Pepsinogens A, C, and D from the Smooth Dogfish*

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ABSTRACT: Four pepsinogens have been separated from the stomach mucosae of the smooth dogfish by chromatography on DEAE-cellulose. Pepsinogen B, which at pH 2 had enzymic activity toward the synthetic substrate, carbobenzoxyglutamyltyrosine, first emerged followed by pepsinogens D, A, and C with potential enzymic activity toward protein substrates. The latter three precursors were purified further by DEAE-cellulose and Sephadex chromatography. Their molecular weights, determined by ultracentrifugal analysis, were found to be approximately 42,000.

Their amino acid composition showed a predominance of acidic over basic residues. In these respects, the dogfish pepsinogens resemble the swine and bovine zymogens. The compositions of pepsinogens A and D were similar to each other and differed from pepsinogen C. The immunological experiments with antipepsinogens A, D, and C also indicated that pepsinogens A and D were similar and that pepsinogen C was distinct.

During the course of the early investigations on swine pepsin and pepsinogen, pepsins with varying specific activities were obtained (*cf.* Northrop *et al.*, 1948). Taylor (1962) and Bovey and Yanari (1960) review the observations made in several laboratories dealing with the multiplicity of proteolytic enzymes isolated from gastric mucosae which are active at acid pH.

At least two reasons for the heterogeneities exist. Rajagopalan *et al.* (1966) showed that it is possible to generate pepsins with different properties from a single highly purified precursor by varying the conditions of activation. They were also able to separate different pepsins from commercial crystalline preparations of the enzyme. Ryle (1960, 1965), Ryle and Hamilton (1966), and Lee and Ryle (1967) extracted swine mucosae in buffers of low ionic strength and isolated at least four different pepsinogens, each of these proteins being the precursor of a different pepsin. The multiplicity of pepsinogens has also been found in the mucosae of other species. Using chromatographic and immunologic techniques, Seijffers *et al.* (1963) were able to separate three distinct pepsinogens from human gastric mucosae. Levchuk and Orekhovich (1963) found three pepsins in chicken stomachs, and Dr. S. T. Donta in this laboratory has evidence for at least four pepsinogens in extracts of chicken mucosae. Using electrophoretic techniques the existence of multiple pepsinogens was shown by Hanley *et al.* (1966) and Etherington and Taylor (1967) in several different mammalian species.

At least three different pepsinogens exist in the stomachs of the smooth dogfish, *Mustelus canis*. The methods of isolations and some properties of three of these precursors, *i.e.*, those that have potential activity toward protein substrates but relatively little activity toward the synthetic substrate, *N*-Cbz-L-Glu-L-Tyr, are the subject of this communication. In order to retain some semblance of order in an already confused terminology, we have continued the nomenclature suggested by Ryle and his coworkers (Ryle, 1965; Ryle and Hamilton, 1966) using as our standard of comparison the order of elution of the proteins from DEAE-cellulose columns at pH 7.5, *i.e.*, pepsinogens B, D, A, and C, respectively. Using this terminology, we find pepsinogen B has enzymatic activity toward synthetic substrates at pH 2 and that the other three, after activation, digest protein substrates (Bar-Eli *et al.*, 1966). Pepsinogens A and D are similar to each other while pepsinogen C is distinct.

Materials and Methods

Materials. Analytical grade reagents were dissolved in distilled deionized water to prepare the buffer solutions. The *N*-Cbz-L-Glu-L-Tyr was purchased from Mann Research Laboratories, Inc., and swine pepsin (three-times crystallized) from the Nutritional Biochemicals Corp. Starch used for electrophoresis was purchased from Connaught Medical Research Laboratories and the acrylamide from Eastman Kodak.

Dogfish Stomachs. Smooth dogfish, *Mustelus canis*, caught at the Woods Hole Marine Biological Laboratories, were killed by a blow on the head, the stomachs were removed and washed, and the mucosae were stripped and stored in a deep freeze. (Inadvertently, one bath of stomachs thawed, but neither the purification nor properties of the enzymes appeared to be affected.)

Enzyme Assays. In the determination of proteolytic activity, hemoglobin was used as a substrate in a modification of the Anson (1938) procedure. To 0.1 ml of zymogen and 0.9 ml of 0.06 N HCl was added 5 ml of 2% hemoglobin that had been extensively dialyzed against 0.06 N HCl. After incubation at 37° for 10 min, the solution was deproteinized by the addition of 5 ml of 5% trichloroacetic acid, incubated a further 10 min, filtered, and read at 280 mμ. The increase in extinction at 280 mμ of the filtrate was a measure of the enzyme activity, and was referred to a standard curve prepared for swine pepsin.

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All assays were carried out in duplicate together with blank reactions in which the hemoglobin was added after the addition of trichloroacetic acid.

In the determination of peptidase activity, *N*-Cbz L-Glu-L-Tyr was used as substrate and the extent of hydrolysis determined by the ninhydrin reaction (Moore and Stein, 1954). The precursors were activated by incubation at pH 2.0 and 37° for 3 min. Activity was determined in a 1.0-ml solution containing 5 μmoles of *N*-Cbz-L-Glu-L-Tyr in 0.02 M NaCl at pH 2. Incubation was at 37° for 15 min. All assays were carried out in duplicate, together with duplicate blank reactions in which ninhydrin reagent was added to activated enzyme prior to the addition of substrate. After the color was developed in the usual manner, the mean increment of the optical densities at 570 mμ was determined and related to a standard tyrosine calibration curve.

Electrophoresis. Electrophoresis in starch gel was performed by the method of Fine and Costello (1963) in citrate–phosphate buffer (pH 7.0) using 150 μg of the protein sample. Separation was carried out for 12 hr at 4° with a voltage gradient of 8 V/cm along the gel. Disc electrophoresis was performed according to the method of Ornstein and Davis (1964) except that the large pore gel has been replaced by a buffered mixture of sample and 0.2 M sucrose, and the ammonium persulfate stock solution was 0.07%. Acrylamide and methylenebisacrylamide were recrystallized according to the procedure of Mengoli and Watne (1966). The bands possessing potential enzyme activities toward hemoglobin were detected by allowing denatured hemoglobin at pH 2 to diffuse into the gel, and then staining the gel in the usual manner with Amido Black. Since the products of digestion are neither fixed nor stained, a weakly colored region against a dark blue background indicated a region of peptic activity. It is necessary for the pepsin to be completely autolyzed if an absolutely clear region is to be obtained. In general, the hemoglobin in acid was allowed to diffuse into the gel for 30 min at 37°, but occasionally variations in the reaction time for different concentrations and for individual pepsinogens was required.

Molecular Weights. Molecular weights were determined in the Spinco Model E analytical ultracentrifuge at 5° by the high-speed sedimentation equilibrium method described by Yphantis (1964), using a six-channel Epon centerpiece and interference optics. Sapphire windows and a T rotor were used at a speed of 31,410 rpm and protein concentrations between 0.2 and 1.1 mg per ml were employed. Photographic plates (Eastman Kodak 11G) were measured in a Gaertner traveling microscope. Zymogen samples were concentrated, if necessary, by ultrafiltration and dialyzed against 0.1 M Tris (pH 7.5). Dilutions were made from the most concentrated samples, and the dialysates were used in the solvent side of the interference cell. A partial specific volume of 0.74 was assumed for each zymogen.

Molecular weights were determined using a Wang Series 370 calculator using programs devised by Dr. Serge Timasheff and Mr. Keith McClelland. Weight-average molecular weights quoted were determined using a least-squares fit program of ln c *vs.* r^2, and the deviations given represent the limits obtained from a point-average molecular weights program. z-average molecular weights are quoted as the mean figures obtained from a point-average molecular weight program.

Amino Acid Analysis. Amino acid analyses (Moore *et al.*, 1958) were performed with a Beckman amino acid analyzer, Model 120B, following hydrolysis in 6 N HCl at 108° for 24 or 48 hr. The number of threonine and serine residues was obtained by extrapolation to zero time, and the isoleucine, leucine, and valine values shown were for 48-hr hydrolyses since samples were not completely hydrolyzed in 24 hr. The total cysteine plus cystine was determined from performic acid oxidized samples (Moore, 1963). The number of tryptophan residues was determined by the method of Beaven and Holiday (1952).

Preparation of Antisera. New Zealand white rabbits were immunized with purified preparations of pepsinogens A, C, and D by injecting them *via* the toepad and muscle with 2–5 mg of material in 0.1 M Tris (pH 7.5) mixed with an equal volume of Freund's adjuvant. The rabbits were bled 3 weeks later and, if necessary, booster doses were administered in a similar manner and bleedings were taken at 7–10-day intervals until satisfactory antibody titers were obtained as judged by double immunodiffusion in two dimensions (Ouchterlony, 1949).

Preparation of Dogfish Pepsinogens. All operations were carried out at 4° and at pH 7.5 unless otherwise stated. In a typical run, the mucosae stripped from seven dogfish stomachs (210 g) were partially thawed, and homogenized for 30 sec with 360 ml of 0.05 M Tris buffer.

The homogenate was stirred for 1 hr, passed through gauze, and the filtrate was centrifuged at 7500 rpm for 45 min. The supernatant was further clarified by ultracentrifugation for 45 min at a speed of 35,000 rpm and then dialyzed overnight against 0.03 M Tris. The pepsinogens were adsorbed batchwise from the dialyzed solution onto 250 ml of DEAE-cellulose (type 11) previously equilibrated with buffer. After stirring for 30 min, the DEAE-cellulose was collected by centrifugation, washed with 400 ml of buffer, and added to a column of DEAE-cellulose (24 × 5 cm) and elution was started using the same 0.03 M Tris buffer; 500 ml of buffer was sufficient to elute a large peak of inactive protein and a gradient of increasing molarity was applied by connecting a cylindrical bottle with 0.5 M Tris buffer (2000 ml) with a mixing cylindrical chamber containing the starting buffer (2000 ml). Assays using hemoglobin and *N*-Cbz-L-Glu-L-Tyr as substrates were performed on every fifth tube and the peaks were pooled accordingly. The partially purified pepsinogens were dialyzed against 0.03 M Tris, and each was further purified by a repeat of the DEAE-cellulose chromatography. Prior to gel filtration on Sephadex G-100, it was necessary to concentrate the individual pepsinogens contained in approximately 400 ml of solution by adsorbing them onto a small DEAE-cellulose column (30 × 3 cm) in 0.03 M Tris and eluting it with 0.6 M Tris buffer. The proenzymes were further concentrated about tenfold, to a volume of 5 ml by ultrafiltration; each was applied to a Sephadex column by the sucrose density layering method and eluted with 0.1 M Tris buffer. Enzyme assays were performed and the potentially active fractions were pooled, concentrated by ultrafiltration, and filtered once again through the Sephadex column. The extraction procedure resembles that of Ryle (1960, 1965) and Lee and Ryle (1963) in that the pepsinogens were extracted from the mucosae by dilute buffer and isolated by chromatography. Ryle (1960) had previously reported that the use of buffers containing $(NH_4)_2SO_4$ (Herriott, 1948) was unsuitable for extracting the various pepsinogens from the mucosae since some of the zymogens remained in the precipitate with the inactive protein.

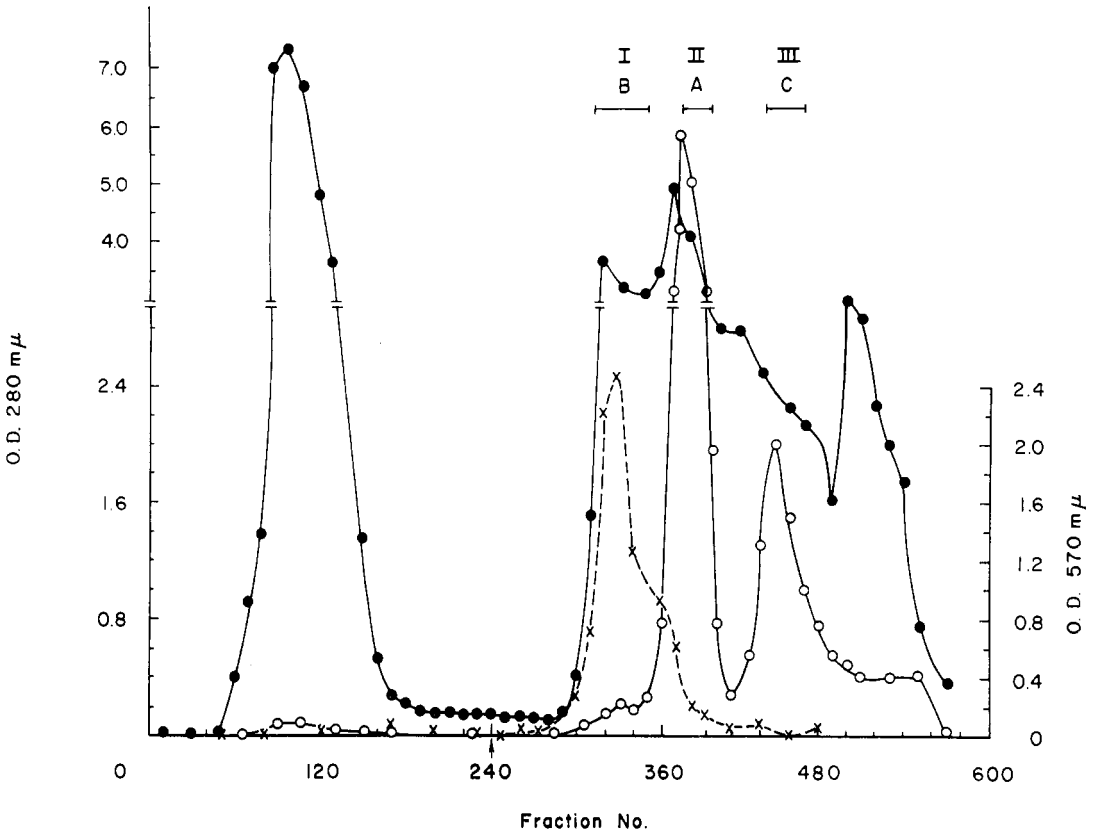

FIGURE 1: Chromatography of crude dogfish pepsinogens extracted from seven stomaches on a DEAE-cellulose column in 0.03 M Tris (pH 7.5). Tris gradient from 0.03 to 0.5 M applied at tube 240. Flow rate was 40 ml/hr and fractions of 14 ml were collected. The bars show the fractions pooled for further purification. (●—●) Optical density at 280 mμ. (O—O) Activity against hemoglobin expressed as increase of optical density at 280 mμ of digested trichloroacetic acid soluble material. (×—×) Ninhydrin assay used to follow digestion of *N*-Cbz-L-Glu-L-Tyr (optical density at 570 mμ).

Results

The protein content of crude mucosal extracts in 0.05 M Tris buffer at pH 7.5 varied in different preparations from 4 to 6% of the initial tissue weight. Approximately 4% of the total protein was represented by enzyme(s) that digested the blocked dipeptide, *N*-Cbz-L-Glu-L-Tyr, and another 8% by precursors of enzymes that readily digested hemoglobin. High-speed centrifugation of the extract prior to chromatography was essential in order to maintain satisfactory flow properties with DEAE-cellulose columns. The initial purification by the ion exchanger yielded three peaks which had no enzymic activity when assayed for their ability to clot milk at pH 5.5. At acid pH (Figure 1), the protein in the first peak hydrolyzed *N*-Cbz-L-Glu-L-Tyr while the proteins in the second and third peaks were active against hemoglobin. The proenzymes in the second and third peaks were purified further by repeating twice the DEAE-cellulose chromatography and it was then that the second peak split into two peaks, both of which were capable of digesting hemoglobin after activation (Figure 2). The proteins were termed pepsinogens B, D, A, and C, respectively, in order to maintain the terminology suggested by Ryle (1965) for the swine system. The final purification steps were performed using Sephadex G-100 (two times). Figure 2 is typical of the separations obtained with the individual pepsinogens, and it is worth noting that under the conditions of assay the purified pepsinogens D, A, and C showed no ability to digest the dipeptide after activation, although pepsinogen B was capable of limited digestion of the protein substrate (Figure 1). This activity disappeared as pepsinogen B was purified.

Each proenzyme was stored frozen in 0.1 M Tris buffer at pH 7.5. However, some caution should be exercised in handling pepsinogens of different species, if the pepsin which is generated is stable at neutral pH and/or if the autocatalytic conversion of the precursor can take place at elevated pH. For example, dogfish pepsins are somewhat more stable than swine pepsin at neutral pH, yet the precursors can be stored for long periods of time without encountering activation. However, the storage of chicken pepsinogen under identical conditions leads to the progressive generation of enzyme activity (S. T. Donta, personal communication, 1968). Chicken pepsin was found to be markedly stable at neutral and slightly alkaline pH (S. T. Donta, personal communication, 1968), confirming an earlier observation made by Herriott (1938).

Starch gel electrophoresis of pepsinogens A and C in citrate–phosphate buffer at pH 7 yielded single spots that migrated toward the anode (Figure 3). Swine pepsinogen and pepsinogen B which had been purified further were also included in this run.

Pepsinogens A, C, and D were also separable on electrophoresis at pH 8.5 on acrylamide gels. The order of their mobilities toward the anode were C > A > D. Polyacrylamide disc electrophoresis (Figure 4) of pepsinogens A and D yield

predominantly one central band outlined with two fainter bands, while pepsinogen C shows one band. Care with the interpretation of the patterns obtained is necessary since a homogeneous protein, as judged by the phase rule definition, may give rise to more than one band due to different molecular variants of the same protein, or a rapid reversible interaction between the macromolecule and an unchanged constituent of the solvent medium (Cann and Good, 1964). In the early runs a protein band moved with the interacting dye in every case but it was eliminated by the use of recystallized acrylamide and preelectrophoresing the gels for 30 min.

Results of molecular weight studies are given in Figure 5 and Table I. The plots of $M_w(r)$ *vs.* $b^2 - r^2$ show that pepsinogens A, C, and D are reasonably homogeneous with respect to molecular weight. It is of interest that pepsinogens A and D were eluted from Sephadex G-100 in the same volume but pepsinogen C was eluted slightly later.

TABLE I: Molecular Weights of Dogfish Pepsinogens in 0.1 M Tris (pH 7.5).[a]

	Concn (mg/ml)	Wt-Av Mol Wt $\times 10^{-3}$	*z*-Av Mol Wt $\times 10^{-3}$
Pepsinogen A	0.2	49.0 ± 2.0	45.5 ± 2.5
	0.5	42.8 ± 0.9	47.2 ± 2.0
	0.9	43.7 ± 0.9	41.3 ± 2.3
Pepsinogen C	0.3	44.1 ± 1.5	47.0 ± 2.4
	0.7	40.1 ± 1.6	39.6 ± 2.2
	1.1	41.8 ± 1.8	42.3 ± 4.1
Pepsinogen D	0.3	50.6 ± 2.3	51.7 ± 2.1
	0.7	41.4 ± 1.3	43.9 ± 3.9
	0.9	43.5 ± 0.8	45.4 ± 0.2

[a] Experimental conditions: 12 hr at 31,410 rpm; pH 7.5, 0.1 M Tris; six-channel Epon centerpiece.

The amino acid analysis of the pepsinogens are listed in Table II, and represent the average values correct to the nearest whole residue for 24- and 48-hr acid hydrolyses. Molecular weights of pepsinogens A, D, and C were assumed to be 41,500 for the calculation of the analysis. The ratios of the amino acids found in the hydrolysates approached whole numbers and the recovery of the amino acids was in fair agreement with the amount of protein used for the analysis. The finding of seven cysteic acid residues per mole of performic acid oxidized protein would suggest that at least one cysteine residue is present but independent quantitative assays for cystine and cysteine are necessary before any definitive statement can be made.

As shown in Figure 6 pepsinogens A, C, and D each gave a single precipitin band with the homologous antibody. Pepsinogen C did not react with either of the heterologous antibodies. The serologic reactions of pepsinogens A and D were indistinguishable with antipepsinogen A; there were no signs of spurring between pepsinogens A and D.

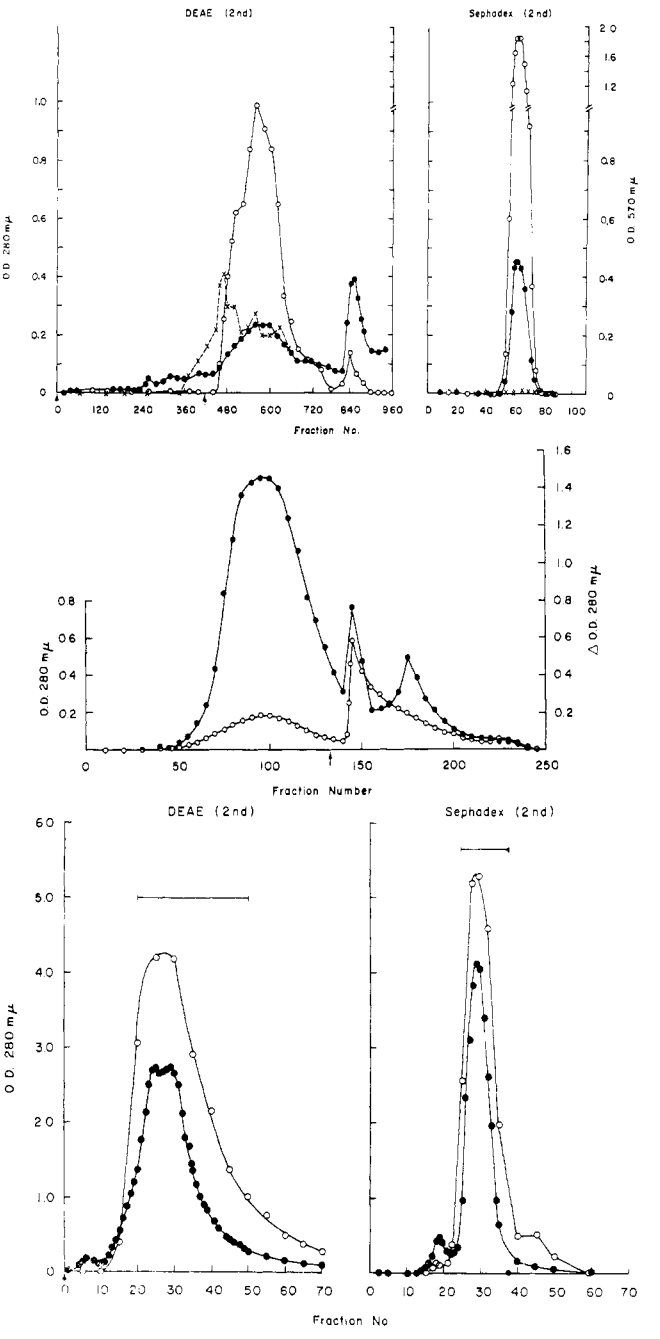

FIGURE 2: (Top left) rechromatography of pepsinogen A on a DEAE-cellulose column (5 × 36 cm) in 0.03 M Tris (pH 7.5). The elution was carried out with a linear gradient of 0.03–0.25 M Tris applied at tube 1 followed by a gradient of 0.25–0.5 M Tris applied at tube 420. Fraction volume was 10 ml. (Right) final gel filtration of pepsinogen A on Sephadex G-100 column (3 × 60 cm) in 0.1 M Tris (pH 7.5). Fraction volume was 3 ml and flow rate 16 ml/hr. (●—●) Optical density at 280 mμ. (○—○) Activity against hemoglobin. (×—×) Activity against *N*-Cbz-L-Glu-L-Tyr. (Middle) Rechromatography of leading edge of pepsinogen A peak from DEAE 2nd (above), on a the DEAE-cellulose column (3 × 36 cm) in 0.03 M Tris (pH 7.5). The elution was carried out with a linear gradient of 0.03–0.25 M Tris followed by a gradient of 0.25–0.5 M Tris. The arrow indicates the application of the second gradient. The first peak represents pepsinogen D, followed by pepsinogen A and pepsinogen C. Fraction volume was 10 ml. (○—○) Optical density at 280 mμ. (●—●) Activity against hemoglobin. (Bottom left) rechromatography of pepsinogen C on a DEAE-cellulose column (3 × 36 cm) in 0.3 M Tris (pH 7.5), Tris gradient from 0.3 to 0.6 M applied at tube 1. (Right) second gel filtration of pepsinogen C on Sephadex G-100 (2 × 90 cm) in 0.1 M Tris (pH 7.5). Fraction volume was 3 ml and flow rate 10 ml/hr. (●—●) Optical density at 280 mμ. (○—○) Activity against hemoglobin.

TABLE II: Amino Acid Compositions of Pepsinogens.

	Dogfish Pepsinogens			Swine Pepsinogens		Bovine Pepsinogens
	A	C	D	A[a]	C[b]	A[c]
Lys	14	5	11	10	12	8
His	7	4	7	3	2	2
Arg	14	8	15	4	7	6
Asp	44	44	45	44	30	40
Thr	23	27	24	26	25	27
Ser	43	51	38	46	35	50
Glu	39	41	38	28	47	32
Pro	19	22	17	19	20	15
Gly	40	48	43	35	35	35
Ala	18	18	16	19	23	16
½-Cys	7	7	7	6	6	6
Val	23	27	25	23	22	25
Met	7	6	7	4	5	4
Ile	22	18	22	25	16	32
Leu	28	24	25	33	40	25
Tyr	19	20	21	17	22	18
Phe	17	17	18	15	24	15
Trp	5			6	6	6
NH_3				27		37
Mol wt	41,500	41,500	41,500	38,944	41,000	38,943

[a] Rajagopalan *et al.* (1966). [b] Ryle and Hamilton (1966). [c] Chow and Kassell (1968).

Discussion

Four precursors of acid-activated enzymes have been isolated from the gastric mucosae of dogfish stomachs. These zymogens seem to be analogous to the various pepsinogens isolated from swine stomachs by Ryle (1960, 1965), Ryle and Hamilton (1966), and Lee and Ryle (1967).

The weight-average molecular weights of the pepsinogens as determined in the ultracentrifuge are consistent with those normally obtained for pepsinogens from other species, *i.e.*, approximately 42,000 (Williams and Rajagopalan, 1966). However, Figure 5 and Table I do indicate some concentration dependence which is interpreted as an indication that the pepsinogens form aggregates in solution.

Pepsinogens A and D have similar though not identical amino acid compositions. Compared with pepsinogen D and neglecting single residue differences, pepsinogen A has 3 Lys, 5 Ser, 2 Pro, 2 Ala, and 2 Leu residues in excess. It lacks 3 Gly, 2 Val, and 2 tyrosine residues. Considering the size of these proteins, the comparative aspects of the analysis (*e.g.*, assumption of equivalent molecular weights for the proteins), the necessity to still obtain tryptophan, amide NH_3, and phosphate contents for each protein, at present we consider only differences beyond 10% as significant. The immunological reactions indicate that they have very similar conformations since they react equally well with the heterologous antisera both by immunodiffusion and by quantitative complement fixation (Merrett *et al.*, 1970). The inability of the sensitive immunological techniques to detect differences provides us with the best evidence of the similarities of these two proteins. Swine pepsinogen D appears to be a dephosphorylated A (see Lee and Ryle, 1967), but we do not yet have sufficiently good data to make a statement about the phosphate content of the dogfish proenzymes.

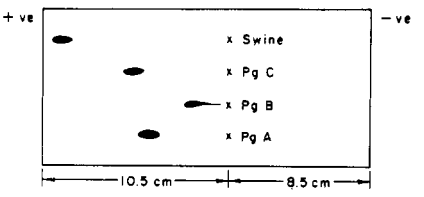

FIGURE 3: Starch gel electrophoresis of swine pepsinogen and dogfish pepsinogens A, B, and C in citrate–phosphate buffer (pH 7.0).

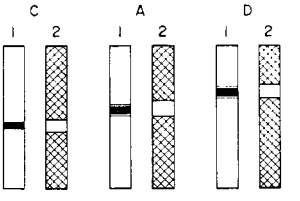

FIGURE 4: Schematic drawing of polyacrylamide disc gels of pepsinogens C, A, and D stained for protein with Amido Black (clear gels, no. 1 of each pair) and for enzyme activity by perfusing the gels with hemoglobin solution at pH 2 and then staining with Amido Black (dark gels, no. 2 of each pair). Direction of electrophoresis from top to bottom is cathode to anode. Note the relative mobilities C > A > D.

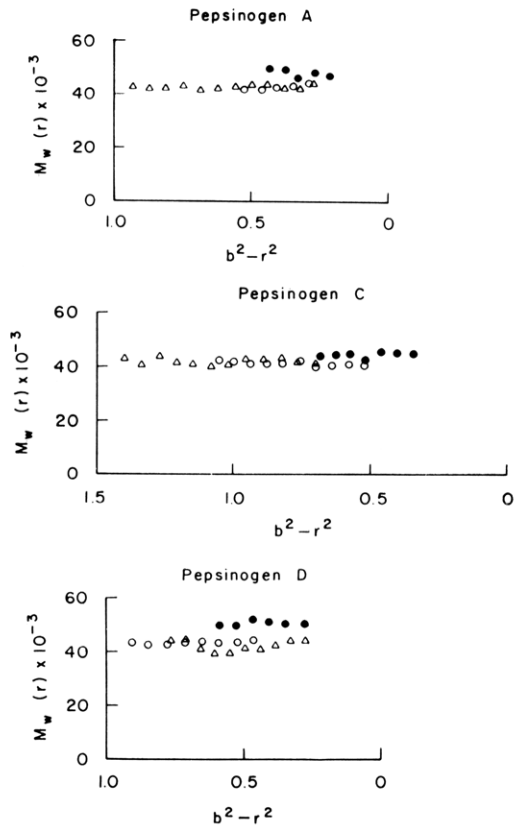

FIGURE 5: Direct comparison of the weight-average molecular weights, $M_w(r)$, observed in three channels of equilibrium experiments on pepsinogens A, C, and D. There are three different initial loading concentrations for each pepsinogen: △ represents the highest initial concentration, ○ the intermediate concentration, and ● the lowest concentration. See Table I for further experimental conditions.

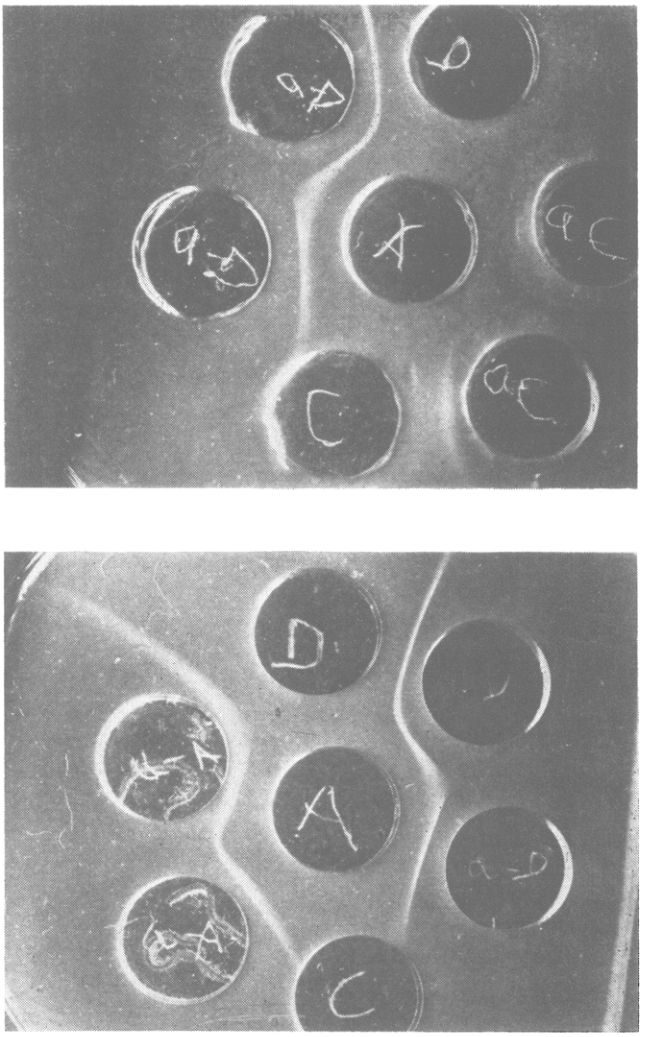

FIGURE 6: Immunodiffusion analysis of pepsinogens A, D, and C versus antipepsinogens A, D, and C (a-A, a-D, and a-C). Top: the central antigen containing wells are flanked by antipepsinogen C and antipepsinogen D. Note that antipepsinogen C reacts only with pepsinogen C and also the pattern of identity between pepsinogens A and D. Bottom: the central antigen wells are flanked by a-A and a-D. Note the lack of any reaction between pepsinogen C and a-A and a-D.

Significant amino acid differences exist between pepsinogens A and C. Pepsinogen A contains a greater number of basic amino acids (14 Lys, 7 His, and 14 Arg) than pepsinogen C (5 Lys, 4 His, and 8 Arg) and is also richer in the Leu, Ile residues. If these were the only differences, one might suggest that the structure of pepsinogen C is encompassed in the slightly larger molecule, pepsinogen A. However, pepsinogen C contains significant excess of Thr (4), Ser (9), Pro (3), Gly (8), and Val (4). The total lack of cross-reaction between pepsinogen C and the antisera directed toward pepsinogens A and D and the reverse, *i.e.*, pepsinogens A and D, do not react with antipepsinogen C support the view that they are unrelated proteins. Ryle and Hamilton (1966) also believe that swine pepsinogens A and C are distinct proteins and base their conclusions on the finding of inversions in the contents of aspartic acid with glutamic acid and leucine with isoleucine as well as significant differences in the content of other amino acids.

The amino acid composition of dogfish pepsinogen A when compared with that of swine pepsinogen A (Arnon and Perlmann, 1963; Rajagopalan *et al.*, 1966) and bovine pepsinogen A (Chow and Kassell, 1968) reveals that it possesses a greater content of the three basic amino acids and that additional differences, although to a smaller extent, exist among several other amino acids. Typical of all pepsinogens analyzed thus far, the content of acidic amino acids is markedly greater than the basic amino acids. The qualitative and quantitative features of the peptide lost during conversion into the enzyme remains to be studied in the dogfish system. However, in preliminary experiments the molecular weights of dogfish pepsins A, D, and C, obtained from ultracentrifugal analyses, account for 34,000 to 36,000 of the weight of each pepsinogen molecule, indicating that a fragment of 5000 to 8000 daltons is split off during the activation procedure. The peptide lost during the conversion of swine pepsinogen is quantitatively of this size (Herriott, 1962; Arnon and Perlmann, 1963; Rajagopalan, 1966; Koehn and Perlmann, 1968; Ong and Perlmann, 1968). The interesting finding has been made, however, that during the conversion of chick pepsinogen, the enzyme which is formed has a molecular weight indistinguishable from the precursor by ultracentrifugal analysis and by elution from a calibrated Sephadex G-100 column (S. T. Donta, personal communication 1968). The loss of the majority of

the basic amino acids in a peptide of 5,000–10,000 molecular weight does not appear to be a general characteristic of the autocatalytic reaction with pepsinogens of all species.

While some basis for comparison of pepsinogens of various species is necessary, the exclusive use of electrophoretic mobility as suggested by Etherington and Taylor (1967) might lead to even greater confusion. The chromatographic and electrophoretic behavior of the pepsinogens of the dogfish system are in agreement but swine pepsinogen C migrates more slowly toward the anode on electrophoresis than does swine pepsinogen A, although it is the last enzyme to be eluted from DEAE-cellulose.

In our experiments, pepsinogen A was the major component and is analogous to the pepsinogen most actively studied in the swine and bovine species by other investigators. Pepsinogens D and C occur in lesser but approximately equal amounts. Since there appears to be some evidence for the localization of the various pepsinogens in different anatomical parts of the stomach (Seijffers *et al.*, 1963; Hanley *et al.*, 1966; Ryle and Hamilton, 1966), this distribution may reflect the part of the stomach mucosae used for the extractions. The availability of specific antisera for dogfish pepsinogens A and D and for dogfish pepsinogen C will permit the distribution of these precursors to be determined in different parts of individual dogfish stomachs.

The evidence in favor of homogeneity of the dogfish pepsinogens is based on the superposition of activity and optical density curves in the elution patterns obtained from DEAE-cellulose and Sephadex chromatography, the close approach to whole numbers of the ratios of amino acids found in the hydrolysates, the ultracentrifugal evidence that any heterogeneities that exist are aggregates of pepsinogen, the fact that the antibodies to pepsinogens A, C, and D give a single precipitin band both with the homologous purified antigen and a crude extract of the mucosae, the migration of each protein as a single spot on starch gel electrophoresis and in the case of pepsinogen C, a single band on disc gel electrophoresis. The only evidence for heterogeneity of these proteins in the analysis thus far carried out is the appearance of two faint bands on either side of the main band in the disc gel electrophoresis of pepsinogens A and D. After acid activation in the gel, all of the bands are capable of digesting hemoglobin. In the small number of individual swine stomachs that were analyzed, genetic variants of pepsinogens were not detected (Ryle, 1965). Since our preparations consisted of a pool of stomach mucosae, the possibility that the multiplicity of bands observed on polyacrylamide gel electrophoresis of pepsinogens A and D is due to genetic variants must be considered along with the possibility of the Cann effects. End-group analysis will further define the state of the precursors and may be useful in determining the direction of the hydrolysis to form the active enzymes.

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