Purification and partial characterization of canine pepsinogen A and B

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

Objective—To purify and partially characterize various isoforms of canine pepsinogen (PG) from gastric mucosa.

Sample Population—Stomachs obtained from 6 euthanatized dogs.

Procedure—Mucosa was scraped from canine stomachs, and a crude mucosal extract was prepared and further purified by use of weak anion-exchange chromatography, hydroxyapatite chromatography, size-exclusion chromatography, and strong anion-exchange chromatography. Pepsinogens were characterized by estimation of molecular weights, estimation of their isoelectric points (IEPs), and N-terminal amino acid sequencing.

Results—Two different groups of canine PG were identified after the final strong anion-exchange chromatography: PG A and PG B. Pepsinogens differed in their molecular weights and IEP. Pepsinogen B appeared to be a dimer with a molecular weight of approximately 34,100 and an IEP of 4.9. Pepsinogen A separated into several isoforms. Molecular weights for the various isoforms of PG A ranged from 34,200 to 42,100, and their IEPs ranged from 4.0 to < 3.0. The N-terminal amino acid sequence for the first 25 amino acid residues for PG A and B had good homology with the amino acid sequences for these proteins in other species.

Conclusions and Clinical Relevance—Canine PG B and several isoforms of canine PG A have been purified. Availability of these PGs will facilitate development of immunoassays to measure PG in canine serum as a potential diagnostic marker for gastric disorders in dogs. (*Am J Vet Res* 2002;63:1585–1590)

Pepsinogen (PG) is the precursor of pepsin, an aspartic proteinase that is synthesized mainly by gastric chief cells. Pepsinogen has been found to be heterogeneous in many of the species in which it has been isolated.[1-6] Two main groups of PG have been identified: PG A and PG C. Other members of the family of aspartic proteinases include the intracellular proteinase PG B (Cathepsin E) and prochymosin (neonatal PG).[7] The main groups, PG A and PG C, contain various isoforms. Human PG A includes 5 isoforms, whereas human PG C contains 2 isoforms.[8] Immunologic cross-reactivity is reported to exist between isoforms of the same group, but not between isoforms of different groups.[6] Human PG A and PG C are synthesized in the gastric fundus, whereas PG C is also synthesized in the pyloric region and the duodenum. This variation in cellular localization of PG A and PG C is important, as alterations in their serum concentrations are correlated with various gastric and duodenal disorders in humans (eg, gastric and duodenal ulceration, gastritis, and gastric adenocarcinoma).[7] Although some of these disorders have little or no clinical importance in canine patients, these findings suggest that immunoassays for the quantification of PG in canine serum may prove to be practical, economical, and minimally invasive for the diagnosis of gastric disorders in dogs. Immunoassays for quantification of PG in serum are some of the few serum markers currently available in human gastroenterology to assess function or pathologic changes of the stomach. A catalytic assay for peptic activity in serum has been used as a tool to diagnose ostertagiasis in cattle and gastric ulcers in foals.[9-11] Such indirect catalytic assays have limitations when used to quantify precursors, as they can only detect the active form of PG, pepsin, and thus require activation of the zymogen. In contrast, immunoassays directly measure the concentration of protein by detecting antigenic determinants of the protein that are shared with the zymogen and, therefore, easier quantification of the zymogen.

Canine PG has been purified previously.[12-15] However, protocols described in those publications yielded only a single isoform of PG. The aim of the study reported here was to purify and identify various groups and isoforms of canine PG as a prelude to the development of immunoassays for their measurement in serum as a potential marker for gastric disorders in dogs.

Materials and Methods

Assay for proteolytic activity—Fractions examined for PG content were treated to activate PG to pepsin and were assayed for proteolytic activity by use of a modified method originally described by Anson.[16] Briefly, a volume of 125 µL of each fraction that was obtained after each purification step was incubated with 125 µL of a sodium citrate buffer (pH, 2.0) and 1.25 mL of an acidified 20% bovine hemoglobin[a] solution (pH, 2.0) for 10 minutes at 37°C. After the incubation period, 2.5 mL of a 5% trichloroacetic acid[a] solution was added to precipitate undigested hemoglobin, and the samples were centrifuged for 15 minutes at 3,300 × *g* and 4°C. The absorbance of the supernatant was measured at 280 nm to determine the quantity of tyrosine released during proteolysis. One activity unit is defined as an increase in absorbance of 0.001 U/min.

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From the Gastrointestinal Laboratory, Department of Small Animal Medicine and Surgery, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843-4474. Dr. Boari's present address is Dipartimento di Science Veterinarie e Agroalimentari–Sezione di Medicina Interna, Via F. Crispi, 212 64100 Teramo, Italy.
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Address correspondence to Dr. Suchodolski.

Purification of canine PG—Stomachs were collected from healthy dogs euthanatized for unrelated projects, rinsed with distilled water, and stored at –20°C. One dog stomach per purification run was slowly thawed at room temperature (approx 20°C) for 1 hour, then transferred into a 0.1M sodium phosphate buffer (pH, 8.0) until completely thawed. The stomach was repeatedly dried with paper towels and washed with buffer to remove any mucus. The mucosa was scraped off with a razor blade (approx 45 g of mucosal tissue), transferred immediately into the same buffer (3 mL buffer/g of mucosal tissue), and homogenized with a tissue grinder.[b] The homogenate was centrifuged at 15,000 × *g* for 60 minutes at 4°C. Ammonium sulfate was slowly added to the supernatant until 35% saturation (0.8M) was reached. During the addition of ammonium sulfate, the pH was kept constant at 7.4 by addition of dilute NaOH. The solution was centrifuged at 15,000 × *g* for 50 minutes at 4°C. The supernatant was collected and ammonium sulfate added until a 75% saturation (2.0M) was reached. After being stirred for 30 minutes, the solution was once again centrifuged at 15,000 × *g* for 50 minutes at 4°C. The precipitate was dissolved in a small volume of 0.1M sodium phosphate, pH 7.4, (buffer A) and dialyzed for 12 hours against the same buffer for desalting. Buffer exchange was performed every 2 hours for 3 changes. The solution was filtered through a series of filters with a decreasing pore size from 10 to 0.45 µm and loaded onto a 1.6 × 10-cm **diethylaminoethyl (DEAE)** Sepharose column[c] that had been equilibrated with buffer A. Proteins were eluted by use of a linear gradient of 0.2 to 0.7M NaCl in buffer A for 120 minutes at a flow rate of 1 mL/min.

Fractions containing proteolytic activity against bovine hemoglobin were pooled and dialyzed against 5mM sodium phosphate, pH 7.0, (buffer B) for 12 hours. This solution was applied to a 0.7 × 5.2-cm hydroxyapatite column[d] that had been calibrated with buffer B. Fractions containing proteolytic activity did not bind to the column. To remove proteins bound to the column during consecutive chromatography runs, the hydroxyapatite column was cleaned with 0.5M sodium phosphate, pH 7.0 (buffer C). Fractions containing proteolytic activity were pooled and dialyzed against 0.1M sodium phosphate, 0.15M NaCl, pH 7.4, (buffer D) for 12 hours. The protein solution was concentrated[e] to a 10-mL volume, and size-exclusion chromatography was performed at a flow rate of 0.5 mL/min on a 2.6 × 60-cm column[f] that had been equilibrated with buffer D. Fractions containing proteolytic activity were pooled and dialyzed against buffer A for 12 hours. The solution was applied to a 1.0 × 10-cm strong anion-exchange column[g] and eluted with a linear gradient of 0.25 to 1.0M NaCl in buffer A for 120 minutes at a flow rate of 1 mL/min.

To denature dimers, fractions believed to contain PG B were dialyzed overnight against 4.0M urea and gel-filtered on a 1.6 × 60-cm size-exclusion column[h] at a flow rate of 0.5 mL/min. Fractions containing proteolytic activity were dialyzed against buffer A and applied to a strong anion-exchange column[g] as described.

Characterization of canine PG—Various isoforms of canine PG were analyzed by SDS-PAGE under reducing conditions on 1-mm-thick 10% Bis-Tris gels.[i] The molecular weights of PG were estimated by interpolation on a plotted standard curve that was generated by measuring the migration distance from the well to the protein bands of a commercially available molecular weight mixture.[j] The standard curve was calculated by use of a power curve-fit: $y = cx$.[b] Isoelectric points were estimated by polyacrylamide electrophoresis on a polyacrylamide gel, pH 3 to 7,[k] by use of a commercially available isoelectric focusing calibration kit[l] for the generation of a standard curve. All gels were stained with a silver stain kit.[m] The N-terminal amino acid sequences for the first 25 residues for 5 distinct isoforms of canine PG were obtained by use of the Edman degradation method and a gas phase amino acid sequencer.[n]

Sheep were vaccinated with 100 µg of canine PG A by use of 1 mg of saponin[o] in 1 mL of PBS solution[p] as an adjuvant. Injections were given every 3 weeks. Serum was collected after 4 vaccinations. Fractions with proteolytic activity against bovine hemoglobin during the purification procedure were applied to agarose immunodiffusion plates[q] and evaluated for reactivity against these sheep anti-PG antibodies.

Results

All 6 stomachs were purified individually but had essentially the same results (**Table 1**). Chromatography on a DEAE-Sepharose column separated the mucosal extract into 2 peaks with proteolytic activity against bovine hemoglobin (**Fig 1**). The peak that eluted first had a lower proteolytic activity than the second peak. Pepsinogen did not bind to the hydroxyapatite column and was found in the wash-out phase (**Fig 2**). On size-exclusion chromatography, 2 peaks with proteolytic activity against bovine hemoglobin were separated, indicating isoforms of PG with a significantly different molecular weight (**Fig 3**). Finally, strong anion-exchange chromatography separated the

Table 1—Purification of canine pepsinogen (PG) A from 1 g of gastric mucosa

	Measurements after completion of a step			
Steps	Protein content (mg)	Total activity (AU*)	Specific activity (AU/mg of protein)	Recovery (%)
Mucosal extract	21.9	2420.4	110.5	100.0
DEAE	6.5	1546.5	237.9	63.9
Hydroxyapatite	2.7	1170.9	433.7	48.4
Gel-filtration	1.6	863.8	539.9	35.7
Strong anion-exchange chromatography	0.4	279.8	699.5	11.6

*One activity unit (AU) is defined as the increase of 0.001 U/min in the absorbance of the hemoglobin activity assay.
DEAE = Diethylaminoethyl.

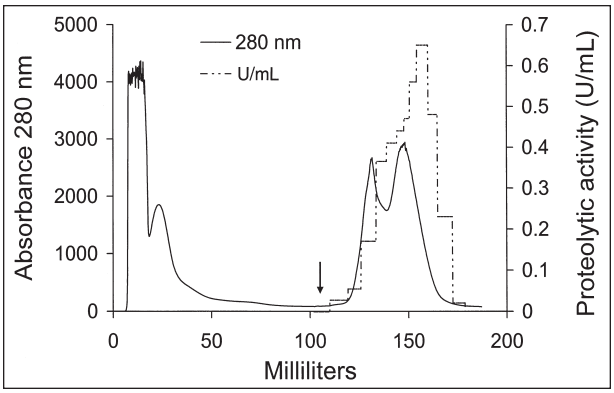

Figure 1—Chromatogram of canine mucosal extract obtained by use of weak anion-exchange chromatography on a diethylaminoethyl-Sepharose column. Protein solution after ammonium sulfate precipitation was applied to the column and washed with buffer A. A linear gradient of .2 to .7M NaCL in buffer A was applied over a period of 120 minutes (starting at the arrow). Fractions that had proteolytic activity against bovine hemoglobin were pooled and dialyzed against buffer B.

protein solution into several peaks that contained proteolytic activity against bovine hemoglobin (**Fig 4**). The first peak that eluted from the strong anion-exchange column had proteolytic activity against bovine hemoglobin and had a molecular weight, estimated by SDS-PAGE analysis, of 68,200. Incubation with 4.0M urea overnight followed by size-exclusion chromatography and strong anion-exchange chromatography yielded a single band with a molecular weight of 34,100 (**Fig 5**). Results of SDS-PAGE analysis revealed single bands for various peaks (**Fig 6**). The N-terminal amino acid sequence for the first 25 residues was determined for 5 peaks (**Table 2**). A comparison with the N-terminal amino acid sequence of these proteins from other species indicated that the first peak that eluted from a strong anion-exchange column[g] resembles porcine PG B. The other 4 peaks sequenced had a similar N-terminal amino acid sequence to PG A from other species.

Estimation of molecular weights by SDS-PAGE

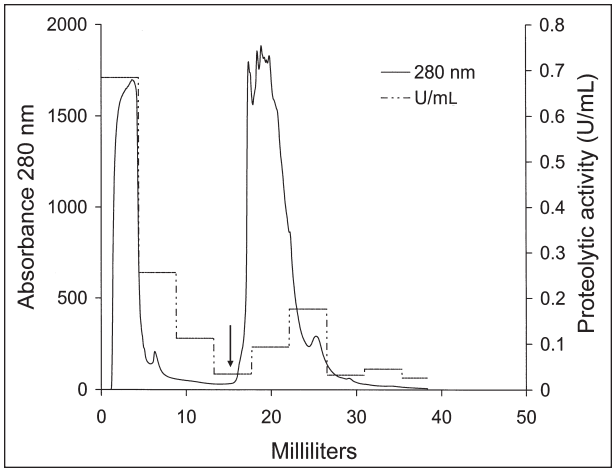

Figure 2—Chromatogram of canine mucosal extract obtained by use of weak anion-exchange chromatography on a hydroxyapatite column. Fractions collected between 2 and 5 mL had the highest proteolytic activity, indicating that canine pepsinogen (PG) does not bind to the hydroxyapatite column. These fractions were pooled and dialyzed against buffer D. Proteins that bound to the column were eluted (starting at the arrow) with 100% buffer C and discarded.

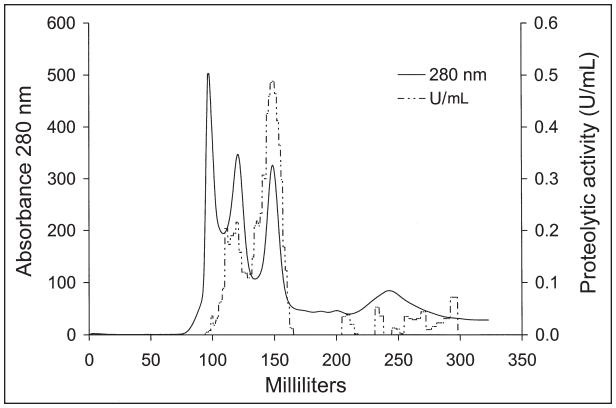

Figure 3—Chromatogram of canine mucosal extract obtained by use of size-exclusion chromatography on a Sephacryl S-100 HR column. Two peaks had proteolytic activity indicating 2 different molecular weights for canine PG.

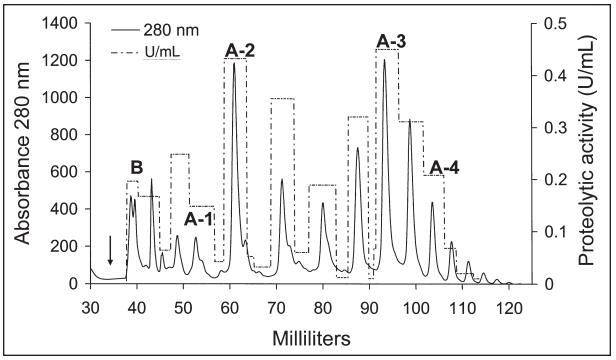

Figure 4—Chromatogram of canine mucosa extract obtained by use of strong anion-exchange chromatography. A linear gradient of .25 to 1.0M NaCl buffer A was applied over a period of 120 minutes (starting at the arrow). Several peaks with proteolytic activity were eluted. The N-terminal amino acid sequence of peaks labeled B, A-1, A-2, A-3, and A-4 was determined and had homology with PG B (peak B) and PG A (A-1 to A-4) from other species. Canine PG A was labeled in order of elution. Except for canine PG B, all peaks with proteolytic activity had immunologic cross-reactivity with canine PG A-3, indicating that they are all isoforms of PG group A.

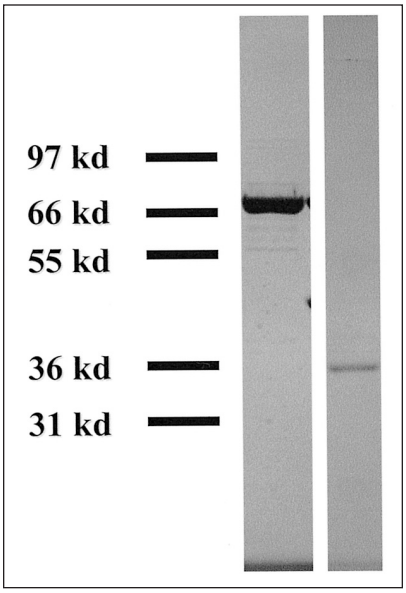

Figure 5—Results of SDS-PAGE analysis of canine PG B. Separation on a strong anion-exchange column[g] yields a protein band with a molecular weight of approximately 68,200. Denaturing with 4.0M urea yields a protein band of approximately 34,100, indicating that PG B is a dimer.

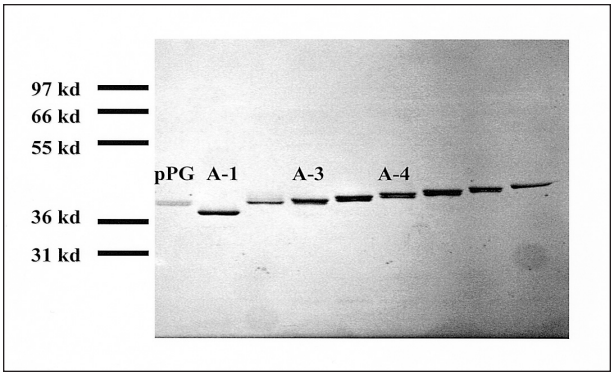

Figure 6—Results of SDS-PAGE analysis of various isoforms of canine PG A (A-1, A-3, A-4) and porcine PG A (pPG). Protein bands that are not labeled represent several canine isoforms of PG A that have not been sequenced but had immunologic cross-reactivity with canine PG A-3. The nonlabeled PG eluted between 87 and 115 mL on strong anion-exchange chromatography (*See* Figure 4).

Table 2—N-terminal amino acid sequence for the 25 first residues of canine PG A and B

Pepsinogens	First 25 residues of PG B																								
	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
Canine PG B	Val	Glu	Arg	Ile	Ile	Leu	Lys	Lys	Gly	Lys	Ser	Ile	Arg	Gln	Val	Met	Glu	Glu	Arg	Gly	Val	Leu	Glu	Thr	Phe
Porcine PGB	***Met***	Glu	Arg	Ile	Ile	Leu	***Arg***	Lys	Gly	Lys	Ser	Ile	Arg	***Glu***	***Ala***	Met	Glu	Glu	***Gln***	Gly	Val	Leu	Glu	***Lys***	Phe
	First 25 residues of PG A																								
Canine PG A-1	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
Canine PG A-2	Ala	Ile	Val	Lys	***Val***	Pro	Leu	Val	Arg	Lys	Lys	Ser	Leu	Arg	Gln	Asn	Leu	Ile	Glu	His	Gly	Leu	Leu	Asn	Asp
Canine PG A-3	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
Canine PG A-4	Ala	Ile	Val	Lys	Ile	Pro	Leu	Val	Arg	Lys	Lys	Ser	Leu	Arg	Gln	***Lys***	Leu	Ile	Glu	His	Gly	Leu	Leu	Asn	Asp
Canine PG A*	Ala	Ile	Val	Lys	Val	Pro	Leu	Val	Arg	Lys	Lys	Ser	Leu	Arg	Gln	Asn	Leu	Ile	Glu	His	Gly	Leu	Leu	Asn	Asp
Human PG 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
Bovine PG A	—	***Val***	Val	Lys	Val	Pro	Leu	Val	***Lys***	Lys	Lys	Ser	Leu	Arg	Gln	Asn	Leu	Ile	Glu	***Asn***	Gly	***Lys***	Leu	***Lys***	***Glu***
Rabbit PG A	—	Ile	***His***	Lys	Val	Pro	Leu	Val	Arg	Lys	Lys	Ser	Leu	Arg	***Lys***	Asn	Leu	Ile	Glu	***Lys***	Gly	Leu	Leu	***Gln***	Asp

Residues in bold indicate differences in the amino acid sequence of other species compared with that of canine PG B and canine PG A-1, respectively.
*N-terminal amino acid sequence of canine PG A inferred from coding DNA by Narita et al.[26]

Table 3—Molecular weights and isoelectric points of canine PG A and B

Pepsinogens	Measurements	
	MW	IEP
Canine PG B	34,100	4.9
Canine PG A-1	34,200	4
Canine PG A-2	34,200	3.9
Canine PG A-3	37,200	< 3.0
Canine PG A-4	38,500	< 3.0

MW = Molecular weight. IEP = Isoelectric point.

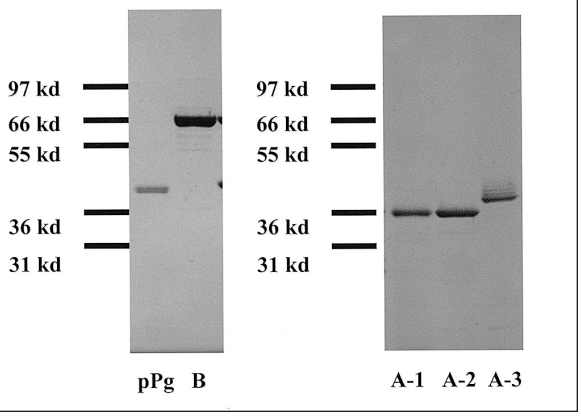

Figure 7—Comparison of molecular weights of porcine PG (pPG) and canine PG (PG B and A-1, A-2, A-3 isoforms of PG A) by use of SDS-PAGE analysis.

analysis revealed different molecular weights for the various isoforms of canine PG (**Table 3**). Pepsinogen B had a molecular weight of approximately 34,100. The molecular weights of the various isoforms of PG A ranged between 34,200 and 42,100 (**Fig 7**). Estimation of the **isoelectric points (IEPs)** indicates that PG B has an IEP of approximately 4.9, and the IEPs for PG A ranged from 4.0 to < 3.0 (**Fig 8**).

Results of immunodiffusion indicated no immunocross-reactivity between antiserum from sheep vaccinated with PG A-3 and PG B. All other isoforms of PG had immunocross-reactivity with PG A-3. In addition, all peaks with proteolytic activity against

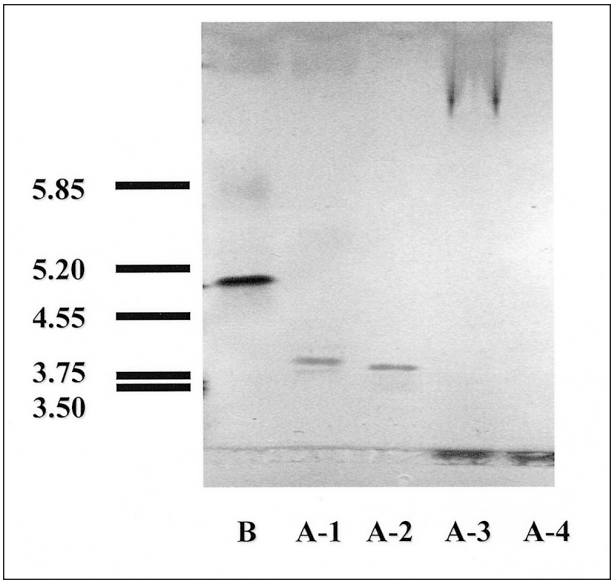

Figure 8—Isoelectric focusing of canine PG A (isoforms A-1, A-2, A-3, and A-4) and PG B by use of polyacrylamide electrophoresis.

bovine hemoglobin that had not been sequenced had immunocross-reactivity with PG A-3.

Discussion

The purification of canine PG by use of weak anion-exchange chromatography on a DEAE-cellulose Sepharose column and gel-filtration has been reported previously.[12-15] These authors independently reported the purification of a single isoform of canine PG. Also, these authors described similar molecular weights and amino acid composition for canine PG.[13,14] The first 3 residues of the N-terminal amino acid sequence were stated as Ala-Ile-Val.[13,15] It is difficult to determine whether the PG purified by those investigators represent the same or various isoforms of canine PG. One would expect canine PG to have similar heterogeneity as reported in other species. Results of electrophoretic studies of canine gastric mucosa reveal the presence of at least 8 zymogens.[17] In our effort to identify and characterize additional isoforms of canine PG, we used

strong anion-exchange chromatography. This technique allows a better resolution between proteins with marginal differences in their electrochemical behavior. The final purification step on a strong anion-exchange column[g] led to the isolation of several peaks with proteolytic activity against bovine hemoglobin. On average, up to 16 peaks could be identified. N-terminal amino acid sequencing and immunologic studies[1,2,4] identified those peaks as a single isoform of PG B and several isoforms of PG A.

The results of SDS-PAGE analysis indicated that the various isoforms of PG A had a molecular weight between 34,200 and 42,100, which is comparable to the results of previous studies[13,14] that reported molecular weights for canine PG of 41,667 and 45,000. Our results are also in accordance with reported molecular weights of PG in other species, such as humans, goats, Asiatic black bears, and bullfrogs, which range from 32,000 to 42,000.[18-21] The IEPs of the various isoforms of canine PG A purified here ranged from 4.0 to < 3.0. An IEP for canine PG has not previously been reported in literature. However, our results are similar to IEPs determined for PG in other species: 4.6 for bovine PG A and 3.7 in humans.[18,22]

One of the peaks purified after strong anion-exchange chromatography had an N-terminal amino acid sequence that resembled that of porcine PG B.[1] The molecular weight was 68,200. After incubation overnight with 4.0M urea and subsequent gel-filtration, it formed a single band with a molecular weight of 34,100. Thus, canine PG B appears to be purified as a dimer. It is not known whether dimer formation occurs in vivo or only in vitro. Human PG B has been shown to be a homodimer that consists of 2 monomers linked by an intermolecular disulphide bond.[23] Similar to PG B, Cathepsin D is an intracellular proteinase. Purification of Cathepsin D from human gastric mucosa also yielded a protein that consisted of 2 monomeric subunits.[24] Purification of canine PG B has not previously been reported. Canine PG B had a 76% sequence homology for the first 25 N-terminal residues with porcine PG B (Table 2).

The N-terminal amino acid sequence for the first 25 residues of 4 different peaks revealed similarity with human, bovine, and rabbit PG A (Table 2).[2,4,25] Two isoforms had an identical amino acid sequence for their first 25 residues, A-1 and A-3. Both eluted at different molar concentrations of NaCl from a strong anion-exchange column[g] and differed in their molecular weights and IEPs. Because of their electrochemical differences, it is likely that they would have variances in their amino acid sequence at a later stage than the 25 residues that were analyzed here and are, therefore, believed to be 2 different isoforms. Two isoforms of PG A (A-2 and A-4) differed in a single residue (96% homology) from canine PG isoforms A-1 and A-3. Canine PG A-1 and A-3 had 60, 75, and 84% homology with human, bovine, and rabbit PG A, respectively. The N-terminal amino acid sequence of our purified PG A-2 is identical to the amino acid sequence previously deduced from DNA.[26]

Pepsinogen C is the second largest group of PG that has been purified from different species, such as humans, Japanese monkeys, and goats.[21,27,28] Pepsinogen A is produced in the gastric body and fundus, whereas PG C is also produced in the pyloric region and duodenum. Despite its wider distribution in the stomach, PG C occurs at smaller concentrations in the gastric mucosa than PG A in all species in which it has been isolated. The different cellular origins of PG A and PG C are important, because differential alterations in their serum concentrations are specific for various gastric disorders in humans.[18] After chromatography by use of a strong anion-exchange column,[g] we examined all peaks that had proteolytic activity for cross-reactivity against antiserum from sheep vaccinated with canine PG A-3. Immunologic cross-reactivity is reported to exist between isoforms of the same PG group, but not between isoforms of different groups. In our study, except for PG B, all peaks had immunocross-reactivity with canine PG A-3 identifying them as isoforms of PG A. Fractions which eluted from the hydroxyapatite column and had residual proteolytic activity were analyzed by SDS-PAGE. The protein bands obtained from those fractions had molecular weights either < 21,000 or > 150,000. These values are outside the reported molecular weight range for PG C, which is similar to PG A.[21,27,28] The residual activity may be the result of formation of complexes between PG A and other molecules that resulted in their binding to the hydroxyapatite column. Our purification protocol yielded no evidence for the existence of canine PG C. We cannot exclude, however, the possibility that PG C is synthesized in dogs. Pepsinogen C may be present in minute amounts or may be more sensitive to degradation during the purification process than in other species. Our results support the findings of a recent publication, in which, in contrast to other species, the authors were unable to isolate the coding DNA for PG C in dogs.[26] Narita et al[26] hypothesize that the gene for PG C is inactivated in dogs and that PG B takes a greater role in this species than in species that have synthesis of PG C. In our purification protocol, the overall yield for PG A was 11.6% (0.4 mg/g of mucosa), which was comparable to the yield reported for other species.[21] The overall yield for PG B was low. From 45 g of mucosal tissue, only 250 µg of protein could be purified, suggesting that PG A might be the predominant form of PG in dogs. However, yields from protein purification do not necessarily reflect tissue abundance of the proteins, and PG B maybe more labile than PG A during purification precluding higher yields.

[a]Sigma Chemicals, St Louis, Mo.
[b]Polytron PT-2100, Kinematica AG, Luzern, Switzerland.
[c]DEAE HiPrep 16/10, Amersham Biosciences, Piscataway, NJ.
[d]Bio-Scale CHT2-I column, BioRad, Hercules, Calif.
[e]Centripep YM-10, Amicon Bioseparations, Bedford, Mass.
[f]Sephacryl S-100, Amersham Biosciences, Piscataway, NJ.
[g]Mono-Q, Amersham Biosciences, Piscataway, NJ.
[h]Superdex S-200 HR, Amersham Biosciences, Piscataway, NJ.
[i]NuPAGE 10% Bis-Tris gel, Invitrogen, Carlsbad, Calif.
[j]Mark12 MW standard, Invitrogen, Carlsbad, Calif.
[k]pH 3-7 IEF gel, Invitrogen, Carlsbad, Calif.

[l]IEF calibration kit, Amersham Biosciences, Piscataway, NJ.
[m]GelCodeSilverSNAP, Pierce, Rockford, Ill.
[n]Procise-492 Sequencer, Applied Biosystems, Foster City, Calif.
[o]Quill A, Accurate Chemical, Westbury, NY.
[p]PBS, Pierce, Rockford, Ill.
[q]Immunodiffusion plate agarose, Pierce, Rockford, Ill.

References

1. Nielsen PK, Foltmann B. Purification and characterization of porcine pepsinogen B and pepsin B. *Arch Biochem Biophys* 1995;322:417–422.

2. Harboe M, Andersen PM, Foltmann B. The activation of bovine pepsinogen. Sequence of the peptides released, identification of a pepsin inhibitor. *J Biol Chem* 1974;249:4487–4494.

3. Bohak Z. Purification and characterization of chicken pepsinogen and chicken pepsin. *J Biol Chem* 1969;244:4638–4648.

4. Kageyama T, Tanabe K, Koiwai O. Structure and development of rabbit pepsinogens. *J Biol Chem* 1990;265:17031–17038.

5. Muto N, Tani S. Purification and characterization of rat pepsinogens whose contents increase with developmental progress. *J Biochem* 1979;85:1143–1149.

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

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

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

9. Berghen P, Dorny P, Vercruysse J. Evaluation of a simplified blood pepsinogen assay. *Am J Vet Res* 1987;48:664–669.

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

11. Pletschke BI, Naudé RJ, Oelofsen W, et al. Ostrich pepsinogens I and II: purification, activation and chemical and immunochemical characterization of the enzymes from the proventriculus. *Int J Biochem Cell Biol* 1995;27:613–624.

12. Kassell B, Wright CL, Ward PH. Canine pepsinogen and pepsin. *Methods Enzymol* 1976;45:452–459.

13. Marciniszyn JP, Kassell B. Purification and characterization of canine pepsinogen. *J Biol Chem* 1971;246:6560–6565.

14. Twining SS, Huibregtse, K, Glick, DM. A pepsinogen from dog stomach. *Comp Biochem Physiol B* 1983;75:103–107.

15. Cavadore JC, Cataldi M, Steffens R, et al. On the activation of the canine pepsinogens. *Biochimie* 1979;61:355–360.

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

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

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

19. 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.

20. Yakabe E, Tanji M, Ichinose M, et al. Purification, characterization, and amino acid sequences of pepsinogens and pepsins from the esophageal mucosa of bullfrog. *J Biol Chem* 1991;33:22436–22443.

21. Suzuki M, Narita S, Moriyama A, et al. Purification and characterization of goat pepsinogens and pepsins. *Comp Biochem Physiol B* 1999;122:453–460.

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

23. Fowler SD, Kay J, Dunn BM, et al. Monomeric human cathepsin E. *FEBS Lett* 1995;366:72–74.

24. Kageyama T, Takahashi K. A cathepsin D-like acid proteinase from human gastric mucosa. *J Biochem* 1980;87:725–735.

25. Evers MP, Zelle B, Bebelman JP, et al. Nucleotide sequence comparison of five human pepsinogen A (PGA) genes: evolution of the PGA multigene family. *Genomics* 1989;4:232–239.

26. 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.

27. Hengels KJ, Strohmeyer G. Pepsinogens A and C: purification from human gastric mucosa and determination in serum by optimized radioimmunoassays. *Z Gastroenterol* 1989;27:406–411.

28. Kageyama T, Takahashi K. Pepsinogen C and pepsin C from gastric mucosa of Japanese monkey—purification and characterization. *J Biochem* 1976;80:983–992.