, Westbury, NY), and delipidated with the use of organic solvents. The solvents used were acetone, chloroform, 1-butanol, and ethyl ether. Prior to the delipidation procedure, all solvents were kept at 4°C. All organic solvents used were handled in a well ventilated fume hood. Three large feline pancreata were soaked in 100 ml acetone and minced with a tissue grinder. The pancreatic material was then transferred to a medium Buchner funnel and filtered to dryness with vacuum suction. The solvent was discarded. The filtrate was mixed two more times in 100 ml of acetone and filtered to dryness each time with a medium Buchner funnel. Each time the solvent was discarded. The filtrate was further delipidated by immersing the pancreatic material in 200 ml of a 9 to 1 mixture of chloroform and 1-butanol and mixing thoroughly with a tissue grinder followed by filtration to dryness with a medium Buchner funnel. This process was completed two more times, with the solvent being discarded after each filtration step. The pancreatic material was further delipidated by

mixing with 200 ml of a 4 to 1 mixture of chloroform and 1-butanol with a tissue grinder and filtered to dryness. This step was completed one more time as described above. Lastly, the filtrate was carefully dissolved in ethyl ether and mixed with a spatula for approximately two minutes and then filtered to dryness through the medium Buchner funnel. Once the filtrate was completely dry, it was broken up into a fine powder and this delipidated pancreatic extract was kept at -20°C until the protein extraction procedure.

Protein extraction - Two grams of delipidated pancreatic extract was weighed out and combined with 250 µl of 100 mM PMSF in isopropyl alcohol. Immediately, 25 ml of 20 mM Tris-HCl, 2 mM benzamidine, 150 mM NaCl, pH 7.5 was added to this mixture in a centrifuge tube and mixed thoroughly for 1 min with a tissue homogenizer. Benzamidine and PMSF were both used as protease inhibitors. Then the mixture was put onto a rocking plate and mixed for 20 min at 4°C. The mixture was then centrifuged for 15 min at 10,000 g and 4°C. The supernatant was evaluated for absorbance at 280 nm, evaluated for lipolytic activity, and saved in the refrigerator. Another 25 ml of 20 mM Tris-HCl, 2 mM benzamidine, 150 mM NaCl, pH 7.5 and 250 µl of 100 mM PMSF in isopropyl alcohol was added to the precipitate and mixed again with the tissue homogenizer, the rocking plate, and centrifuged under the same conditions as above. The supernatant was again evaluated for absorbance at 280 nm and lipolytic activity and then combined with the supernatant from the first extraction step. This extraction procedure was carried out a third time and the supernatant was analyzed as above and

combined with the supernatant from the first two extraction steps. The final precipitate was discarded.

Buffer exchange - The buffer of the pooled supernatant was changed to 10 mM Tris-HCl, 2 mM benzamidine, 1 mM PMSF, pH 8.0 (buffer A) before application to the anion-exchange column. This was accomplished by using a disposable gel-filtration column (PD-10®, Amersham Pharmacia Biotech, Piscataway, NJ) at 4°C following the manufacturer's instructions. The column was first drained of the storage solution and then washed with 20 ml of buffer A. Non-specific binding sites were then blocked by washing the column with 20 ml of buffer A containing 0.1 g/ml bovine serum albumin, and washed again with 20 ml of buffer A. The next step was to add 3 ml of supernatant and discard the eluent, followed by the addition of 4 ml of buffer A and collection of the eluent. The column was washed again with 20 ml of buffer A and the eluent was discarded. After this, the column was ready for another cycle of buffer exchange. This process was carried out until all of the pooled supernatant had been applied to the column. To speed up the process four columns were used simultaneously.

Anion-exchange chromatography - For further purification by anion-exchange chromatography, feline PL was applied to a pre-packed Mono Q® column (bed height 100 mm, column diameter 10 mm) in buffer A. The column was washed until the absorbance monitor at a wavelength of 280 nm had returned to base-line. Then the feline PL mixture was eluted using a continuous NaCl gradient from 0 to 80 mM NaCl in buffer A at a flow rate of 1 ml/min for 30 min. Fractions were collected in 5 ml aliquots and those fractions suspected of lipase activity were analyzed. Fractions with

high lipolytic activity were collected, pooled, concentrated, and the buffer was changed to 10 mM MES, 2 mM benzamidine, 1 mM PMSF, pH 6.0 using a concentrating device (Ultrafree® 10K concentrator; Millipore Corporation, Bedford, MA). The concentrated fraction was then saved for further purification.

Size-exclusion chromatography - The next purification step of feline PL was carried out on a Sephacryl S-300 HR® size-exclusion chromatography column (bed height 600 mm, column diameter 16 mm). Feline PL was applied to the column in 10 mM MES, 2 mM benzamidine, 1 mM PMSF, pH 6.0 at a flow rate of 1 ml/min. Fractions were collected in 5 ml aliquots and those fractions suspected of lipase activity were analyzed. Fractions with high lipolytic activity were collected, pooled, concentrated, and the buffer was changed to 10 mM MES, pH 6.0 by means of an Ultrafree® 10K concentrator. This fraction was then saved for further purification.

Cation-exchange chromatography - The final purification step utilized cation-exchange chromatography to further purify feline PL. This was done with a self-packed Source S® column (bed height 100 mm, column diameter 10 mm). The feline PL preparation from the previous purification step was applied to the column in 10 mM MES, pH 6.0 at a flow rate of 1 ml/min. After the absorbance monitor at a wavelength of 280 nm had returned to the base-line a linear NaCl gradient from 0 to 200 mM NaCl in 10 mM MES, pH 6.0 was applied over 60 min at a flow rate of 1 ml/min to elute the feline PL mixture. For this last purification step 2.5 ml fractions were collected and analyzed for lipase activity. The fraction determined to have the greatest lipase activity was collected, concentrated, and the buffer was changed to phosphate buffered saline

(100 mM sodium phosphate, 150 mM NaCl, pH 7.2) using an Ultrafree® 10K concentrator. Once the buffer had been changed, the absorbance of the purified feline PL was adjusted to $A_{280} = 0.59$, then aliquotted and frozen at -80°C.

Partial characterization - The molecular mass of the purified feline PL was estimated using a SDS-PAGE protocol following manufacturer's directions. The gel was stained with a Coomassie blue type stain (GelCode® blue, Pierce Chemical Company, Rockford, IL) for estimation of molecular mass. For a more exact determination of molecular mass a sample was sent to the Mass Spectrometry Laboratory, Department of Chemistry, Texas A&M University, College Station, TX. The specific absorbance of fPL was determined by measurement of the protein concentration of a serial dilution of a solution of fPL in PBS, pH 7.2 with a known absorbance at a wavelength of 280 nm using a BCA protein assay.[58] An Edman degradation procedure utilizing an automated amino acid sequence analyzer was used in order to determine the N-terminal amino acid

sequence of the first 25 amino acid residues. The Edman degradation procedure was conducted by the Department of Biochemistry, Purdue University, West-Lafayette, IN.

RESULTS

Feline PL was successfully purified from feline pancreatic tissue by delipidation, protein extraction, anion-exchange chromatography (Fig 1), size-exclusion chromatography (Fig 2), and cation-exchange chromatography (Fig 3). The overall yield of the purification protocol was 18.6% (Table 1). The approximate specific absorbance at 280 nm of fPL was 1.18 for a 1 mg/ml sample (Table 2). Analysis of the purified fPL upon sodium dodecyl sulfate polyacrylamide gel electrophoresis showed a single band with an estimated molecular mass of 52.5 kDa (Fig 4). The exact molecular mass was determined to be 52.4 kDa by mass spectrometry. Edman degradation analysis determined the N-terminal amino acid sequence of the first 25 amino acid residues as Lys–Glu–Ile-?–Phe–Pro–Arg–Leu–Gly-?–Phe–Ser–Asp–Asp–Ala–Pro–Trp–Ala–Gly-Ile-Ala-Gln-Arg-Pro-Leu (positions indicated with ? could not be determined; Table 3).

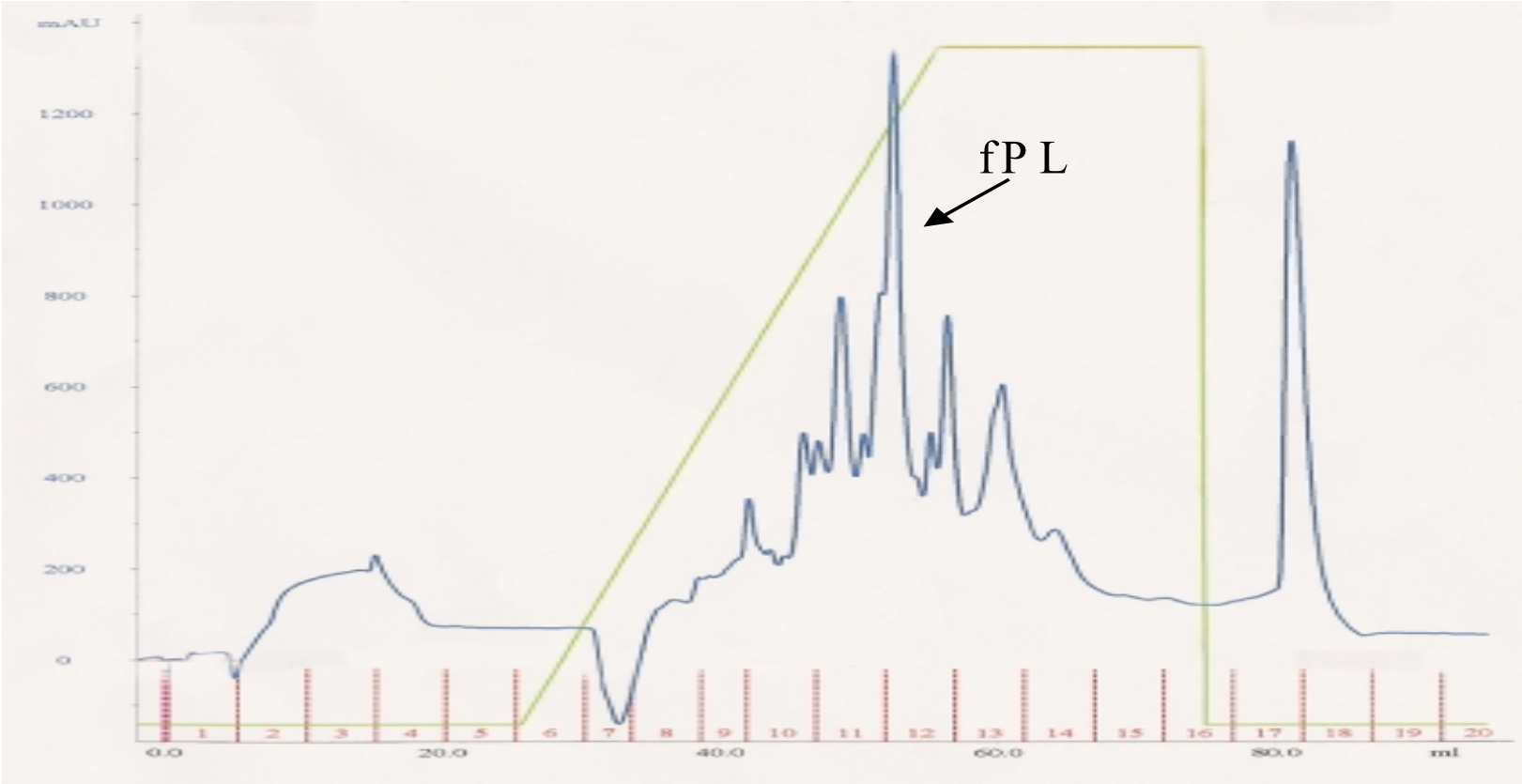

Figure 1-Anion-exchange chromatography of aqueous feline pancreatic extract. This figure shows a representative chromatogram after anion-exchange chromatography of the aqueous pancreatic extract after buffer exchange using a PD-10® column. The blue line depicts the absorbance at a wavelength of 280 nm during chromatography. The green line shows the concentration of NaCl. The pink numbers show the beginning of each collected fraction. The arrow marked fPL points to the only peak that contained lipolytic activity. Column: Mono Q® (pre-packed); flow rate: 1 ml/min; mobile phase: 10 mM Tris-HCl, 2 mM benzamidine, 1 mM PMSF, pH 8.0.

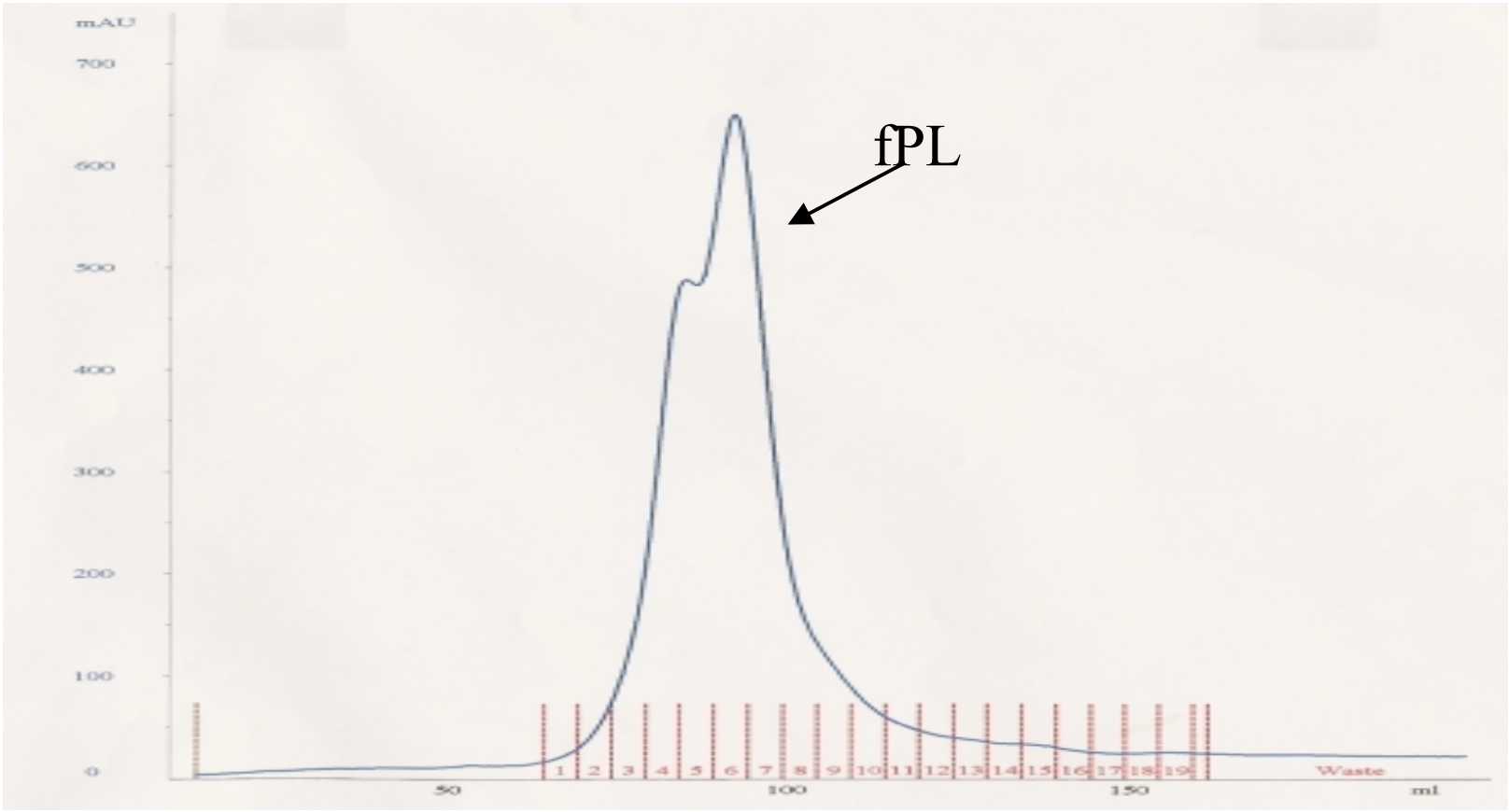

Figure 2-Size-exclusion chromatography of partially purified feline pancreatic lipase. This figure shows a representative chromatogram after size-exclusion chromatography of partially purified feline PL. The blue line depicts the absorbance at a wavelength of 280 nm during chromatography. The pink numbers show the beginning of each fraction collected. The arrow points to the peak with lipolytic activity. Column: Sephacryl S-300 HR® (pre-packed); flow rate: 1 ml/min; mobile phase: 10 mM MES, 2 mM benzamidine, 1 mM PMSF, pH 6.0.

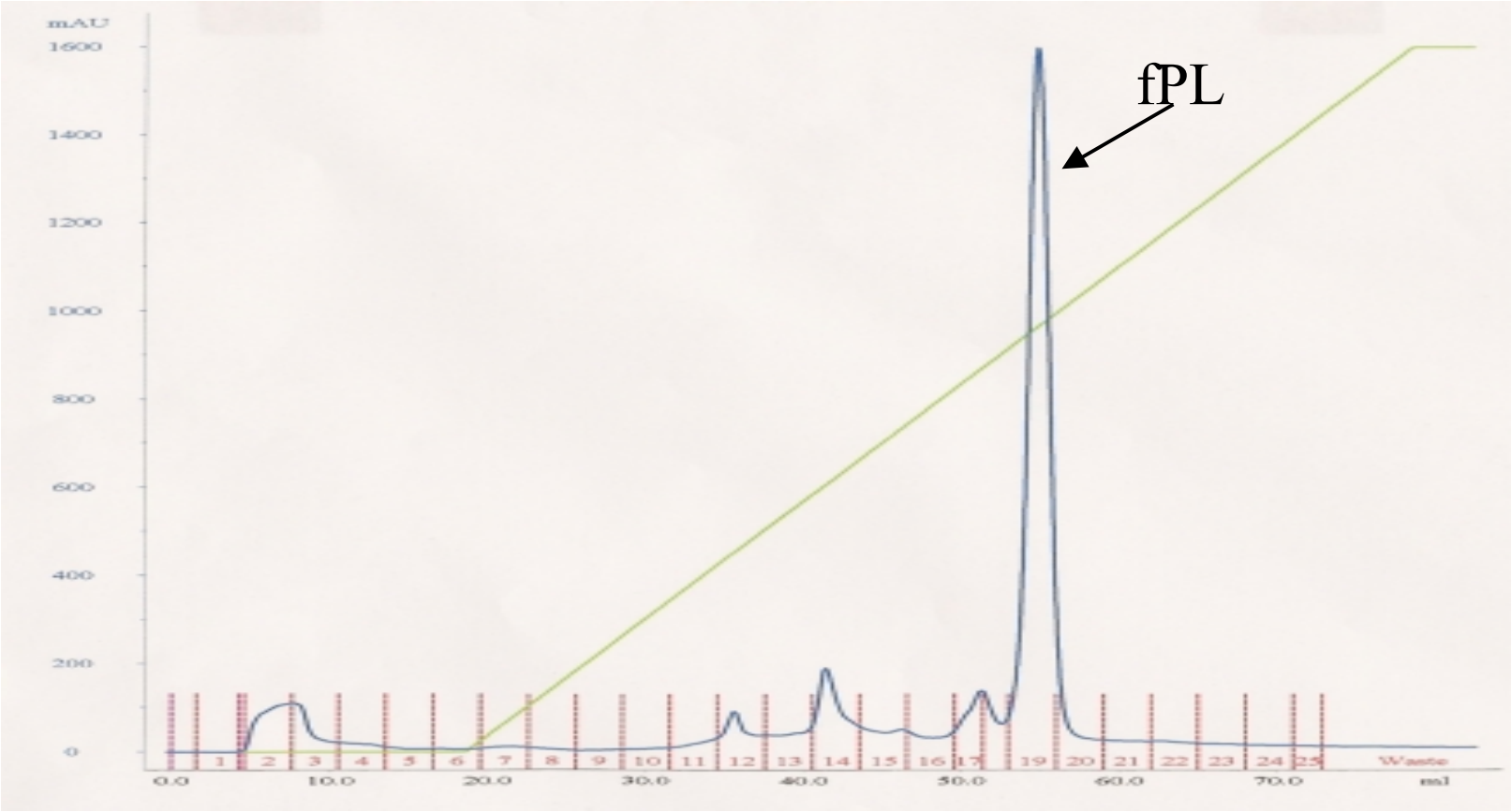

Figure 3-Cation-exchange chromatography of partially purified feline pancreatic lipase. This figure shows a representative chromatogram after cation-exchange chromatography of partially purified feline PL. The blue line depicts the absorbance at a wavelength of 280 nm during chromatography. The green line shows the concentration of NaCl. The pink numbers show the beginning of each fraction collected. The arrow points to the peak with lipolytic activity. Column: Source S® (self-packed); flow rate: 1 ml/min; mobile phase: 10 mM MES, pH 6.0.

Table 1-Purification of fPL. This table presents the data obtained throughout the purification procedure of fPL. With this data it is possible to see how much lipolytic activity is present after each step in the process. The protein content was estimated using a specific absorbance of A_{280}=1.18 for feline PL. The data shown for the post cation-exchange chromatography row was obtained after the concentration procedure, buffer change to PBS, pH 7.2, and the adjustment of the remaining sample to A_{280}=0.550.

Purification Stage	Volume (ml)	A_{280}	Protein Content (mg)	Specific Lipolytic Activity (U/mg)	Total Lipolytic Activity (U)	Yield (%)
Aqueous pancreatic extract	136.5	6.821	789.0	126	99,474	100.0
Post buffer exchange	228.0	2.746	530.5	147	77,963	78.4
Post anion-exchange chromatography	32.5	0.798	22.0	2732	60,105	60.4
Post-gel-filtration chromatography	10.0	0.582	4.9	6001	29,405	29.6
Post cation-exchange chromatography	3.85	0.550	1.8	10,261	18,470	18.6

Table 2-Specific absorbance of fPL. This table shows the results for the estimation of the specific absorbance for fPL. A solution of fPL in PBS, pH 7.2 with and absorbance of A_{280}=0.55 was used as the starting solution and was analyzed in a BCA protein assay.

Dilution Factor	A_{280}	Assay Result (μg/ml)	Adjusted Result (μg/ml)	Specific Absorbance
1	0.550	472.86	472.86	1.163
2	0.275	227.67	455.34	1.208
4	0.1375	118.30	473.20	1.162
Mean				1.178

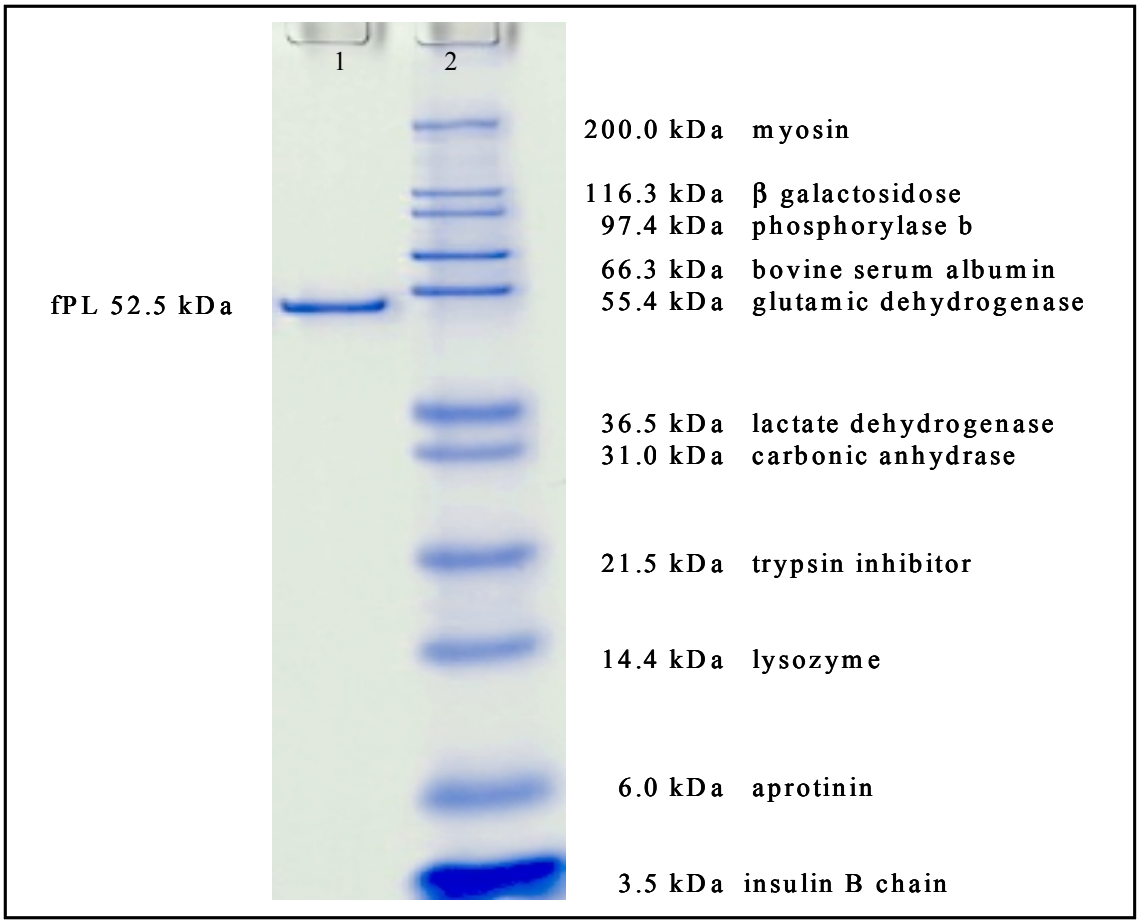

Figure 4-SDS-PAGE of feline classical pancreatic lipase. This NuPAGE 10% Bis-Tris gel was stained with a Coomassie blue stain. Purified feline classical PL (fPL) was loaded in lane 1. A molecular weight standard was loaded in lane 2. The molecular weight of the purified feline classical PL was estimated to be 52.5 kDa.

Table 3-N-terminal amino acid sequence comparison of fPL with other species. This table shows the N-terminal amino acid sequence of feline PL (fPL), porcine pancreatic lipase (pPL), canine pancreatic lipase (cPL), canine pancreatic lipase related protein 1 (cPLRP1), equine pancreatic lipase (ePL), and human pancreatic lipase (hPL). The amino acid sequences for ePL and hPL were predicted from cDNA sequence analysis. The three-letter code is used for amino acid identification. Amino acid residues that could not be identified are represented with '?'. The amino acid residues shown in blue are non-homologous with feline PL.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
fPL	Lys	Glu	Ile	?	Phe	Pro	Arg	Leu	Gly	?	Phe	Ser	Asp	Asp	Ala	Pro	Trp	Ala	Gly	Ile	Ala	Gln	Arg	Pro	Leu
pPL	Ser	Glu	Val	Cys	Phe	Pro	Arg	Leu	Gly	Cys	Phe	Ser	Asp	Asp	Ala	Pro	Trp	Ala	Gly	Ile	Val	Gln	Arg	Pro	Leu
cPL	Lys	Glu	Val	Cys	Phe	Pro	Arg	Leu	Gly	Cys	Phe	Ser	Asp	Asp	Ser	Pro	Trp	Ala	Gly	Ile	Val	Glu	Arg	Pro	Leu
cPLRP1	Lys	Glu	Val	Cys	Tyr	Glu	Gln	Ile	Gly	Cys	Phe	Ser	Asp	Ala	Glu	Pro	Trp	Ala	Gly	Thr	Ala	Ile	Arg	Pro	Leu
ePL	Asn	Glu	Val	Cys	Tyr	Glu	Arg	Leu	Gly	Cys	Phe	Ser	Asp	Asp	Ser	Pro	Trp	Ala	Gly	Ile	Val	Glu	Arg	Pro	Leu
hPL	Lys	Glu	Val	Cys	Tyr	Glu	Arg	Leu	Gly	Cys	Phe	Ser	Asp	Asp	Ser	Pro	Trp	Ser	Gly	Ile	Thr	Glu	Arg	Pro	Leu

DISCUSSION

This is the first detailed description of the purification of feline PL. Classical PL has previously been purified from many other vertebrate species.[59-66] However, many of the previous purification protocols described the purification of PL from pancreatic juice. This method was initially developed because aqueous extractions of pancreatic tissue led to the formation of a macromolecular form of PL, known as fast lipase. Fast lipase is a macromolecular aggregate consisting of PL, colipase, and lipids.[30] In order to collect the pancreatic juice, the pancreatic duct must be cannulated, which raises animal use concerns, especially when a non-invasive alternative such as the purification from pancreatic tissue collected from cats euthanized for unrelated projects exists. Using the delipidation procedure described in the materials and methods section it was possible to purify fPL from pancreatic tissue without the formation of fast lipase.

Reports of the specific absorbance of PL in any species could not be found. A BCA-based protein assay was used to estimate the protein content in a solution of pure fPL with a known absorbance at a wavelength of 280 nm.[58] Bovine serum albumin was used as a standard in this assay. Although, different proteins may have a different relative response in the BCA assay the determination of specific absorbance of fPL through the use of this assay may be inaccurate.[58] The estimated specific absorbance of fPL reported here can be used as a reproducible reference point for future studies involving feline PL.

A sample of purified feline PL showed a single band on SDS-PAGE when stained with a Coomassie blue stain. The molecular mass of fPL as determined by mass

spectrometry was 52.4 kDa. This value is comparable to other values reported for PL such as 52 kDa reported for porcine PL, 50.7 kDa reported for canine PL, 48 kDa reported for both human and chicken PL, and 45 kDa reported as the molecular mass of dromedary PL.[63,65-68]

The first 25 amino acid residues of the N-terminal amino acid sequence were identified except for two residues. A comparison of the sequence for the 23 identified amino acid residues of feline classical PL showed a sequence homology of 87% with porcine classical PL, 83% with canine classical PL, 74% with equine classical PL, 70% with human classical PL, and 61% with canine pancreatic lipase related protein 1 (Table 3).[19,66,69-71] The two amino acid residues that could not be determined were in positions 4 and 10. In pigs, dogs, horses, and human beings these two residues have both been shown to be cysteine residues. Cysteines can form disulfide bridges to form cystine that cannot easily be elucidated during amino acid sequencing. Therefore, it is hypothesized that the amino acid residues in positions 4 and 10 of fPL are cysteine as well. However, this hypothesis has yet to be proven by repeated amino acid sequencing.

In conclusion, this is the first detailed report of the purification of feline classical PL from cat pancreas. Feline PL as reported here shares many characteristics with classical PL previously purified in other species.

CHAPTER III

DEVELOPMENT AND VALIDATION OF A RADIOIMMUNOASSAY FOR FELINE PANCREATIC LIPASE IMMUNOREACTIVITY (fPLI)

INTRODUCTION

The development of radioimmunoassay technology by Yalow and Berson in 1960 was a major step for the diagnostic capabilities of physicians and also veterinarians.[72] Yalow and Berson conducted research on diabetes mellitus that led to the discovery of the formation of anti-insulin antibodies in diabetic patients treated with exogenous insulin.[73] This discovery led to the development of radioimmunoassays. Since then over 80,000 scientific papers have been written on this topic and many radioimmunoassays for the measurement of different analytes have been developed. A common feature that these assays share is that the target molecule has a relatively high molecular mass, thus facilitating the production of antiserum. The general principles of radioimmunoassays are simple. The target substance is radiolabeled with ^{125}I to achieve efficient detection. The radioactive tracer is commonly produced using the chloramine T method first described by Hunter and Greenwood.[74] For the assay a known amount of the radioactive tracer and a known amount of antiserum are placed into a test tube and either a known amount of unlabeled target substance (standard) or a plasma, serum, or other biological fluid sample with an unknown amount of the target substance is added. The radioactively labeled tracer competes with the unlabeled target substance in the standard or sample and forms immune complexes. Bound and unbound tracer are then

separated and the radioactivity of the bound fraction is determined by measuring the radioactivity of the sample with a gamma counter. When the concentration of target substance in the standard or sample solution is low the number of bound counts is high. In contrast, when the concentration of target substance in the standard or sample solution is high the number of bound counts is low. After evaluating a range of standard solutions, a standard curve can be generated and unknown samples can be measured by comparison with the standard curve.

Pancreatitis used to be diagnosed infrequently in cats. Recent studies have shown that pancreatitis occurs frequently in cats, which has led to an increased level of suspicion by veterinary clinicians.[49] However, definitive diagnosis of feline pancreatitis remains elusive and a minimally-invasive diagnostic test that is both highly sensitive and specific for feline pancreatitis is needed.

This is the first detailed report of the development and validation of a radioimmunoassay for the measurement of concentrations of feline PL in biological samples.

MATERIALS AND METHODS

Materials - All commonly used research materials and supplies were purchased from VWR Scientific, West Chester, PA. Radioactive iodine (^{125}I) was from NEN® Life Sciences Products, Inc., Boston, MA. Precipitation solution (N6) was from Diagnostic Products Corporation, Los Angeles, CA. The gamma counter (Riastar) used was from Packard Instrument Company, Meriden, CT.

Antibody production - Polyclonal antibody production was conducted at Lampire Biological Laboratories, Inc. (LBL) in Pipersville, PA following their Express-Line PLUS protocol using New Zealand White rabbits. Feline PL was prepared in phosphate buffered saline, pH 7.2 and sent to LBL for antibody production. Two rabbits were used and both were inoculated with 0.25 mg fPL in 0.5 ml PBS emulsified with 0.5 ml Complete Freund's adjuvant. Three weeks after the first injection, each rabbit received another injection of 0.25 mg fPL in 0.5 ml PBS emulsified with an equal volume of Incomplete Freund's adjuvant. After another three week period, each rabbit was again injected with 0.25 mg fPL in 0.5 ml PBS emulsified with 0.5 ml Incomplete Freund's adjuvant. A production bleed was completed one week after the last booster and produced 47 ml of serum between the two rabbits. An exsanguination bleed was performed two weeks after the last booster and the yield was 181 ml of serum between the two rabbits.

Radioiodination - For the radioimmunoassay, tracer was produced by iodination of feline PL with ^{125}I, using the chloramine T method.[74] A mini stir bar (8 mm x 1.5 mm) was placed in a polypropylene test tube (75 mm x 12 mm) that was situated over a stir plate. Then 10 µl of free ^{125}I (NaI, 0.1 mCi/µl at time of production) were added to the test tube using a Hamilton syringe and mixed with 10 µl 250 mM sodium phosphate buffer, pH 7.5. This was followed by the addition of 10 µl of purified feline PL in 10 µl PBS, pH 7.2, 10 µl of 2 mg/ml chloramine T in 50 mM sodium phosphate, pH 7.5, 100 µl of 400 mg/ml sodium metabisulfite in 50 mM sodium phosphate, pH 7.5, and 860 µl of 2 mg/ml potassium iodide in 50 mM sodium

phosphate, pH 7.5 in rapid succession. The iodinated protein fraction was separated from the free iodide by size-exclusion chromatography on a disposable column (PD-10®, Amersham Pharmacia Biotech, Piscataway, NJ) following the manufacturer's directions. Radioimmunoassay buffer (RIAB, 50 mM sodium phosphate, pH 7.5, with 5 g/L bovine serum albumin and 0.2 g sodium azide) was used as the mobile phase. Fractions of 1 ml each were collected and the fraction containing the peak protein concentration was collected. The tracer was adjusted to approximately 40,000 counts/minute/100 µl tracer and kept in a lockable refrigerator at 4°C until further use.

fPLI-RIA procedure - Polypropylene tubes were labeled in duplicate fashion. The first two tubes were labeled TC (total count) and each received 100 µl tracer. The next two tubes labeled NB (nonspecific binding) received 100 µl tracer and 200 µl radioimmunoassay buffer (RIAB), followed by two tubes labeled B_0 (reference) that received 100 µl tracer, 100 µl anti-fPL antibody solution (approximately 1 mg/ml purified anti-fPL antibody diluted at 1 in 16,000 with RIAB), and 100 µl RIAB. The following 14 tubes were used for the standards and received 100 µl tracer, 100 µl antibody solution, and 100 µl standard solution of 128, 64, 32, 16, 8, 4, or 2 µg/L fPL in RIAB. All of the following tubes were used for unknown samples and received 100 µl tracer, 100 µl antibody solution, and 100 µl of the unknown sample. Tubes were vortexed and incubated for 2 hours at room temperature. After the incubation, all tubes, except the tubes labeled TC, received 100 µl rabbit carrier serum (1 ml normal rabbit serum mixed with 99 ml RIAB) and 1 ml of a commercially available precipitation

solution (N6) containing anti-rabbit IgG antibodies. Again, all tubes were vortexed and then centrifuged at 3000 g and 4°C for 20 min. The supernatant of all tubes, except the tubes labeled TC, was carefully decanted and all tubes were counted for 1 min in a gamma counter. A standard curve was calculated using a log/logit curve fit. The fPL concentrations were plotted along the x-axis in a logarithmic fashion. Values on the y-axis were calculated using the formula $y = \log_e((B_{standard}/B_0)/(1-(B_{sample}/B_0))$ with $B_{standard}$ being the counts per minute (CPM) for each standard and B_0 being the CPM for the reference.

fPLI-RIA validation - This assay was validated by determination of assay sensitivity, control range, linearity, accuracy, precision, and reproducibility by testing assay sensitivity, dilutional parallelism, spiking recovery, intra-assay variability, and inter-assay variability. Assay sensitivity was determined by setting up 10 duplicates of B_0 and calculating the standard deviation of the raw counts of these 10 duplicates. Three standard deviations were subtracted from the mean count and the resulting value evaluated on the standard curve. The sensitivity also served as the lower limit of the working range. Serum samples were selected from random feline serum samples to fall into different areas of the working range of the assay. Because a volume of 1 ml or greater was not available for most serum samples, samples used for validation were generated from a pool of several serum samples. For dilutional parallelism, 4 serum samples were evaluated at full strength and at dilutions of 1 in 2, 1 in 4, and 1 in 8. Spiking recovery was determined by adding 0, 2, 4, 6, 8, 16, 32, and 64 µg/L fPL in RIAB to each one of 4 serum samples. Intra-assay variability was determined by

evaluating four different serum samples 10 times within the same assay run. Finally, inter-assay variability was determined by evaluating four different samples in 10 consecutive assay runs. The control range for the radioimmunoassay for fPLI was determined from the central 95th percentile (2.5th to the 97.5th percentile) of serum fPLI concentration in 30 clinically healthy cats.

RESULTS

A typical standard curve used for the analysis of feline pancreatic lipase immunoreactivity (fPLI) by radioimmunoassay is shown in Figure 5. The sensitivity of the assay was calculated to be 1.2 μg/L, leading to a lower limit of the working range of 1.2 μg/L. Observed to expected ratios for dilutional parallelism of 4 serum samples ranged from 58.0 to 164.3% (Table 4). Observed to expected ratios for spiking recovery of 4 serum samples ranged from 76.0 to 156.5% (Table 5). Coefficients of variation for intra-assay variability of the 4 serum samples were 10.1, 4.5, 2.2, and 3.9% (Table 6). Finally, coefficients of variation for inter-assay variability of the 4 serum samples were 24.4, 15.8, 16.6, and 21.3% (Table 7). The control range established from the central 95th percentile of serum fPLI in 30 clinically healthy cats was 1.2 to 3.8 μg/L (Fig 6).

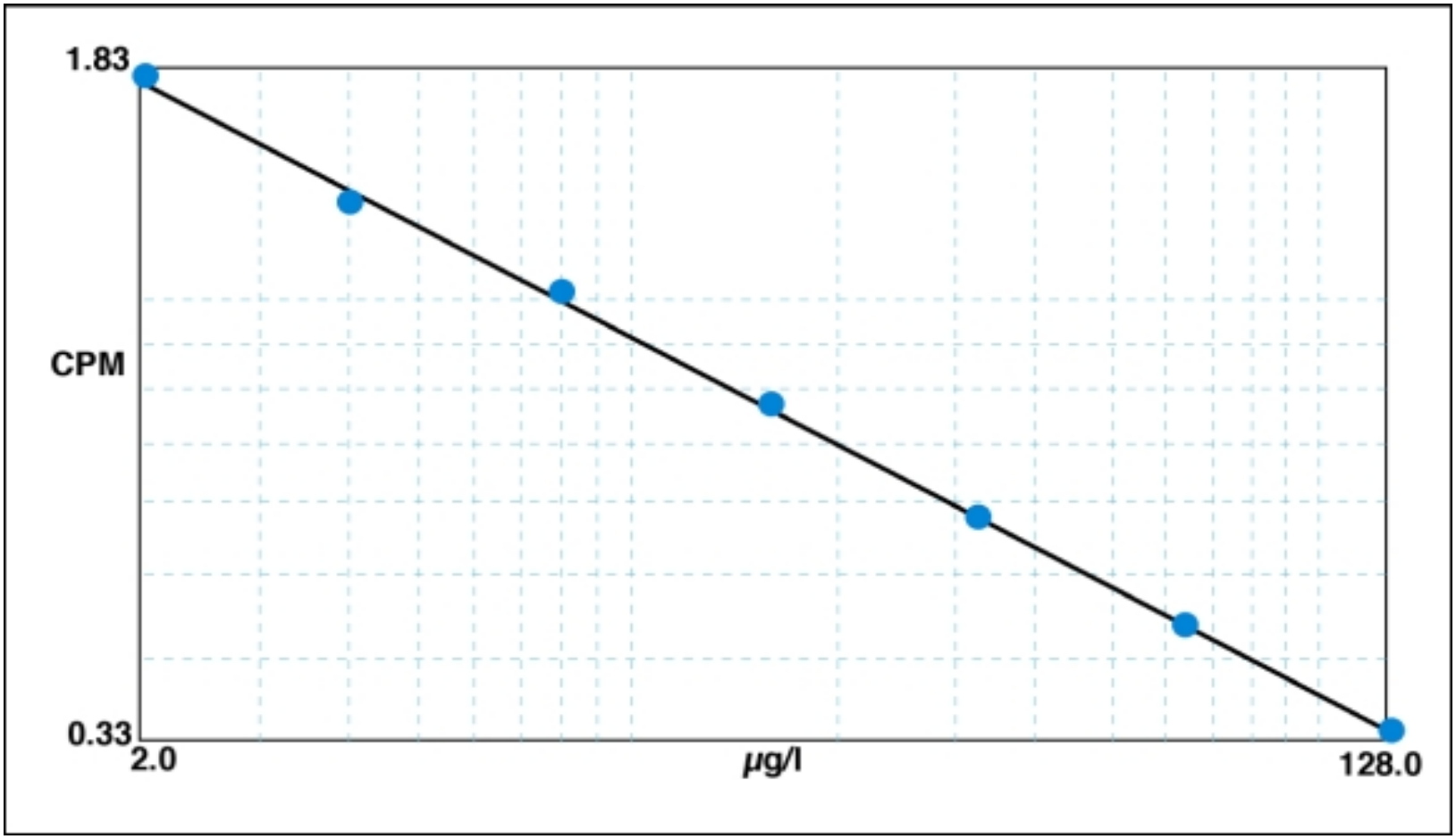

Figure 5-Typical standard curve of fPLI-RIA. This figure shows a typical standard curve for a radioimmunoassay for fPLI. The seven standard concentrations are 2, 4, 8, 16, 32, 64, and 128 µg/L, which are marked on the x-axis in a logarithmic fashion. $\%B/B_0$ values are displayed on the y-axis as counts per minute (CPM) and are calculated from $y = \log_e((B_{standard}/B_0)/(1-(B_{sample}/B_0)))$. The CPM values must be multiplied by a factor of 10,000 to obtain the actual results.

Table 4-Dilutional parallelism of radioimmunoassay for fPLI. This table shows data from serial dilution of 4 serum samples throughout the control range of the assay. All 4 serum samples were measured in dilutions of 1 in 1, 1 in 2, 1 in 4, and 1 in 8. The observed to expected ratios (O/E) are shown as % in bold print.

	Dilution	**Observed Value (µg/L)**	**Expected Value (µg/L)**	**O/E (%)**
Sample 1	1 in 1	4.48		
	1 in 2	3.5	2.24	**156.3**
	1 in 4	1.28	1.12	**114.3**
	1 in 8	0.92	0.56	**164.3**
Sample 2	1 in 1	7.53		
	1 in 2	3.95	3.77	**104.9**
	1 in 4	2.2	1.88	**116.9**
	1 in 8	0.93	0.94	**98.8**
Sample 3	1 in 1	45.06		
	1 in 2	24.45	22.53	**108.5**
	1 in 4	11.9	11.27	**105.6**
	1 in 8	6.47	5.63	**114.9**
Sample 4	1 in 1	115.79		
	1 in 2	53.04	57.9	**91.6**
	1 in 4	20.69	28.95	**71.5**
	1 in 8	8.4	14.47	**58**

Table 5-Spiking recovery of radioimmunoassay for fPLI. This table shows the data obtained from the spiking recovery performed on 4 different serum samples throughout the control range. The observed to expected ratios (O/E) are shown as % in bold print.

	Concentration added (µg/L)	**Observed (µg/L)**	**Expected (µg/L)**	**O/E (%)**
Sample 1	0	2.7		
	2	5.1	4.7	**108.6**
	4	8	6.7	**119.9**
	8	13	10.7	**121.6**
	16	26.3	18.7	**141**
	32	48.8	34.7	**140.8**
	64	104.4	66.7	**156.5**
Sample 2	0	3		
	2	4.1	5	**81.3**
	4	5.8	7	**82.2**
	8	9.2	11	**83.1**
	16	14.6	19	**76.9**
	32	27.9	35	**79.6**
	64	51	67	**76**
Sample 3	0	16.9		
	2	22.3	18.9	**118**
	4	26.2	20.9	**125.4**
	8	29.8	24.9	**119.8**
	16	43.7	32.9	**132.8**
	32	72.2	48.9	**147.6**
	64	124.5	80.9	**153.9**
Sample 4	0	35.9		
	2	51.1	37.9	**134.8**
	4	53.4	55.1	**96.9**
	8	62.8	61.4	**102.3**
	16	85.1	78.8	**108**
	32	121.9	117.1	**104.1**
	64	214.7	185.9	**115.1**

Table 6-Intra-assay variability of radioimmunoassay for fPLI. This table shows the results obtained from the intra-assay variability of the radioimmunoassay for fPLI using 4 different serum samples. (SD = standard deviation; CV = coefficient of variation; CV(%) = (SD/Mean)*100).

	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**
Number of Repeats	10	10	10	10
Mean (µg/L)	3.35	7.51	19.82	39.78
SD (µg/L)	0.34	0.33	0.44	1.54
CV (%)	**10.1**	**4.5**	**2.2**	**3.9**

Table 7-Inter-assay variability of radioimmunoassay for fPLI. This table shows the results obtained from the inter-assay variability of the radioimmunoassay for fPLI using 4 different serum samples. (SD = standard deviation; CV = coefficient of variation; CV(%) = (SD/Mean)*100).

	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**
Number of Repeats	10	10	10	10
Mean (µg/L)	3.8	6.98	27.75	57.4
SD (µg/L)	0.93	1.1	4.62	12.23
CV (%)	**24.4**	**15.8**	**16.6**	**21.3**

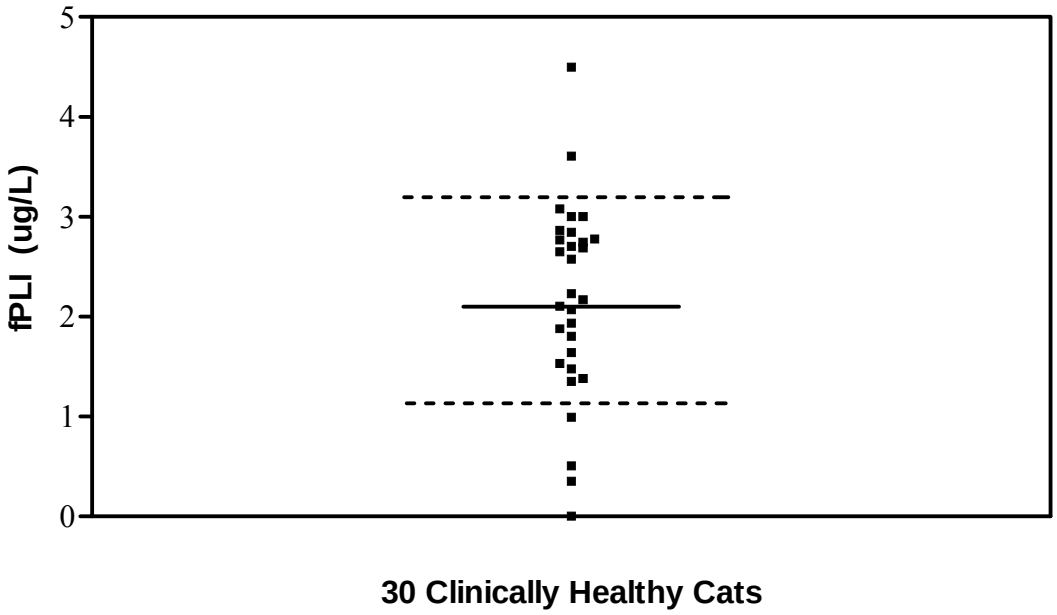

Figure 6-Mean serum concentration for fPLI in 30 clinically healthy cats The mean serum concentration for fPLI as measured in 30 clinically healthy cats was 2.18 +/- 0.975 µg/L. The control range was determined from the central 95th percentile to be 1.2 to 3.8 µg/L. The mean fPLI concentration is depicted by the solid line. The control range is shown between the dotted lines.

DISCUSSION

A radioimmunoassay for the measurement of feline pancreatic lipase immunoreactivity (fPLI) in cat serum was successfully established. Although many radioimmunoassays have been developed for use in both human and veterinary medicine, there is still very little known about the minimal performance characteristics for a clinically useful assay.[75,76,77-79] Most investigators simply state the performance characteristics of the assay they develop and validate.[80,81,82] Other investigators have targeted observed to expected ratios for dilutional parallelism and spiking recovery between 80 to 120% but references are not provided as to the reason for these targeted values.[76] Also, some authors target coefficients of variation for intra-assay and inter-assay variability to reach a maximum of 10 to 15%, but again there is no rationale given for this target value.[76] However, while there is little evidence to suggest the validity of these targeted performance criteria many radioimmunoassays that have proven to be clinically relevant have shown performance criteria meeting these values.[76]

Observed to expected ratios for serial dilution ranged between 58.0 and 164.3%. The observed to expected ratios for sample 1 were elevated above 120% for the 1 in 2 and 1 in 8 dilution. The dilution of 1 in 4 for sample 1 was between the performance indices. The observed to expected ratios for sample 2 and sample 3 fall into the targeted range of values for a clinically relevant assay. The first dilution step for sample 4 falls nicely into the desired range of observed to expected ratios, but the second and third dilution steps have ratios less than 80%. The data presented suggests that the assay has limited linearity.

Observed to expected values for spiking recovery were between 76.0 and 156.5%. The observed to expected ratios for sample 1 are within the range of 80 to 120% for the first three spiking steps, but begin to increase outside the range when larger amounts of protein are added to the sample. The observed to expected ratios for sample 2 are within the desired range for the first three spiking steps, and drop slightly below the 80% marker for the last three steps when larger amounts of protein are added. The data obtained for sample 3 is very similar to sample 1 in that the first three steps are very close to the desired range, while the last three steps have elevated observed to expected ratios. All of the observed to expected ratios for sample 4 fall into the desired range except the first sample that was spiked with 2 µg/L of fPL protein. This value for the first step of sample 4 could be attributed to assay variability because the data shows that 51.1 µg/L was observed when only 37.9 µg/L was present. Granted, some of these values are outside the range of 80 to 120%, but this is usually only the case when large amounts of fPLI are added to the samples. The data presented for spiking recovery suggest that the assay is accurate.

The control range of the assay was calculated to be 1.2 to 3.8 µg/L from the central 95th percentile of serum fPLI concentrations in 30 clinically healthy cats.

The coefficients of variation (CVs) for the intra-assay variability for 3 of the 4 serum samples were well below 10% and the fourth value was just out of range at 10.1%. These values suggest that the assay is sufficiently precise for clinical use. The CVs for inter-assay variability of the four serum samples were 24.4, 15.8, 16.6, and 21.3%. The CV for sample 1 is higher than desired, but was accepted because the value

of sample 1 falls in the low area of the working range. The CV for sample 4 was also higher than desired and accepted because the value of sample 4 falls in the high area of the working range. The data obtained from inter-assay variability suggests that the assay has a limited reproducibility in the upper and lower areas of the working range.

In conclusion, the radioimmunoassay described here is sufficiently accurate and precise for clinical use, with a limited linearity and reproducibility in the upper and lower areas of the working range. Clinical studies will be necessary in order to determine whether this degree of assay linearity and reproducibility will be sufficient to definitively distinguish cats with pancreatitis from clinically healthy cats.

CHAPTER IV

MEASUREMENT OF SERUM fPLI AND fTLI CONCENTRATIONS IN CATS WITH EXPERIMENTALLY INDUCED PANCREATITIS

INTRODUCTION

Several hundred thousand cases of pancreatitis are diagnosed each year in the United States alone. It has been estimated that almost 90% of all the human pancreatitis cases remain undiagnosed. Pancreatitis also commonly occurs in both dogs and cats. It can be assumed, that as in human beings, canine and feline pancreatitis remain undiagnosed in many cases. This could be because of the nonspecific clinical symptoms presented by canine and feline patients with pancreatitis combined with the lack of a clinically useful diagnostic test that is both highly sensitive and specific for pancreatitis.

Studies performed in dogs and human beings with pancreatitis have shown increases in serum amylase and lipase activities.[83,84] However, serum amylase and serum lipase activities have been shown to have no clinical usefulness for the diagnosis of feline pancreatitis. Many of the reports that have been published regarding cats with pancreatitis describe a very vague clinical picture.[50,85,86]

Recently, a new diagnostic test for exocrine pancreatic disease in cats, feline trypsin-like immunoreactivity assay (fTLI) has been described.[87] The reference range for fTLI is between 12.0 – 82.0 μg/L. A serum fTLI concentration equal to or below 8.0 μg/L is diagnostic for EPI, while concentrations between 8.0 and 12.0 μg/L are equivocal.[88] A serum fTLI in excess of 100.0 μg/L is consistent with either acute or

chronic pancreatitis or decreased renal excretion caused by severe renal insufficiency.[89] The measurement of serum fTLI concentration is highly specific for pancreatitis, but the sensitivity of the assay is less than optimal. Therefore, a new diagnostic test for feline pancreatitis that is both highly sensitive and specific is highly desirable.

As described previously, the control range for the radioimmunoassay for the measurement of serum fPLI concentration in cat serum was established as 1.2-3.8 μg/L.

This study was completed in order to compare the clinical utility of serum fPLI and fTLI concentrations for the diagnosis of pancreatitis in the cat.

MATERIALS AND METHODS

Serum samples - All serum samples that were used for this comparison study were obtained from remaining serum from a previous research project relating to feline pancreatitis. The samples that were used for this project came from cats with experimentally induced pancreatitis. The pancreatitis was induced by retrograde injection of oleic acid into the pancreatic duct. Cat 1, cat 2, and cat 3 had serum samples

collected at 0, 8, 24, 48, 72, 96, 120, 144, 192, 240, 288, 384, and 480 hours after induction of pancreatitis. Cat 4 had serum samples collected at 0, 8, 24, 48, 72, 96, 120, 144, 192, and 240 hours after induction of pancreatitis.

Assays - All of the serum samples were previously analyzed for feline trypsin-like immunoreactivity (fTLI) at the Gastrointestinal Laboratory, Department of Small Animal Medicine and Surgery, Texas A&M University, College Station, TX using an in-house enzyme-linked immunosorbent assay (ELISA). All of the serum samples were also analyzed for feline pancreatic lipase immunoreactivity (fPLI) using the newly developed and validated radioimmunoassay using the protocol described in Chapter III.

RESULTS

Serum fTLI concentration as measured by ELISA and serum fPLI concentration as measured by RIA were successfully measured in 4 cats with experimentally induced pancreatitis (Table 8). Serum fPLI and fTLI concentrations were compared for each time point and each cat (Figs 7 to 10).

Table 8-Serum fPLI (µg/L) and fTLI (µg/L) concentrations in 4 cats with experimentally induced pancreatitis. Serum fPLI concentration as measured by RIA and serum fTLI concentration as measured by ELISA were measured in 4 cats with experimentally induced pancreatitis and compared over time. There was no serum sample available to analyze the fPLI concentration for cat 4 for the 120 hour collection time and the 240 hour data point was the last time serum was collected from cat 4. The control range for serum fPLI is 1.2-3.8 µg/L, the reference range for serum fTLI is 12.0-82.0 µg/L.

Time	0 h	8 h	24 h	48 h	72 h	96 h	120 h	144 h	192 h	240 h	288 h	384 h	480 h
Cat 1 fPLI	0.1	17.1	29.3	18.7	13.6	11.8	10.8	8.4	9.0	4.9	3.7	1.4	0.2
Cat 1 fTLI	47	230	168	80	37	55	39	38	24	30	38	24	25
Cat 2 fPLI	1.0	20.1	25.8	17.6	12.7	7.5	5.2	4.6	6.2	6.7	3.0	3.0	2.8
Cat 2 fTLI	57	336	256	37	109	14	15	18	72	81	41	48	34
Cat 3 fPLI	0.8	34.8	50.9	34.5	12.5	4.6	3.7	3.1	2.3	2.2	1.7	2.6	2.5
Cat 3 fTLI	54	1856	5008	159	67	32	18	8	11	5	8	20	19
Cat 4 fPLI	0.1	28.9	25.1	15.9	22.5	17.1		19.5	13.6	2.4			
Cat 4 fTLI	94	972	212	50	130	92	164	210	129	54			

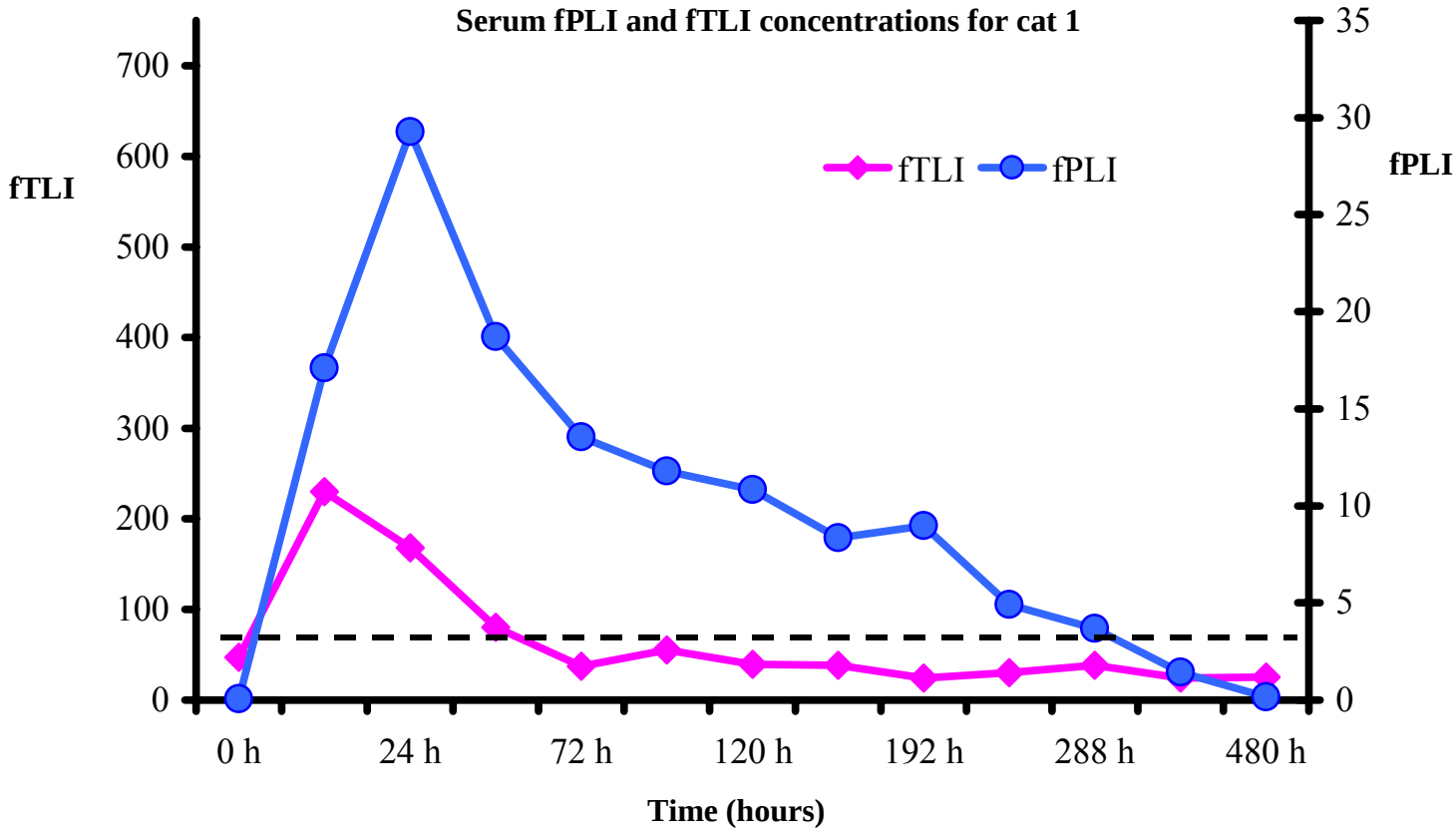

Figure 7-Serum fPLI and fTLI concentrations for cat 1. This graph shows the comparison of serum fPLI concentration to serum fTLI concentration for cat 1 over time. The dotted line depicts the upper limit of the normal range for fTLI and fPLI. Serum fPLI concentration remains elevated above the upper limit of the control range much longer than serum fTLI concentration.

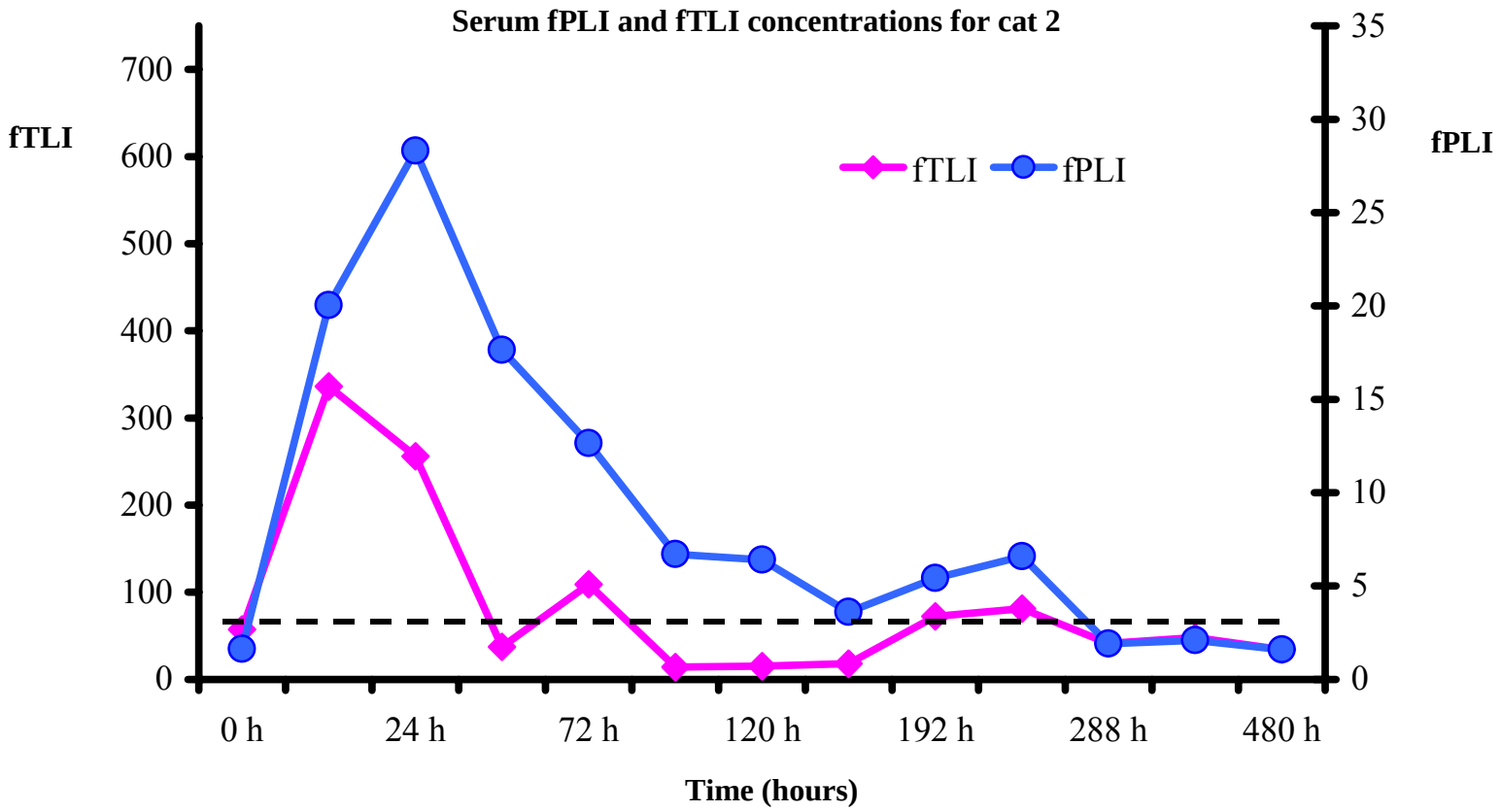

Figure 8-Serum fPLI and fTLI concentrations for cat 2. This graph shows the comparison of serum fPLI concentration to serum fTLI concentration for cat 2 over time. The dotted line depicts the upper limit of the normal range for fTLI and fPLI. Serum fPLI concentration remains elevated above the upper limit of the control range much longer than serum fTLI concentration.

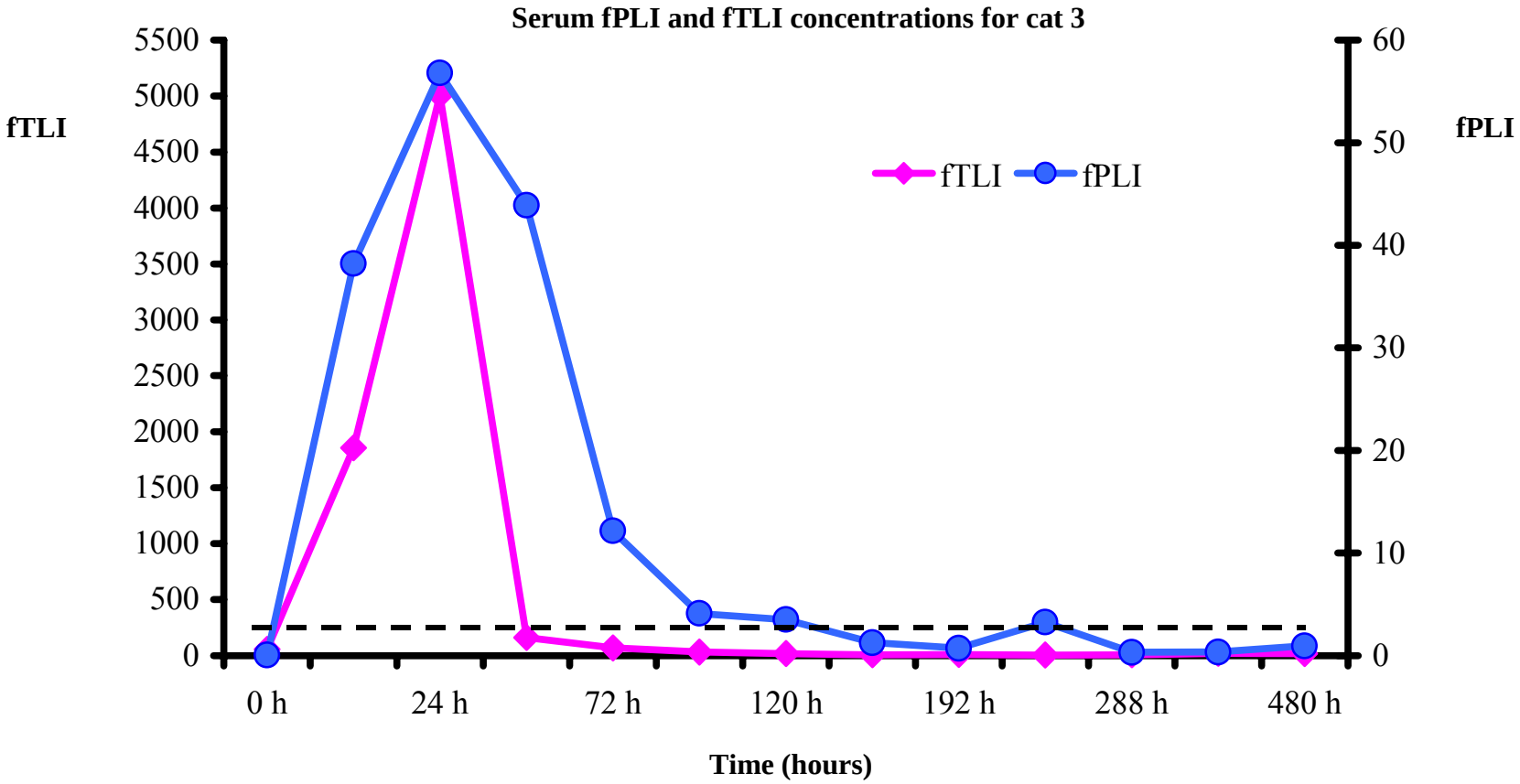

Figure 9-Serum fPLI and fTLI concentrations for cat 3. This line graph shows the comparison of serum fPLI concentration to serum fTLI concentration for cat 3 over time. The dotted line depicts the upper limit of the normal range for fTLI and fPLI. Serum fPLI concentration remains elevated above the upper limit of the control range much longer than serum fTLI concentration.

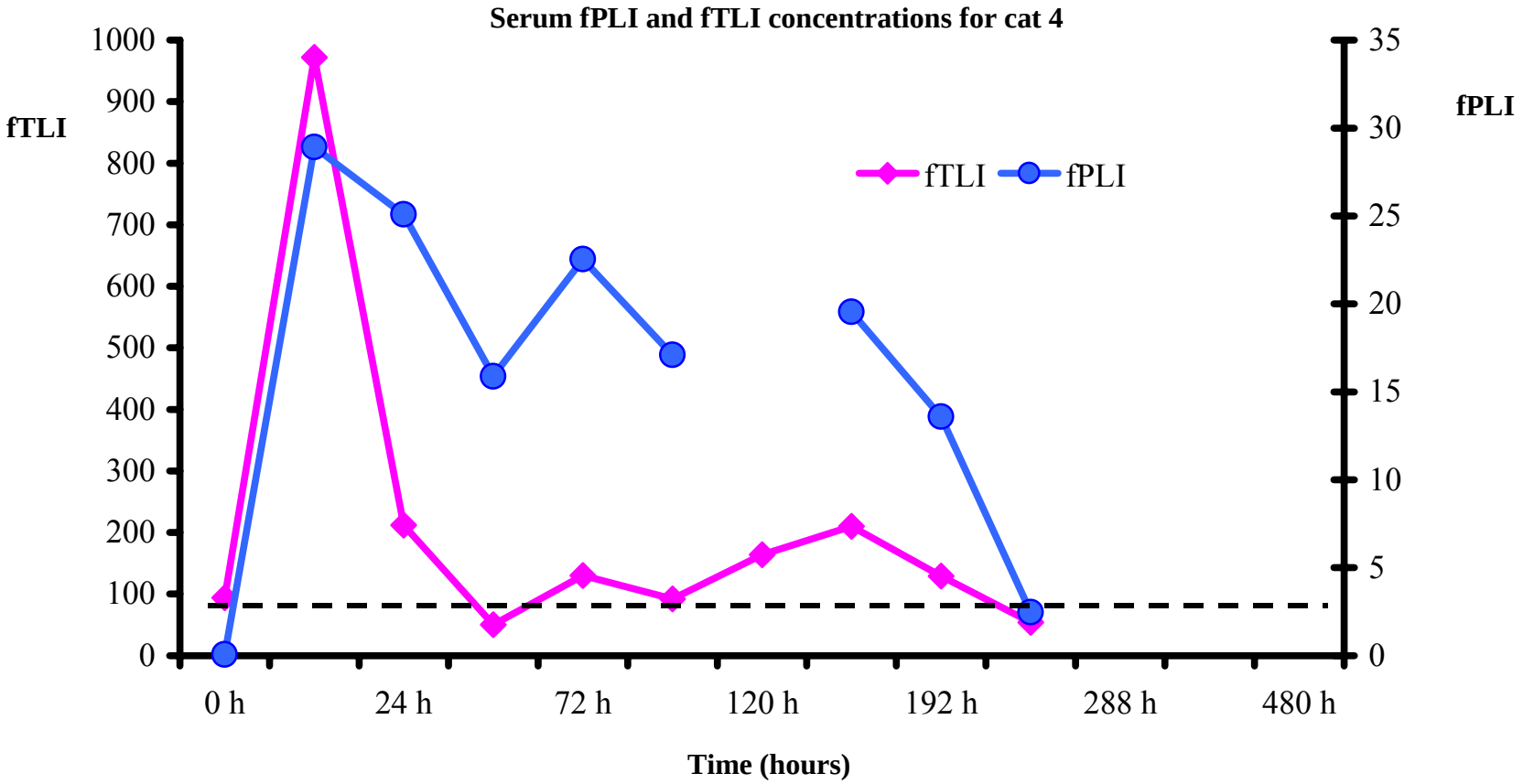

Figure 10-Serum fPLI and fTLI concentrations for cat 4. This graph shows the comparison of serum fPLI concentration to serum fTLI concentration for cat 4 over time. The dotted line depicts the upper limit of the normal range for fTLI and fPLI. Serum fPLI concentration remains elevated above the upper limit of the control range much longer than serum fTLI concentration.

DISCUSSION

One can draw two important conclusions from comparison of serum fPLI and fTLI concentrations in cat 1 (Fig 7). The first conclusion is that both serum fPLI and fTLI concentrations exhibit the same trend over time. That is, both parameters increase initially and then decrease. Secondly, and more importantly, serum fPLI concentrations remain elevated above the upper limit of the fPLI control range much longer than do serum fTLI concentrations. Serum fTLI concentrations drop back into the normal range after approximately 48 hours, while serum fPLI concentrations do not reenter the normal range until 288 hours. This is very important clinically because it prolongs the duration of time that a diagnosis of feline pancreatitis can be made and substantially increases the sensitivity for serum fPLI when compared to serum fTLI.

Similar findings can also be observed for cat 2. Figure 8 shows that the fTLI concentration returns back into the normal range after the initial peak after approximately 48 hours, whereas the fPLI concentration remains elevated above the upper limit of the normal range for approximately 288 hours after the initial peak. Thus, as for cat 1, serum fPLI is much more sensitive for pancreatitis than is serum fTLI concentration.

The concentrations of serum fPLI and fTLI for cat 3 are shown in Figure 9. The same conclusions drawn before are also valid here for cat 3. The one noted difference in the concentrations of serum fPLI and fTLI for cat 3 is that the fTLI concentration remains above the normal range until approximately 60 hours (2.5 days), and the fPLI concentrations drop below the upper limit of the normal range at approximately 110

hours (4.6 days), which is much sooner than any of the other three cats analyzed. These data still show that the fPLI concentration remains elevated above the normal range longer than the fTLI value, which is important from a clinical point of view.

The data shown for cat 4 (Fig 10) also show that as for the previous three cats the fTLI concentration drops below the upper limit of the normal range at approximately 48 hours, while the fPLI concentration remains elevated above the upper limit of the normal range for approximately 240 hours, once again indicating a higher sensitivity of the fPLI assay for feline pancreatitis.

In conclusion, serum fPLI concentration is increased in cats with experimentally induced pancreatitis. Also, serum fPLI concentration decreased back into the reference range many days after serum fTLI concentration. This suggests that the measurement of serum fPLI concentration is likely to be more sensitive for a diagnosis of feline pancreatitis than the measurement of serum fTLI. These encouraging initial data need to be confirmed in cats with spontaneous pancreatitis.

CHAPTER V

CONCLUSIONS

The objective of this project was to test the hypothesis that the measurement of serum feline pancreatic lipase immunoreactivity (fPLI) is useful in the diagnosis of pancreatitis. To test this hypothesis, feline classical PL was purified from pancreatic tissue, antiserum against this protein was raised and purified, a radioimmunoassay for the measurement of fPLI in cat serum was developed and validated, and the assay was evaluated in clinically healthy cats and cats with experimentally induced pancreatitis.

This is the first detailed description of the purification of feline classical PL. Feline PL was extracted and purified from feline pancreatic tissue. Partial characterization of feline PL revealed a molecular mass of 52.4 kDa, an approximate specific absorbance at 280 nm of 1.18 for a 1 mg/ml solution, a specific lipase activity of 10,261 U/mg using tributyrin as a substrate, and a N-terminal amino acid sequence for the first 25 amino acid residues that showed a very high sequence homology with previously reported sequences of classical PL purified in other species (Table 3).[19,66,69-71]

Antiserum directed against fPL was raised in New Zealand White rabbits by repeated injection of the purified protein emulsified with complete or incomplete Freund's adjuvant. The antibody production was conducted at Lampire Biological Laboratories, Inc. (LBL) in Pipersville, PA following their Express-Line PLUS protocol using New Zealand White rabbits.

A radioimmunoassay (RIA) for the measurement of feline pancreatic lipase immunoreactivity (fPLI) in serum was successfully developed and validated. The assay was determined to be sufficiently accurate and precise for clinical use, with a limited linearity and reproducibility in the upper and lower areas of the working range. After evaluating samples from 30 clinically healthy cats with the validated RIA the control range was established as 1.2 to 3.8 μg/L (Fig 6).

Serum fPLI concentration was measured in serum collected from an unrelated research project involving four cats that were experimentally induced with pancreatitis. Cats 1, 2, and 3 had thirteen serum samples taken at specific time points. Cat 4 had ten serum samples taken at specific time points. All samples were evaluated for serum fPLI and fTLI concentrations so that a comparison could be made between the two concentrations over time. The data presented show that serum fPLI concentration decreased back into the reference range many days after serum fTLI concentration (Table 8, Figs 7 to 10). This suggests that the measurement of serum fPLI concentration is likely to be more sensitive for a diagnosis of feline pancreatitis than the measurement of serum fTLI. The presented data are also promising in regards to the fact that the window of diagnosis of feline pancreatitis is much larger when measuring serum fPLI (approximately 10 days) versus measuring serum fTLI (approximately 2 days). Future studies need to be conducted with a larger sample size of cats known to have pancreatitis in order to systematically evaluate the sensitivity and specificity of serum fPLI concentration as measured by radioimmunoassay for the diagnosis of pancreatitis in the cat.

In summary, feline classical PL was successfully purified, antibodies against fPL were produced, and a radioimmunoassay for the measurement of feline pancreatic lipase immunoreactivity (fPLI) was developed and validated. The work completed during this project and presented in this thesis supports the hypothesis that serum fPLI concentration is useful for the diagnosis of feline pancreatitis.

REFERENCES

1. Peterson SB, Drabløs F. A sequence analysis of lipases, esterases, and related proteins. In: Woolley P, Peterson SB, eds. *Lipases – their structure, biochemistry, and application.* Cambridge: Cambridge University Press, 1994;23-48.

2. Suzuki A, Mizumoto A, Rerknimitr R, et al. Effect of bacterial or porcine lipase with low- or high-fat diets on nutrient absorption in pancreatic-insufficient dogs. *Gastroenterology* 1999;116:431-437.

3. Jaeger KE, Ransac S, Dijkstra BW, et al. Bacterial lipases. *FEMS Microbiology Reviews* 1994;15:29-63.

4. Lawson DM, Brzozowski AM, Dodson GG, et al. Three-dimensional structures of two lipases from filamentous fungi. In: Woolley P, Peterson SB, eds. *Lipases – their structure, biochemistry, and application.* Cambridge: Cambridge University Press, 1994;77-94.

5. Mukherjee KD. Plant lipases and their application in lipid biotransformations. *Progress in Lipid Research* 1994;33:165-174.

6. Mukherjee KD, Hills MJ. Lipases from plants. In: Woolley P, Peterson SB, eds. *Lipases – their structure, biochemistry, and application.* Cambridge: Cambridge University Press, 1994;49-75.

7. Lin YH, Yu C, Huang AH. Substrate specificities of lipases from corn and other seeds. *Archives of Biochemistry & Biophysics* 1986;244:346-356.

8. Derewenda ZS, Sharp AM. News from the interface: the molecular structures of triacylglyceride lipases. *Tren Bioc Sci* 1993;18:20-25.

9. Antonian E. Recent advances in the purification, characterization and structure determination of lipases. *Lipids* 1988;23:1101-1106.

10. Mickel FS, Weidenbach F, Swarovsky B, et al. Structure of the canine pancreatic lipase gene. *J Biol Chem* 1989;264:12895-12901.

11. Svendson A. Sequence comparisons within the lipase family. In: Woolley P, Peterson SB, eds. *Lipases – their structure, biochemistry, and application.* Cambridge: Cambridge University Press, 1994;1-21.

12. Carrière F, Withers-Martinez C, Van Tilbeurgh H, et al. Structural basis for the substrate selectivity of pancreatic lipases and some related proteins. *Biochim Biophys Acta Rev Biomembr* 1998;1376:417-432.

13. Hirata K, Dichek HL, Cioffi JA, et al. Cloning of a unique lipase from endothelial cells extends the lipase gene family. *J Biol Chem* 1999;274:14170-14175.

14. Jaye M, Lynch KJ, Krawiec J, et al. A novel endothelial-derived lipase that modulates HDL metabolism. *Nat Genet* 1999;21:424-428.

15. Carrière F, Bezzine S, Verger R. Molecular evolution of the pancreatic lipase and two related enzymes towards different substrate selectivities. *Journal of Molecular Catalysis B: Enzymatic* 1997;3:55-64.

16. Winkler FK, Gubernator K. Structure and mechanism of human pancreatic lipase. In: Woolley P, Peterson SB, eds. *Lipases – their structure, biochemistry, and application.* Cambridge: Cambridge University Press, 1994;139-157.

17. Rinderknecht H. Pancreatic secretory enzymes. In: Go VLW, DiMagno EP, Gardner JD, et al, eds. *The pancreas: biology, pathobiology and disease.* New York:Raven Press, 1993;219-251.

18. Borgström B. The action of bile salts and other detergents on pancreatic lipase and the interaction with colipase. *Biochim Biophys Acta* 1977;488:381-391.

19. Winkler FK, D'Arcy A, Hunziker W. Structure of human pancreatic lipase. *Nature* 1990;343:771-774.

20. Bourne Y, Martinez C, Kerfelec B, et al. Horse pancreatic lipase. The crystal structure refined at 2•3 Å resolution. *J Mol Biol* 1994;238:709-732.

21. Chaillan C, Kerfelec B, Foglizzo E, et al. Direct involvement of the C-terminal extremity of pancreatic lipase (403-449) in colipase binding. *Biochemical & Biophysical Research Communications* 1992;184:206-211.

22. Abousalham A, Chaillan C, Kerfelec B, et al. Uncoupling of catalysis and colipase binding in pancreatic lipase by limited proteolysis. *Protein Engineering* 1992;5:105-111.

23. Ayvazian L, Crenon I, Granon S, et al. Recombinant C-terminal domain of pancreatic lipase retains full ability to bind colipase. *Protein Engineering* 1996;9:707-711.

24. Van Tilbeurgh H, Sarda L, Verger R, et al. Structure of the pancreatic lipase-procolipase complex. *Nature* 1992;359:159-162.

25. Van Tilbeurgh H, Gargouri Y, Dezan C, et al. Crystallization of pancreatic procolipase and of its complex with pancreatic lipase. *J Mol Biol* 1993;229:552-554.

26. Bezzine S, Carrière F, De Caro J, et al. Human pancreatic lipase: an exposed hydrophobic loop from the C-terminal domain may contribute to interfacial binding. *Biochemistry* 1998;37:11846-11855.

27. Lowe ME. The catalytic site residues and interfacial binding of human pancreatic lipase. *J Biol Chem*1992;267:17069-17073.

28. Lowe ME. Mutation of the catalytic site Asp177 to Glu177 in human pancreatic lipase produces an active lipase with increased sensitivity to proteases. *Biochim Biophys Acta* 1996;1302:177-183.

29. Carrière F, Gargouri Y, Moreau H, et al. Gastric lipases: cellular, biochemical and kinetic aspects. In: Woolley P, Peterson SB, eds. *Lipases – their structure, biochemistry, and application.* Cambridge: Cambridge University Press, 1994;181-205.

30. Leger C, Charles M. Pancreatic lipase. *World Review of Nutrition & Dietetics* 1980;35:96-128.

31. Lowe ME. Molecular mechanisms of rat and human pancreatic triglyceride lipases. *J Nutr* 1997;127:549-557.

32. Jensen RG, Clark RM, DeJong FA, et al. The lipolytic triad: human lingual, breast milk, and pancreatic lipases: physiological implications of their characteristics in digestion of dietary fats. *Journal of Pediatric Gastroenterology & Nutrition* 1982;1:243-255.

33. Borovicka J, Schwizer W, Mettraux C, et al. Regulation of gastric and pancreatic lipase secretion by CCK and cholinergic mechanisms in humans. *Am J Physiol* 1997;273:G374-G380.

34. Duan RD, Erlanson-Albertsson C. Evidence of a stimulatory effect of cyclic AMP on pancreatic lipase and colipase synthesis in rats. *Scand J Gastroenterol* 1992;27:644-648.

35. Thiruvengadam R, DiMagno EP. Inactivation of human lipase by proteases. *Am J Physiol* 1988;255:G476-G481.

36. Holtmann G, Kelly DG, Sternby B, et al. Survival of human pancreatic enzymes during small bowel transit: effect of nutrients, bile acids, and enzymes. *Am J Physiol* 1997;273:G553-G558.

37. Lessinger JM, Ferard G. Plasma pancreatic lipase activity: from analytical specificity to clinical efficiency for the diagnosis of acute pancreatitis. *European Journal of Clinical Chemistry & Clinical Biochemistry* 1994;32:377-381.

38. Kok PJ, Holtkamp HC, Seidel B. Calibration of a turbidimetric assay of serum lipase activity. *Clin Chim Acta* 1978;83:123-128.

39. Strombeck DR, Farver T, Kaneko JJ. Serum amylase and lipase activities in the diagnosis of pancreatitis in dogs. *Am J Vet Res* 1981;42:1966-1970.

40. Brobst D, Ferguson AB, Carter JM. Evaluation of serum amylase and lipase activity in experimentally induced pancreatitis in the dog. *J Am Vet Med Assoc* 1970;157:1697-1702.

41. Mia AS, Koger HD, Tierney MM. Serum values of amylase and pancreatic lipase in healthy mature dogs and dogs with experimental pancreatitis. *Am J Vet Res* 1978;39:965-969.

42. Eto K, Pairent FW, Appert HE, et al. Renal excretion of amylase and lipase by dogs. *Arch Surg* 1969;98:241-244.

43. Simpson KW, Simpson JW, Lake S, et al. Effect of pancreatectomy on plasma activities of amylase, isoamylase, lipase and trypsin-like immunoreactivity in dogs. *Res Vet Sci* 1991;51:78-82.

44. Ventrucci M, Gullo L, Daniele C, et al. Comparative study of serum pancreatic isoamylase, lipase, and trypsin-like immunoreactivity in pancreatic disease. *Digestion* 1983;28:114-121.

45. Kolars JC, Ellis CJ, Levitt MD. Comparison of serum amylase pancreatic isoamylase and lipase in patients with hyperamylasemia. *Digestive Diseases & Sciences* 1984;29:289-293.

46. Flamion B, Delhaye M, Horanyi Z, et al. Comparison of elastase-1 with amylase, lipase, and trypsin-like immunoreactivity in the diagnosis of acute pancreatitis. *American Journal of Gastroenterology* 1987;82:532-535.

47. Bedrak E. Blood serum enzyme activity of dogs exposed to heat stress and muscular exercise. *J Appl Physiol* 1965;20:587-590.

48. Roberts IM, Mercer D. Radioimmunoassay for human pancreatic lipase in acute pancreatitis. *Dig Dis Sci* 1987;32, No.4:388-392.

49. Steiner JM, Williams DA. Feline exocrine pancreatic disorders. *The Veterinary Clinics of North America* 1999;29:551-575.

50. Hill RC, Van Winkle TJ. Acute necrotizing pancreatitis and acute suppurative pancreatitis in the cat. A retrospective study of 40 cases (1976-1989). *J Vet Int Med* 1993;7:25-33.

51. Saunders HM, Van Winkle TJ, Drobatz K, et al. Ultrasonographic findings in cats with clinical, gross pathologic, and histologic evidence of acute pancreatic necrosis: 20 cases (1994-2001). *Journal of the American Medical Association* 2002;221:1724-1730.

52. Gerhardt A, Steiner JM, Williams DA, et al. Comparison of the sensitivity of different diagnostic tests for pancreatitis in cats. *J Vet Intern Med* 2001;15:329-333.

53. Parent C, Washabau RJ, Williams DA, et al. Serum trypsin-like immunoreactivity, amylase and lipase in the diagnosis of feline acute pancreatitis. *J Vet Intern Med* 1995;9:194

54. Swift NC, Marks SL, MacLachlan NJ, et al. Evaluation of serum feline trypsin-like immunoreactivity for the diagnosis of pancreatitis in cats. *J Am Vet Med Assoc* 2000;217:37-42.

55. Borgström B, Erlanson C. Pancreatic juice co-lipase: Physiological importance. *Biochim Biophys Acta* 1971;242:509-513.

56. Maylie MF, Charles M, Astier M, et al. On porcine pancreatic colipase: large scale purification and some properties. *Biochemical and Biophysical Research Communications* 1973;52:291-297.

57. Borgström B, Wieloch T, Erlanson-Albertsson C. Evidence for a pancreatic pro-colipase and its activation by trypsin. *FEBS Lett* 1979;108:407-410.

58. Smith PK, Krohn RL, Hermanson GT, et al. Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985;150:76-85.

59. Vandermeers A, Chroistophe J. Alpha-amylase and lipase of rat pancreas. Chromatographic purification and research on molecular weight and amino acid composition. *Biochim Biophys Acta* 1968;154:110-129.

60. Verger R, Haas GH, Sarda L, et al. Purification from porcine pancreas of two molecular species with lipase activity. *Biochim Biophys Acta* 1969;188:272-282.

61. Shahani KM, Khan IM, Chandan RC. Bovine pancreatic lipase. I. Isolation, homogeneity, and characterization. *J Dairy Sci* 1976;59:369-375.

62. Rathelot J, Julien R, Bosc-Bierne J, et al. Horse pancreatic lipase. Interaction with colipase from various species. *Biochimie* 1981;63:227-234.

63. Bosc-Bierne I, Rathelot J, Perrot C, et al. Studies on chicken pancreatic lipase and colipase. *Biochim Biophys Acta* 1984;794:65-71.

64. Gieseg SP, Forrester IT, Carne A. The purification of ovine pancreatic lipase that is free of colipase using an improved delipidation method. *Pancreas* 1992;7:45-51.

65. Mejdoub H, Reinbolt J, Gargouri Y. Dromedary pancreatic lipase: Purification and structural properties. *Biochim Biophys Acta Lipids and Lipid Metab* 1994;1213:119-126.

66. Steiner JM. Canine Digestive Lipases. Texas A&M University PhD Dissertation.

67. De Caro A, Bonicel J, Pieroni G, et al. Comparative studies of human and porcine pancreatic lipases: N-terminal sequences, sulfhydryl groups and interfacial activity. *Biochimie* 1981;63:799-801.

68. Bousset-Risso M, Bonicel J, Rovery M. Limited proteolysis of porcine pancreatic lipase. Lability of the Phe 335-Ala 336 bond towards chymotrypsin. *FEBS Lett* 1985;182:323-326.

69. Bianchetta JD, Bidaud J, Guidoni AA, et al. Porcine pancreatic lipase. Sequence of the first 234 amino acids of the peptide chain. *Eur J Biochem* 1979;97:395-405.

70. Kerfelec B, Foglizzo E, Bonicel J, et al. Sequence of horse pancreatic lipase as determined by protein and cDNA sequencing-Implications for *p*-nitrophenyl acetate hydrolysis by pancreatic lipases. *Eur J Biochem* 1992;206:279-287.

71. De Caro J, Carrière F, Barboni P, et al. Pancreatic lipase-related protein 1 (PLRP1) is present in the pancreatic juice of several species. *Biochim Biophys Acta* 1998;1387:331-341.

72. Yalow RS, Berson SA. Immunoassay of endogenous plasma insulin in man. *Obes Res* 1960;4:583-600.

73. Berson SA, Yalow RS. Peptide hormones in plasma. *Harvey Lecture* 1966;62:107-163.

74. Hunter WM, Greenwood FC. Preparation of ^{131}iodine-labeled human growth hormone of high specific activity. *Nature* 1962;194:495-496.

75. Berson SA, Yalow RS. Radioimmunoassay in gastroenterology. *Gastroenterology* 1972;62:1061-1084.

76. Steiner JM, Medinger TL, Williams DA. Development and validation of a radioimmunoassay for feline trypsin-like immunoreactivity. *Am J Vet Res* 1996;57:1417-1420.

77. Allen WE, Porter DJ. Comparison of radioimmunoassay and enzyme-linked immunoassay for the measurement of progestogen in equine plasma and milk. *Vet Rec* 1987;120:429-431.

78. Williams DA, Scott-Moncrieff JC, Bruner J, et al. Validation of an immunoassay for canine thyroid-stimulating hormone and changes in serum concentration following induction of hypothyroidism in dogs. *J Am Vet Med Assoc* 1996;209:1730-1732.

79. Williams DA, Batt RM. Sensitivity and specificity of radioimmunoassay of serum trypsin-like immunoreactivity for the diagnosis of canine exocrine pancreatic insufficiency. *J Am Vet Med Assoc* 1988;192:195-201.

80. Yalow RS, Berson SA. Radioimmunoassay of gastrin. *Gastroenterology* 1970;58:1-14.

81. Camarillo IG, Thordarson G, Ilkbahar YN, et al. Development of a homologous radioimmunoassay for mouse growth hormone receptor. *Endocrinology* 1998;139:3585-3589.

82. John H, Cammann K, Schlegel W. Development and review of radioimmunoassay of 12-S-hydroxyheptadecatrienoic acid. *Prostaglandins Other Lipid Mediat* 1998;56:53-76.

83. Williams DA. Diagnosis and management of pancreatitis. *J Small Anim Pract* 1994;35:445-454.

84. Levitt MD, Eckfeldt JH. Diagnosis of acute pancreatitis. In Go VLW, DiMagno EP, Gardner JD, et al, eds. *The pancreas: biology, pathophysiology and disease.* 2nd ed. New York:Raven Press, 1993;613-635.

85. Akol KG, Washabau RJ, Saunders HM, et al. Acute pancreatitis in cats with hepatic lipidosis. *J Vet Intern Med* 1993;7:205-209.

86. Dill-Macky E. Pancreatic diseases of cats. *Compend Contin Educ Pract Vet* 1993;15:589-596.

87. Steiner JM, Williams DA, Moeller EM, et al. Development and validation of an enzyme-linked immuno sorbent assay (ELISA) for feline trypsin-like immunoreactivity (fTLI). *Am J Vet Res* 2000;61:620-623.

88. Steiner JM, Williams DA. Serum feline trypsin-like immunoreactivity in cats with exocrine pancreatic insufficiency. *J Vet Intern Med* 2000;14:627-629.

89. Gerhardt A, Steiner JM, Williams DA, et al. Comparison of the sensitivity of different diagnostic tests for pancreatitis in cats. *J Vet Intern Med* 2001;15:329-333.

VITA

Benjamin Gregg Wilson

Academic Record

2001	Texas A&M University	B.S. in biomedical science
2003	Texas A&M University	M.S. in veterinary medical sciences

Personal Data

Address: 415 Thigpen Dr. #1004
Tyler, TX 75703
phone: 979-229-4251

Birth Date: 01-17-1979

Employment

2000-2003	Texas A&M University	Research Assistant Gastrointestinal Laboratory Dept. Small Animal Medicine and Surgery

Publications

Steiner JM, Wilson BG, Williams DA. Purification and partial characterization of feline classical pancreatic lipase. *Comparative Biochemistry and Physiology Part B* 2003;134:151-159.