Purification and partial characterization of feline pepsinogen

Ursula Tress, med vet; Jörg M. Steiner, Dr med vet, PhD; Craig G. Ruaux, BVSc, PhD; Jan S. Suchodolski, Dr med vet; David A. Williams, VetMB, PhD

Objective—To purify and partially characterize feline pepsinogen (fPG) from the gastric mucosa and compare fPG with PGs of other species.

Sample Population—Stomachs of 6 cats.

Procedure—A crude protein extract was prepared from the gastric mucosa of feline stomachs. Feline PG A was purified by ammonium sulfate precipitation, weak-anion-exchange chromatography, size-exclusion chromatography, and strong-anion exchange chromatography. Partial characterization consisted of estimation of molecular weights (MWs) and isoelectric points, N-terminal amino acid sequencing, and investigation of susceptibility to pepstatin inhibition.

Results—Several fPG A-group isoforms were identified. The MWs of the isoforms ranged from 37,000 to 44,820. Isoelectric points were all < pH 3.0. The proteolytic activity of the activated PGs was inhibited completely by pepstatin in a range of equimolar to 10-fold molar excess. The specific absorbance of fPG A was 1.29. The N-terminal amino acid sequence of the first 25 residues of the predominant fPG A7 had 75%, 72%, 64%, and 56% homology with PG A of dogs, rabbits, cattle, and humans, respectively. Sequences of 4 other fPG A-group isoforms were similar to fPG A7. All isoforms were immunologically cross-reactive with sheep anti-fPG A7 antiserum.

Conclusions and Clinical Relevance—PG A is the only identified type of PG in cats and, similar to pg in other species, comprises multiple isoforms. The availability of fPG A may be used to facilitate the development of an immunoassay to quantify serum fPG A as a potential marker for gastric disorders in cats. (*Am J Vet Res* 2004;65:1195–1199)

Pepsinogen (PG) is the zymogen of pepsin, the major proteolytic enzyme in the gastric juice of vertebrates. Pepsinogen is heterogeneous. Multiple isoforms of PG and its active form, pepsin, have been purified from humans and other species.[1-3] On the basis of their biochemical and immunologic properties, isoforms of PG are categorized into different groups. Isoforms within the same PG group have immunologic cross-reactivity, but no cross-reactivity exists between isoforms of different PG groups.[4-6] The number of isolated PG isoforms and their concentration, localization, and enzymatic properties differ between species.[7,8] Pepsinogens A and C are the main PG groups in humans, cows, pigs, and goats.[2,3,9] In rodents, PG C is the only type of PG purified so far.[10] By contrast, in domestic dogs, Asiatic black bears, and rabbits, PG A appears to be the only major type of PG.[11-13] Each PG group often consists of multiple isoforms, especially the PG A group.[8] For example, the PG A group of rabbits, humans, Japanese monkeys, pigs, and goats comprises 6, 5, 4, 3, and 3 isoforms, respectively.[2,3,11,14,15] Two isoforms within the PG C group have been identified for humans, cows, pigs, and goats.[3,7,16] In humans, PGs A and C are produced in the chief cells and mucous neck cells of the gastric body and fundus. Pepsinogen C is further synthesized in the gastric cardia, the pyloric region, and Brunner glands of the proximal portion of the duodenum.[7,17] One study[18] reported the existence of PG A and PG C in the stomach and proximal portion of the duodenum of cats and dogs. This finding has recently been challenged, as no PG C and no cDNA encoding PG C could be identified in dogs.[13,19] Pepsinogen C has not been isolated from the gastric mucosa of any carnivore to date, suggesting that PG A is the only major PG of carnivores.[12,13,19]

Pepsinogens A and C are secretory proteins that enter the circulation. In human medicine, serum concentrations of PGs A and C are used as markers for specific gastric disorders, such as gastritis, gastric and duodenal ulceration, gastric cancer, and *Helicobacter pylori* infection.[20-22] In veterinary medicine, serum PG A activity has been evaluated as a marker for gastric and duodenal ulceration in foals and ostertagiasis in cattle and sheep.[23,24]

The objective of the study reported here was to purify and partially characterize **feline PG (fPG)** from the gastric mucosa. It was of particular interest to find out whether cats synthesize PGs A and C or, like dogs, only produce PG A. The results obtained in this study will enable the future development of an immunoassay for the measurement of fPG A in serum as a potential marker for certain gastric disorders in cats.

Materials and Methods

Tissue specimen collection—Stomachs and approximately 2 cm of the proximal portion of the duodenum were collected from cats euthanized for unrelated research projects at Texas A&M University. The stomach and attached part of the duodenum were opened along the greater curvature, and all ingesta were removed. The mucosa was rinsed thoroughly with 0.1M sodium phosphate buffer (pH, 7.4; buffer A) to remove remaining food particles and mucus. The cleaned stomachs were frozen and stored at –20°C until further use.

Proteolytic activity assay—During the purification process, PGs containing fractions were identified by mea-

Received February 4, 2004.
Accepted March 2, 2004.
From the Gastrointestinal Laboratory, Department of Small Animal Medicine and Surgery, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843-4474.
Presented in part at the 2002 Annual Forum of the American College of Veterinary Internal Medicine, Dallas, Tex, **Month** 2002.
Address correspondence to Dr. Steiner.

surement of proteolytic activity by use of a modification of the method originally described by Anson and Mirsky.[25] Briefly, 125 µL of each protein fraction was mixed with 125 µL of a 0.1M sodium citrate solution (pH, 2.0; to activate the isoforms) and 1.25 mL of acid-denatured 20% bovine hemoglobin solution (pH, 2.0) as a substrate. A blank sample was prepared by use of 125 µL of buffer A instead of protein solution. Mixtures were incubated at 37°C for 10 minutes, then the reaction was stopped and any undigested hemoglobin was precipitated by the addition of 2.5 mL of a 5% trichloroacetic acid solution. Samples were centrifuged[a] at 3,300 × *g* at 4°C for 15 minutes. The absorbances of the supernatants were measured at 280 nm after zeroing the absorbance of the blank sample. One enzymatic activity unit was defined as the amount of enzyme that catalyzed an increase in absorbance of 0.001, compared with the blank.

Purification of fPG from gastric mucosa—Pepsinogen was purified from a total of 6 stomachs. Purification of fPG from the first 2 stomachs was performed individually to assess the repeatability of the purification method used. For subsequent purification of fPG, 2 stomachs were processed simultaneously to increase the purification yield. Stomachs were thawed at room temperature (approx 25°C). The mucosa (approx 5 to 8 g/stomach) was scraped off the muscle layer with a razor blade and transferred into buffer A (2 mL buffer/g mucosa). The mixture was homogenized with a tissue grinder,[b] the homogenate was centrifuged at 15,000 × *g* for 60 minutes at 4°C, and the precipitate was discarded. Ammonium sulfate was added slowly to the supernatant until 35% saturation (0.8M) was obtained. During the procedure, the solution was kept on ice and the pH was maintained at 7.4 by drop wise addition of 1M NaOH. After complete dissolution of the salt crystals, the mixture was centrifuged at 15,000 × *g* for 60 minutes at 4°C and the precipitate was discarded. Again, ammonium sulfate was slowly added to the supernatant until 75% saturation (2M) was reached. The solution was centrifuged as before. The supernatant was decanted, and then the precipitate was dissolved in a small volume (approx 10 mL) of buffer A and dialyzed[c] overnight against this buffer. Subsequently, the dialyzed protein solution was filtered through a series of filters with decreasing pore size (from 10 to 0.45 µm) by use of a vacuum pump. Column chromatography was performed by use of a fast-performance liquid chromatography system.[d] A weak anion-exchange chromatography column[d] was equilibrated with buffer A. The protein extract was loaded onto the column in buffer A at a flow rate of 1 mL/min. Proteins that were not bound were washed off the column with buffer A until a stable baseline was reached. Bound proteins were eluted by applying a linear gradient of sodium chloride[e] in buffer A from 0 to 0.8M for over 80 minutes at a flow rate of 1 mL/min. Fractions with proteolytic activity against bovine hemoglobin[f] were pooled, dialyzed against buffer A, and concentrated to a volume of approximately 7 mL by use of a protein concentrator[g] with a pore size of 10,000 **molecular weight (MW)**. The concentrated protein solution was loaded onto a size-exclusion chromatography column[h] with buffer A as the mobile phase at a flow rate of 0.8 mL/min. Fractions with proteolytic activity against bovine hemoglobin were pooled. The partially purified protein solution was loaded onto a strong-anion exchange column.[i] Proteins that were not bound were washed off the column with buffer A until a stable baseline was reached. Bound proteins were eluted by use of a linear gradient of sodium chloride[e] in buffer A from 0 to 0.8M NaCl for over 90 minutes.

Partial characterization of fPG—The specific absorbance of fPG was determined by measuring the concentration of an aliquot of purified fPG with a bicinchoninic acid assay[j] according to the manufacturer's instructions. Bovine serum albumin was used to generate a standard curve. Pure fPGs were concentrated to approximately 1 mg/mL by use of a protein concentrator with a pore size of 10,000 MW and stored at –20°C until further use. Aliquots of purified fPG were subjected to SDS-PAGE under reducing conditions. Vertical slab precast 10% Bis-Tris gels[k] were used according to the manufacturer's instructions. Gels were stained by use of a commercial silver stain kit.[l] Migration distances of the purified products and the proteins of a commercially available MW standard mix[m] were measured, and a standard curve was generated. The MWs of fPGs were estimated by interpolation from the standard curve. Isoelectric points were estimated by electrophoresis on a polyacrylamide isoelectric focusing gel[n] with a pH gradient from 3.0 to 7.0. A commercially available isoelectric focusing calibration kit[n] was used to generate a standard curve. All gels were stained by use of a silver stain kit. Five purified protein fractions were sent to the Jagiellonian University, Institute of Molecular Biology, BioCenter in Krakow, Poland, for determination of the first 25 N-terminal amino acid residues by use of the Edman degradation method. A modification of the assay for proteolytic activity as already described was used to investigate the susceptibility of the activated fPGs to pepstatin[o] inhibition. Each sample to be tested was adjusted to a concentration of 0.1 mg/mL, which is equivalent to 0.33 nmol/125 µL. A volume of 125 µL of sample was mixed with 125 µL of pepstatin solution of varying concentrations, ranging up to 10-fold molar excess. The subsequent steps were the same as in the regular assay for proteolytic activity, except for a prolonged incubation time of 30 minutes. A baseline value was measured from a test tube containing 125 µL of buffer A instead of pepstatin. Following the incubation, undigested hemoglobin was precipitated and the absorbance of the supernatant was measured as previously described.

Production of anti-fPG A antiserum—Antiserum directed against fPG A was raised in sheep. The sheep was inoculated with 100 µg of fPG A from the seventh peak identified as fPG A (ie, fPG A7) on strong anion-exchange chromatography. The protein was mixed with 1 mg of saponin adjuvant[p] in 500 µL of PBS[q] solution (pH, 7.2). The solution was injected SC into the neck area. Booster injections were given 4 times by use of 50 µg of fPG A7 mixed with 1 mg of the saponin adjuvant in 500 µL of PBS solution every 2 weeks. Blood samples were collected at 2, 4, and 6 weeks after the first booster. The antibody titer was determined by use of an in-house radioimmunoassay with radioiodinated fPG A7 as a tracer. To evaluate immunologic reactivity, all fPGs that eluted separately from the strong anion-exchange chromatography column and the sheep anti-fPG A7 antiserum were loaded onto agarose immunodiffusion plates[r] according to the manufacturer's instructions. Positive immunoreactivity was defined as a clearly visible precipitation line formed between the anti-fPG A7 antiserum and the purified fPG fraction.

Results

Feline pepsinogen A was successfully purified from 6 stomachs by use of ammonium sulfate precipitation, anion-exchange chromatography, and size-exclusion chromatography (**Table 1**). Weak anion-exchange chromatography separated the gastric mucosal extract into 2 peaks with proteolytic activity against acid-denatured bovine hemoglobin (**Figure 1**). Size-exclusion chromatography yielded 1 peak with proteolytic activity (**Figure 2**). The final strong anion-

Table 1—Results of purification of feline pepsinogen A (fPG A) from gastric mucosa (6.8 g) of the stomach and proximal portion of the duodenum of a cat.

Purification step	Measurements			
	Protein (mg)	Total activity (AU)	Specific activity (AU/mg protein)	Recovery (%)
Mucosal extract	618.8	106.8	0.2	100.0
Weak anion exchange	81.6	77.4	0.9	72.4
Gel filtration	19.0	24.6	1.3	23.0
Strong anion exchange	3.6	10.1	2.8	9.4

AU = Activity units, defined as the amount of enzyme that catalyzes an increase in absorbance of 0.001 in the hemoglobin assay.

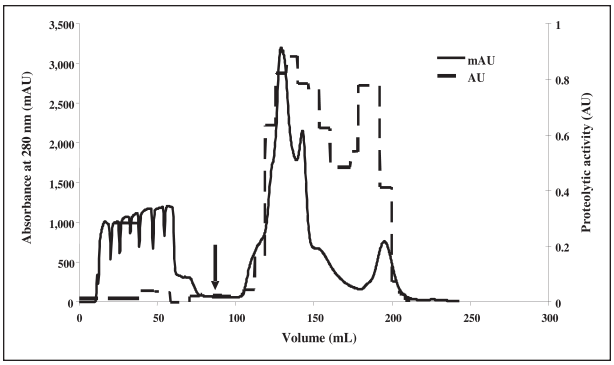

Figure 1—Results of weak anion-exchange chromatography. Crude protein extract of feline gastric mucosa was loaded onto a diethylaminoethyl sepharose column for weak-anion exchange chromatography. Bound proteins were eluted by application of a linear salt gradient (starting at the arrow) of 0 to 0.8M sodium chloride in 0.1M sodium phosphate buffer (pH, 7.4; buffer A) for over 80 minutes at a flow rate of 1 mL/min. mAU = Absorbance units at 280 nm. AU = Proteolytic activity in activity units.

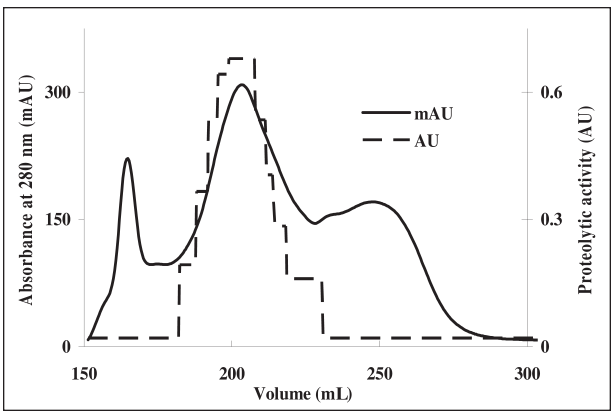

Figure 2—Results of size-exclusion chromatography. Protein fractions with proteolytic activity pooled after weak anion-exchange chromatography were loaded onto a column for size-exclusion chromatography. A 0.1M sodium phosphate buffer (pH, 7.4; buffer A) was used as mobile phase at a flow rate of 1 mL/min. *See* Figure 1 for key.

exchange chromatography separated the protein solution into multiple peaks with proteolytic activity (**Figure 3**). The SDS-PAGE analysis revealed single bands in 8 fractions with proteolytic activity (**Figure 4**). Four fractions with proteolytic activity had bands in the same area and additional contaminant bands. The MWs of fPG A1 and A6 through A12 were

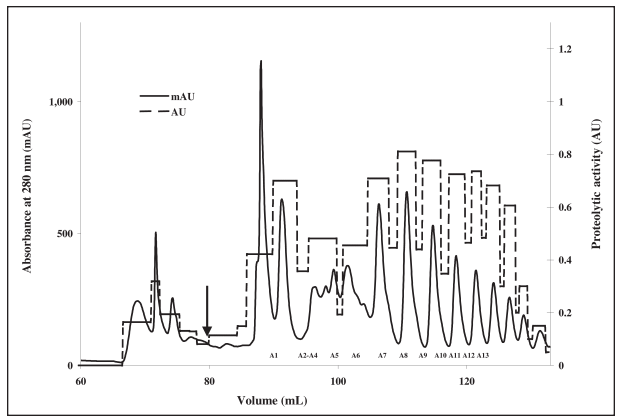

Figure 3—Results of strong anion-exchange chromatography. The partially purified protein solution was loaded on a column for strong anion-exchange chromatography. A linear gradient (starting at the arrow) of 0 to 0.8M sodium chloride in 0.1M sodium phosphate buffer (pH, 7.4; buffer A) over 90 minutes separated partially purified feline pepsinogen A (fPG A) into multiple peaks. Isoforms that were characterized by N-terminal amino acid sequencing are labeled as A6 through A10. All pure fPG fractions, including those that were not characterized by N-terminal amino acid sequencing, were immunologically cross-reactive with fPG A7. *See* Figure 1 for key.

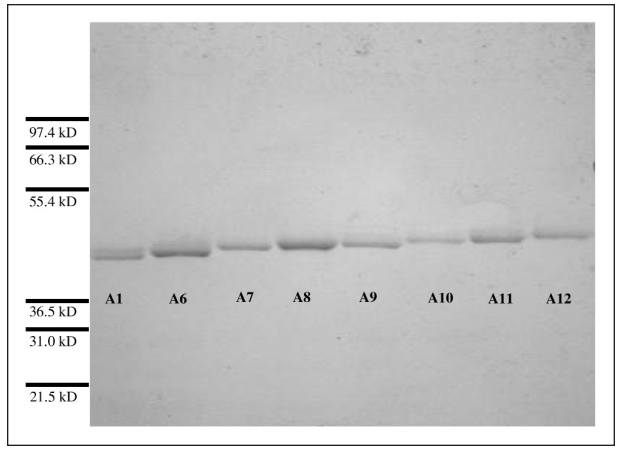

Figure 4—Results of SDS-PAGE analysis of 8 purified fPG A-group isoforms. The gel was silver stained. Bands are labeled according to the order of elution on strong anion-exchange column. Feline pepsinogens A6 to A10 were sequenced and identified as fPG A-group isoforms. All other purified proteins with proteolytic activity were immunologically cross-reactive with fPG A7.

37,000, 39,500, 40,900, 41,700, 42,400, 44,000, 44,800, and 44,800, respectively. The isoelectric points of these isoforms were all < 3.0; exact values could not be determined. The specific absorbance of 1 mg of fPG A/mL of solution was 1.29. The first 25 N-terminal amino acid residues of 5 pure isoforms (ie, fPG A6 to A10) were determined (**Table 2**). Comparison of the sequence of the predominant isoform fPG A7 to sequences of PG A from dogs, rabbits, cattle, and humans revealed homologies of 75%, 72%, 64%, and 56%, respectively. Immunodiffusion studies resulted in clearly visible precipitation lines between all 13 fPG A-group isoforms and the sheep anti-fPG A7 antiserum. The activity of the activated isoforms was inhibited completely by pepstatin in a range from equimolar to 10-fold molar excess (**Figure 5**).

Table 2—Comparison of N-terminal amino acid sequence of pepsinogen (PG) A among species.

	First 25 amino-acid residues of PG																								
PGs	**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**
fPG A6	Thr	Ile	Ile	Lys	Val	Pro	Leu	Ile	Lys	Lys	Lys	Thr	Leu	**Arg**	Glu	Asn	Leu	Ile	Glu	His	Gly	Leu	Leu	Asp	Asp
fPG A7	Thr	Ile	Ile	Lys	Val	Pro	Leu	Ile	Lys	Lys	Lys	Thr	**Thr**	Leu	Glu	Asn	Leu	Ile	Glu	His	Gly	Leu	**Lys**	**Leu**	Asp
fPG A8	Thr	Ile	Ile	Lys	Val	Pro	Leu	**Leu**	Lys	Lys	Lys	Thr	**Lys**	**Lys**	Glu	Asn	Leu	Ile	Glu	His	Gly	**His**	X	Asp	Asp
fPG A9	Thr	Ile	Ile	Lys	Val	Pro	Leu	Ile	**Ile**	Lys	Lys	Thr	Leu	Leu	Glu	Gly	Leu	**Leu**	**Ile**	His	**His**	Leu	Leu	Asp	Asp
fPG A10	Thr	Ile	Ile	Lys	Val	Pro	Leu	Ile	Lys	Lys	Lys	Thr	Leu	Leu	Glu	Asn	Leu	Ile	Glu	X	Gly	X	Leu	X	Asp
hPG A	Ile	**Met**	**Tyr**	Lys	Val	Pro	Leu	Ile	**Arg**	Lys	Lys	**Ser**	**Phe**	Arg	**Arg**	**Thr**	Leu	**Ser**	Glu	**Arg**	Gly	Leu	Leu	**Lys**	Asp
bPG A	NA	**Val**	**Val**	Lys	Val	Pro	Leu	**Val**	Lys	Lys	Lys	**Ser**	Leu	Arg	**Gln**	Asn	Leu	Ile	Glu	**Asn**	Gly	Lys	Leu	**Lys**	**Glu**
rPG A	NA	Ile	**His**	Lys	Val	Pro	Leu	**Val**	**Arg**	Lys	Lys	**Ser**	Leu	Arg	**Lys**	Asn	Leu	Ile	Glu	**Lys**	Gly	Leu	Leu	**Gln**	Asp
cPG A	Ala	Ile	**Val**	Lys	Ile	Pro	Leu	**Val**	**Arg**	Lys	Lys	**Ser**	Leu	Arg	**Gln**	Asn	Leu	Ile	Glu	His	Gly	Leu	Leu	**Asn**	Asp

Residues that are in bold indicate differences among fPG A-group isoforms and in comparison to PG A of humans, cattle, rabbits, and dogs.
hPG A= PG A of humans. bPG A = PG A of cattle. rPG A = PG A of rabbits. cPG A = PG A of dogs. X = Amino acid could not be determined. NA = Not available.

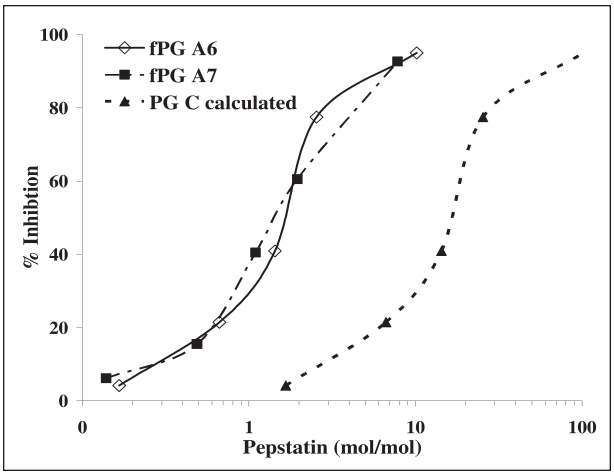

Figure 5—Inhibitory effect of pepstatin on proteolytic activity of activated fPG A-group isoforms. Notice the calculated curve (triangles) that would have been expected for pepsinogen (PG) C, which, in other species, has been shown to need a 100-fold molar excess of pepstatin to be inhibited completely.

Discussion

In our study, several fPG A-group isoforms were successfully purified from the gastric mucosa of cats. The SDS-PAGE analysis of the purified PGs revealed single bands in 8 fractions, indicating purity. Four protein fractions with proteolytic activity had bands in the expected range for PG and additional bands of unidentified proteins.

Similar to PG A of other species, fPG A is heterogeneous. The final separation by strong anion-exchange chromatography resulted in 13 identifiable fPG A-group isoforms. The first 25 N-terminal amino acid residues of 5 of the 13 isoforms were determined. On the basis of the homology that was found with that of sequences of PG A from other species, it was concluded that the sequenced proteins represented different fPG A-group isoforms.

Results of immunodiffusion revealed that all 13 isolated isoforms had immunologic cross-reactivity with antiserum raised against fPG A7. The PGs were therefore designated as belonging to the fPG A group and received Arabic numbers according to their elution profile. The MWs of the fPG A-group isoforms ranged from 37,000 to 44,820. The MWs for fPG A, as determined in our study, are within the range of MWs determined for PG A of humans and other species, which range from 32,000 to 42,100.[2,3,13,26]

The isoelectric points of the purified fPG A-group isoforms were all < pH 3.0. This is slightly lower than the isoelectric points of PG A reported for other species, including 4.6, 3.7, and 4.0 to < 3.0 for bovine, human, and canine PG A, respectively.[13,27-29] This might be the result of a higher content of acidic amino acid residues in the fPG A molecule, compared with PG A in other species. This hypothesis would be confirmed only by sequencing the entire fPG A molecule. The overall yield of fPG A by use of the purification protocol described in our study was 9.4%, which is comparable to yields reported for the purification of PG A in other species.[3,13]

In a previous study,[30] mucosal extracts from cat stomachs were analyzed by use of electrophoretic techniques. The authors reported the existence of at least 8 isoforms of pepsin in feline gastric mucosa without specifying an exact number.[30] In humans, PGs belonging to group C are synthesized in the pyloric part of the stomach and proximal portion of the duodenum. Therefore, in our study, the mucosa of the entire stomach and the proximal portion of the duodenum were used for purification. However, on the basis of immunologic reactivity observed among all isoforms isolated in our study, no evidence was found for the existence of fPG C. We investigated the susceptibility of isolated fPG A-group isoforms to pepstatin inhibition after activation. Pepstatin, isolated from *Streptomyces* spp, is a potent nonselective inhibitor of aspartic proteinases such as pepsin and has been used to distinguish between PGs A and C in other species.[26] Although the proteolytic activity of activated isoforms within the PG A group is inhibited completely with approximately equimolar amounts of pepstatin, a 100-fold molar excess is necessary to fully inhibit the proteolytic activity of isoforms of the pepsin C group.[3,26] The proteolytic activity of all purified isoforms in our study was suppressed with pepstatin in a range from equimolar to 10-fold molar excess, further suggesting that PG C was not purified in our investigation. Our results are contrary to the findings of Liebman and Samloff[18] who suggested the existence of PG C in cats and dogs on the basis of findings by use of immunohistochemistry. However, these authors did not purify fPGs in their study but rather used antibodies raised against human PG A and C, which possibly could have cross-reacted with other proteins within the gastric mucosa of dogs and cats. In agreement with our

results, there has not been any report of the purification of PG C in carnivores to date.[12,13] Furthermore, our findings are similar to those of a recent study performed by Narita et al[19] who did not find cDNA encoding for PG C in dogs. These authors hypothesized that PG C might not be useful for the digestion of proteins from meat sources and that the gene for PG C might thus not be functional in carnivores.

We conclude on the basis of the findings presented here that PG A appears to be the major, if not the only, group of PGs in cats. The purification of fPG A will help facilitate the development of an immunoassay for the measurement of fPG A concentrations in serum and its investigation as a potential diagnostic aid for gastric disorders in cats.

[a]Centrifuge 5810 R, Eppendorf, Westbury, NY.
[b]Polytron PT MR 2100, Brinkmann Instruments Inc, Westbury, NY.
[c]SnakeSkin pleated dialysis tubing, Pierce Biotechnology Inc, Rockford, Ill.
[d]DEAE HiPrep 16/10, Amersham Biosciences Corp, Piscataway, NJ.
[e]NaCl, Sigma Chemical Co, St Louis, Mo.
[f]Centriprep centrifugal filter device, Millipore Corp, Bedford, Mass.
[g]Sephacryl S-100, Amersham Biosciences Corp, Piscataway, NY.
[h]ÄKTA Purifier 10, Amersham Biosciences Corp, Piscataway, NY
[i]Mono Q, Amersham Biosciences Corp, Piscataway, NY.
[j]BCA protein assay reagent kit, Pierce Biotechnologiy Inc, Rockford, Ill.
[k]Novex precast gels, Invitrogen, Carlsbad, Calif.
[l]Gelcode, SilverSNAP kit, Pierce Biotechnology Inc, Rockford, Ill.
[m]Mark 12 MW standard, Invitrogen, Carlsbad, Calif.
[n]Isoelectric focusing kit, Invitrogen, Carlsbad, Calif.
[o]Pepstatin, Sigma Chemical Co, St Louis, Mo.
[p]Quill-A, Accurate Chemicals, Westbury, NY.
[q]BupH, phosphate buffered saline packs, Pierce Biotechnology Inc, Rockford, Ill.
[r]Immunodiffusion plate agarose gelling agent, single pattern, Pierce Biotechnology Inc, Rockford, Ill.

References

1. Samloff IM. Slow moving protease and the seven pepsinogens. *Gastroenterology* 1969;57:659–669.

2. Foltmann B, Drohse HB, Nielsen PK, et al. Separation of porcine pepsinogen A and progastricsin. Sequencing of the first 73 amino acid residues in progastricsin. *Biochim Biophys Acta* 1992;1121:75–82.

3. Suzuki M, Narita Y, Oda S, et al. Purification and characterization of goat pepsinogens and pepsins. *Comp Biochem Physiol B Biochem Mol Biol* 1999;122:453–460.

4. Foltmann B, Pedersen VB. Comparison of the primary structures of acidic proteinases and of their isoforms. *Adv Exp Med Biol* 1976;95:3–22.

5. Foltmann B. Purification, structure and activation of pepsinogens. *Prog Clin Biol Res* 1985;173:1–13.

6. Samloff IM. Pepsinogens, pepsins, and pepsin inhibitors. *Gastroenterology* 1971;60:586–604.

7. Plebani M. Pepsinogens in health and disease. *Crit Rev Clin Lab Sci* 1993;30:273–328.

8. Kageyama T. Pepsinogens, progastricsins, and prochymosins: structure, function, evolution, and development. *Cell Mol Life Sci* 2002;59:288–306.

9. Eckersall PD, Macaskill J, McKellar QA, et al. Multiple forms of bovine pepsinogen: isolation and identification in serum from calves with ostertagiasis. *Res Vet Sci* 1987;43:279–283.

10. Furihata C, Saito D, Fujiki H, et al. Purification and characterization of pepsinogens and a unique pepsin from rat stomach. *Eur J Biochem* 1980;105:43–50.

11. Kageyama T, Takahashi K. Rabbit pepsinogens: purification, characterization analysis of the conversion process to pepsin and determination of the NH_2-terminal amino acid sequences. *Eur J Biochem* 1984;141:261–269.

12. Kageyama T, Moriyama A, Takahashi K. Purification and characterization of pepsinogens and pepsins from Asiatic Black Bear, and amino acid sequence determination of the NH_2-terminal 60 residues of the major pepsinogen. *J Biochem* 1983;94:1557–1567.

13. Suchodolski JS, Steiner JM, Ruaux CG, et al. Purification and partial characterization of canine pepsinogen A and B. *Am J Vet Res* 2002;63:1585–1590.

14. Kageyama T, Takahashi K. Pepsinogens and pepsins from gastric mucosa of Japanese Monkey. Purification and characterization. *J Biochem* 1976;79:455–468.

15. Athauda SB, Tanji M, Kageyama T, et al. A comparative study on the NH2-terminal amino acid sequences and some other properties of six isozymic forms of human pepsinogens and pepsins. *J Biochem* 1989;106:920–927.

16. Martin P, Corre C. Fractionation of the multiple forms of bovine gastric aspartic proteases by chromatofocusing. *Anal Biochem* 1984;143:256–264.

17. Samloff IM, Taggart RT. Pepsinogen, pepsin, and peptic ulcer. *Clin Invest Med* 1987;10:215–221.

18. Liebman WM, Samloff IM. Immunochemical characterization and cellular localization of pepsinogens in cat and dog. *J Histochem Cytochem* 1978;26:1115–1120.

19. Narita Y, Oda S, Takenaka O, et al. Phylogenetic position of eulipotyphla inferred from the cDNA sequences of pepsinogens A and C. *Mol Phylogenet Evol* 2001;21:32–42.

20. Samloff IM, Stemmermann GN, Heilbrun LK. Elevated serum pepsinogens I and II levels differ as risk factors for duodenal ulcer and gastric ulcer. *Gastroenterology* 1986;90:570–676.

21. Borch K, Axelsson CK, Halgreen H, et al. The ratio of pepsinogen A to pepsinogen C: a sensitive test for atrophic gastritis. *Scand J Gastroenterol* 1989;24:870–876.

22. Wagner S, Haruma K, Gladziwa U, et al. Helicobacter pylori infection and serum pepsinogen A, pepsinogen C, and gastrin in gastritis and peptic ulcer: significance of inflammation and effect of bacterial eradication. *Am J Gastroenterol* 1994;89:1211–1218.

23. Wilson JH, Pearson MM. Serum pepsinogen levels in foals with gastric or duodenal ulcers. *Equine Pract* 1986;51:149–155.

24. Hilderson H, Dorny P, Berghen PL. Gastrin and pepsinogen changes during an *Ostertagia ostertagi* challenge infection in calves. *Zentralbl Veterinarmed [B]* 1991;38:25–32.

25. Anson ML, Mirsky AE. The estimation of pepsin with hemoglobin. *J Gen Physiol* 1932;16:59–63.

26. Narita Y, Oda S, Moriyama A, et al. Pepsinogens and pepsins from house musk shrew, *Suncus murinus*: purification, characterization, determination of the amino-acid sequences of the activation segments, and analysis of proteolytic specificities. *J Biochem* 1997;121:1010–1017.

27. Gomes MA, Lima Wdos S, Pesquero JL. A new method for bovine pepsinogen purification. Preparation of a specific antibody. *J Immunoassay* 1994;15:157–170.

28. Gritti I, Banfi G, Roi GS. Pepsinogens: physiology, pharmacology pathophysiology and exercise. *Pharmacol Res* 2000;41:265–281.

29. Gomes MA. A new method for bovine pepsinogen purification. Preparation of a specific antibody. *J Immunoassay* 1994;15:162–170.

30. Shaw B, Wright CL. The pepsinogens of cat gastric mucosa and the pepsins deived from them. *Digestion* 1976;14:142–152.