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Chicken Pepsinogens and Pepsins. Their Isolation and Properties*

Sam T. Donta and Helen Van Vunakis

ABSTRACT: Pepsinogens A, D, and C have been isolated from the gastric mucosae of chickens using ion-exchange and molecular sieve chromatography and were found to have molecular weights of approximately 42,000. Pepsinogens A and D are closely related in their amino acid compositions, electrophoretic mobilities, and stabilities. Pepsinogen C is significantly different in these properties. Unlike swine pepsin, chicken pepsins A and D are stable at neutral pH and have molecular weights similar to their precursors. They contain over twenty of the approximately thirty basic amino acid residues originally present in the respective precursors. Pepsin C has a molecular weight of about 38,500, indicating that a larger peptide fragment is removed from pepsinogen C during the conversion process.

Multiple forms of pepsinogen,[1] which yield enzymes active at acid pH, have been found in the gastric mucosae of several species. In the most extensive studies, three or four pepsinogens have been extracted from the gastric mucosae of the swine (Ryle, 1960, 1965), chicken (Levchuk and Orekhovich, 1963), human (Seijffers *et al.*, 1963), and dogfish (Merrett *et al.*, 1969). The purification and properties of three chicken pepsinogens and the pepsins derived from them are the subject of this communication. Their immunological interrelationships are reported in the accompanying paper (Donta and Van Vunakis, 1970a).

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[1] The nomenclature adopted by various authors for the pepsinogens has not been uniform. Because of the similarities of isolation techniques and elution patterns from ion-exchange columns, we have used the system adopted by Ryle (1965) for the swine pepsinogens, as recommended by the Commission on Enzymes of the International Union of Biochemistry.

Materials and Methods

Unless otherwise stated, materials were similar to those used in the study of the dogfish pepsinogens by Merrett *et al.* (1969). All chromatographic and preparative procedures were carried out at 0–5°. DE-11 and DE-52 ion-exchange cellulose (Whatman) and Sephadex G-100 (Pharmacia) were used for the isolation of the pepsinogens. Sodium phosphate buffer, pH 6.9, was used in most of the purification procedures since it was found to give the best separation of the proteins. However, in one scheme, the protein isolations were carried out in Tris buffer, pH 7.5.

Proteolytic and potential proteolytic activities were determined using hemoglobin (Anson, 1938) or milk (Herriott, 1938) as substrates. Peptidase activity was determined by measuring the extent of hydrolysis of the synthetic substrate Cbz-L-Glu-L-Tyr (CGT) using the ninhydrin assay (Moore and Stein, 1954). Details of these methods, as well as the modifications used, are outlined in Merrett *et al.* (1969).

Proteins were concentrated with an Amicon Corp. Diaflo ultrafiltrator, using a UM-1 membrane (10,000 mol wt cutoff). Analytical acrylamide gel electrophoresis was performed by the method of Ornstein and Davis (1964), except that no sample gel was used. Acrylamide gel electrophoresis in sodium

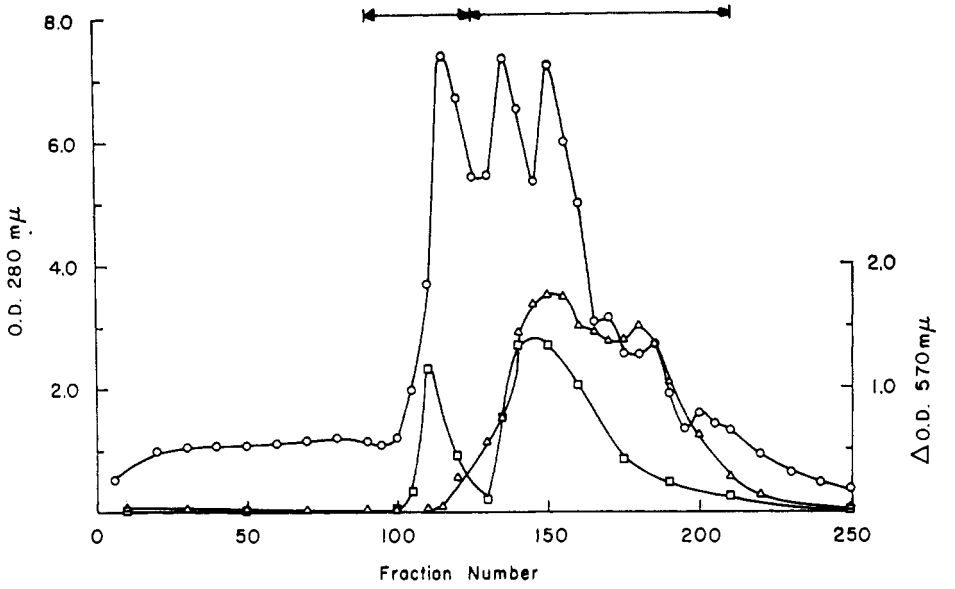

FIGURE 1: Chromatography of original crude stomach extract on DE-11 ion-exchange cellulose. The extract from 72 chicken stomachs was applied to a column (35 × 6 cm) and eluted with a gradient of 2.0 l. of 0.02 M phosphate buffer, pH 6.9, to 2.0 l. of 0.02 M phosphate buffer containing 0.9 M NaCl, pH 6.9; 20-ml fractions were collected. The arrows indicate fractions pooled: (○) optical density at 280 mμ; (△) proteolytic activity at pH 2 using hemoglobin as substrate, expressed as increase of optical density at 280 mμ of digested, trichloroacetic acid soluble material; (□) peptidase activity using the synthetic substrate *N*-Cbz-L-Glu-L-Tyr determined by the ninhydrin assay and expressed as increase in optical density at 570 mμ.

dodecyl sulfate was carried out according to the method of Shapiro *et al.* (1966). Samples for amino acid analysis were dialyzed exhaustively against deionized H_2O (0.001 N NaOH was added to maintain a pH value of 7.5 for the pepsinogens), dried, hydrolyzed in constant-boiling point HCl, and analyzed according to the method of Moore *et al.* (1958) using a Beckman Model 120 C amino acid analyzer.

Molecular weights were determined by high-speed sedimentation to equilibrium in a Beckman Model E ultracentrifuge, according to the method of Yphantis (1964). A six-channel charcoal-filled or aluminum-lined epon certerpiece housed the samples in a cell with sapphire windows. All samples were centrifuged at 31,410 rpm and 5° for at least 16 hr and were analyzed at three or more concentrations in either Tris or phosphate buffer. Under these conditions of centrifugation, the σ_w values were 5.0, assuring maximal accuracy. The molecular weights were determined with the aid of a Wang Series 370 calculator, which was programmed in the laboratory of Dr. S. Timasheff for point average and least-squares-average molecular weights.

Results

Purification of the Pepsinogens and Pepsins. The proventricula (stomach equivalents) of 40–80 freshly sacrificed (Kosher) domestic chickens (*Gallus gallus*) were immediately placed in iced 0.02 M phosphate buffer at pH 6.9. The fat and mucus were removed but the mucosae were too fragile to strip; therefore, the whole stomachs were used as the source of the pepsinogens. After grinding the stomachs in a meat grinder, the mixture was extracted with 1–2 l. of phosphate buffer by stirring for 1–2 hr. The residue was removed by centrifugation at 10,000 rpm for 30 min.

In the initial isolation procedures, the supernatant fraction was stirred in the presence of DE-11 cellulose for 1 hr. The resin on which the pepsinogens were adsorbed was collected by centrifugation. Phosphate buffer was then added to form a thick slurry, which was poured into a column containing a preexisting bed of DE-11 cellulose resin. In later isolation procedures, the supernatant fraction was adsorbed directly onto a DE-11 cellulose column.

One to three liters of starting buffer was used to elute a large peak of inactive protein from the column. A linear salt gradient was then used to elute the pepsinogens. Depending on the gradient and the total amount of starting material, the patterns of elution from DE-11 cellulose varied from one extract to another. However, the bulk of the potential proteolytic activity was always present in the last major peak (Figure 1). All fractions were monitored by the hemoglobin assay at acid pH (to detect peptic and potential peptic activity) and by the milk-clot assay at pH 5.6 (to determine if any activation of the pepsinogens to pepsins had taken place). The Cbz-L-Glu-L-Tyr assay was used to detect the peptidase which is active at low pH on small synthetic substrates but has little or no detectable activity on protein substrates (Donta and Van Vunakis, 1970b). The latter enzyme may correspond to swine pepsinogen B (Ryle, 1965).

The fractions with potential enzymatic activity on protein substrates were pooled, dialyzed against 0.02 M phosphate buffer, pH 6.9, and applied to a second DE-11 cellulose column. Although specific activities of the pepsinogens were not markedly increased by the second resin passage, the flow properties of the eluate on subsequent DE-52 cellulose columns were greatly enhanced. The pepsinogen fraction from the second DE-11 cellulose column was redialyzed and applied to a DE-52 cellulose column. Two protein peaks were resolved (Figure 2a). The smaller peak corresponds to pepsinogen C, whereas the bulk of the potential proteolytic

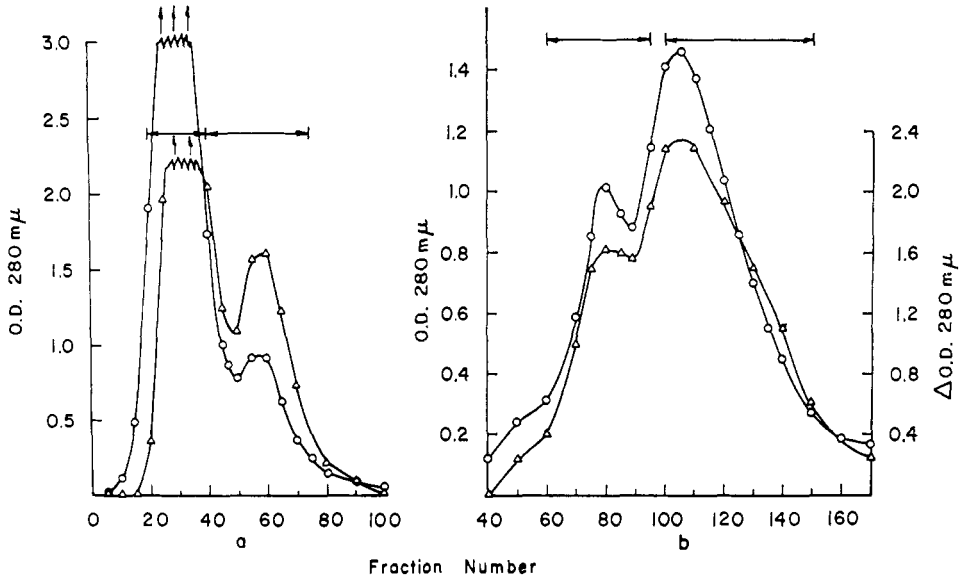

FIGURE 2: Separation of pepsinogens. (a) Separation of pepsinogen C from main pepsinogen fraction with a DE-52 cellulose column (8 × 3 cm) and gradient of 2.0 l., 0.02 M phosphate buffer, pH 6.9, to 2.0 l., 0.02 M phosphate buffer–0.6 M NaCl, pH 6.9; 15-ml fractions were collected. Symbols are the same as in Figure 1. (b) Separation of pepsinogens D and A; DE-52 cellulose column (8 × 3 cm), eluted with gradient of 1.0 l., 0.02 M phosphate buffer, pH 6.9, to 1.0 l., 0.02 M phosphate buffer–0.45 M NaCl, pH 6.9; 10-ml fractions were collected.

activity corresponds to pepsinogens A and D. Acrylamide gel electrophoresis of individual samples of pepsinogen C was necessary prior to the pooling of that zymogen since substantial amounts of pepsinogen A were present in the tubes from the ascending portion of the pepsinogen C peak. To separate pepsinogens D and A, two additional DE-52 column chromatographic steps were necessary; in each step the slope of the salt gradient was progressively lower (Figure 2b). Each pepsinogen (A, D, and C) was rechromatographed on DE-52 cellulose and superimposition of the graphs of protein concentration and potential proteolytic activities was observed. Acrylamide gel electrophoresis of the zymogens at this point revealed single bands for pepsinogens A and C and a minor contaminating band with pepsinogen D.

Each of the three pepsinogens was concentrated, layered on a Sephadex G-100 column (100 × 1.2 cm), and eluted with 0.02 M phosphate–0.15 M NaCl, pH 6.9 buffer. Each pepsinogen was eluted as a single sharp peak; however, a small amount of nonproteolytic material emerged before pepsinogen D. Potential proteolytic activity in each peak paralleled protein concentration, and acrylamide gel analysis revealed only a single band for each pepsinogen.

The pepsins were generated by incubating the purified pepsinogens at a concentration of 1 mg/ml at 37°, pH 2.0, for 4 min; these conditions resulted in maximal activation, as judged by the milk-clot assay. The activation mixtures were dialyzed against 0.1 N acetate buffer, pH 4.0, for 12 hr, at 0° in order to remove any peptide fragments. They were then dialyzed against 0.02 M phosphate buffer, pH 6.9, for 8 hr, at 0°, prior to Sephadex column chromatography. Again, single peaks of elution, similar to those obtained with their respective pepsinogens, were observed. The pepsins could be eluted in 0.1 M Tris (pH 7.5) or 0.02 M phosphate (pH 6.9) buffer without loss of specific enzymatic activity.

Properties of the Purified Protein. The relative electrophoretic mobilities of the three pepsinogens and pepsins in 7% polyacrylamide gels at pH 8.5 are shown in Figure 3. Pepsinogen C moves directly behind the bromophenol blue dye marker and is the most negatively charged of the three pepsinogens. Although pepsinogens A and D appear to migrate to identical positions when they are electrophoresed singly, they can, however, be distinguished by the slightly faster migration of A when they are electrophoresed together.

A diagrammatic representation of the mobilities of the pepsins under the same conditions as those of the pepsinogens in 7% gels is also given in Figure 3. Multiple bands are shown. (Similar patterns were obtained when the pepsins were electrophoresed in a barbiturate buffer system at pH 7.0.) The presence and relative positions of the bands in each of the pepsins could not be altered by either preelectrophoresis of the gels or varying the final ammonium persulfate concentra-

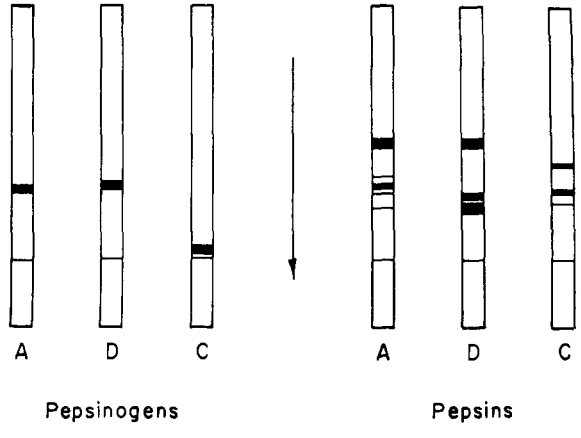

FIGURE 3: Schematic representation of polyacrylamide gel electrophoresis of the pepsinogens and pepsins in Tris-HCl buffer system, pH 8.5. Arrow indicates direction of electrophoresis toward anode; bottom line in each case represents dye front.

TABLE I: Amino Acid Compositions of Chicken Pepsinogens and Pepsins.[a]

	Pepsinogens			Pepsins		
	A	D	C	A	D	C
Lys	17	15	5	11	10	3
His	7	7	3	5	5	1
Arg	7	7	3	6	6	2
Asp	44	44	41	44	44	35
Thr	28	28	33	31	32	32
Ser	39	40	40	44	46	39
Glu	32	29	48	31	29	43
Pro	19	20	22	19	19	17
Gly	33	34	40	35	34	39
Ala	21	20	22	20	19	18
$^1/_2$-Cys[b]	(6)	(6)	(6)	(6)	(6)	(6)
Val	27	28	22	27	28	21
Met	10	10	9	10	10	10
Ile	24	24	24	25	26	23
Leu	30	29	30	29	29	28
Tyr	24	27	24	24	24	20
Phe	25	22	27	23	22	24

[a] We have chosen to base the amino acid compositions on the molecular weight obtained by ultracentrifugal analysis, *i.e.*, 42,000 for pepsinogens A, D, and C, pepsins A and D; 38,500 for pepsin C, realizing that additional data have to be compiled and incorporated into the final analyses. We have not yet determined the contents of tryptophan, amide, carbohydrate, and phosphorus in these proteins. Since approximately fifteen amino acid residues are lost during the conversion of pepsinogens A and D, this change in molecular weight must also be included in the final analyses of pepsins A and D. All amino acid values listed are based on analyses of triplicate hydrolyses (24 hr) except for isoleucine and valine (48 hr) and threonine and serine (values of 24- and 48-hr determinations were extrapolated to zero time). The discrepancies between the serine and threonine contents in pepsins A and D (higher than contents of these residues in their respective precursors) may be due to the corrections applied in each case. [b] Tryptophan and cysteic acid determinations have been performed recently by Dr. Eleanor B. McGowan in this laboratory. Tryptophan determinations were done in 5.6 M Gd·HCl, pH 7.5, according to the method of Edelhoch (1967). Cysteic acid was determined after performic acid oxidation and amino acid analysis as described by Hirs (1956). Reactive sulfhydryl groups were determined by the method of Ellman (1959) in the presence of 5.6 M Gd·HCl or 0.1% sodium dodecyl sulfate, pH 7.5. Pepsinogen A and pepsin A each contain 5 moles of tryptophan and 7 moles of cysteic acid per mole of protein. With Ellman's reagent, 0.89 ± 0.03 mole of sulfhydryl group is reactive per mole of each protein in the presence of either denaturant. These values confirm those reported by Bohak (1969). Pepsinogen C and pepsin C each contain 5 moles of tryptophan and 6 moles of cysteic acid per mole of protein. No free sulfhydryl groups were detected by reaction with Ellman's reagent.

tion. These same bands, which were specific for each pepsin, were reproducible from one pepsin preparation to another and were present in samples from both the ascending and descending portions of an elution peak from Sephadex gel filtration. If the pepsins were treated with sodium dodecyl sulfate, however, and their mobilities examined in gels with 0.1% sodium dodecyl sulfate–0.1 M phosphate, pH 7.0, only one band for each pepsin was observed. Under these conditions, pepsins A and D moved identically, and pepsin C slightly faster. The pepsins appeared homogeneous when analyzed by techniques that distinguish differences in size, *i.e.*, electrophoresis in sodium dodecyl sulfate, molecular-sieve chromatography, and ultracentrifugation. The heterogeneity in the pepsins (Figure 3) cannot be explained simply by differences in charge, since immunoelectrophoresis of the immune systems of pepsins A and D, respectively, reveals only single precipitation arcs (Donta and Van Vunakis, 1970a). Other techniques, *e.g.*, end-group analyses, still need to be used to determine whether the multiple bands on acrylamide electrophoresis represent artifacts (Cann and Good, 1964), the products of slight autodigestion, or subtle structural differences in a population of pepsin molecules generated from a purified precursor.

The amino acid analyses of the three pepsinogens and their pepsins are shown in Table I. In preparing the samples for amino acid analysis, it was noted that the proenzymes could be activated to their pepsins after exhaustive dialysis against deionized water at 4°. This conversion (detected with the milk-clot assay and acrylamide gel electrophoresis) could be prevented by adding 0.001 N NaOH to the water to maintain a pH of 7.5 without affecting the subsequent hydrolysis by HCl or resolution of the amino acids by the analyzer. Pepsinogens that were activated in deionized water yielded pepsins that behaved on acrylamide electrophoresis in a manner similar to the purified pepsins that had been activated at acid pH and subjected to gel filtration chromatography.

Determinations of molecular weight by high-speed sedimentation to equilibrium for the pepsinogens and pepsins were performed in both 0.02 M phosphate–0.15 M NaCl (pH 6.9) and 0.1 M Tris (pH 7.5) buffers except for pepsinogen C and pepsin C, which were sedimented only in the phosphate buffer. No significant differences were observed between results obtained in the two buffer systems. Typical straight line plots of log concentration *vs.* distance from the center of rotation were obtained, for the zymogens and their pepsins reflecting homogeneity of the individual proteins. The averages of several least-squares-average molecular weight determinations for each of the proteins at the various concentrations that were studied are listed in Table II. Except for pepsin C, with values averaging 38,500, the average molecular weights of the three pepsinogens, as well as pepsins A and D, are approximately 42,000. A tendency to aggregate was noticed with pepsins A and D, if determinations were performed on preparations that had been refrigerated in buffer for more than 3 days.

Samples of pepsin A and pepsinogen A placed on the same Sephadex G-100 column emerged in identical volumes (Figure 4). If one assumes no effect of variables such as shape or charge (Whitaker, 1963), the difference in molecular weight between pepsinogen and pepsin would be no greater than 1500.

The comparative specific activities of swine pepsin and chick pepsins D, A, and C toward hemoglobin are 1.0, 3.4,

TABLE II: Molecular Weights of the Pepsinogens and Pepsins.[a]

Pepsinogen	Concentration (%)	Mol Wt	Pepsin	Concentration (%)	Mol Wt
A	0.11	42,800	A	0.08	43,800
	0.06	41,700		0.06	43,750
	0.03	40,300		0.03	42,850
D	0.10	42,400	D	0.11	46,750
	0.08	43,250		0.07	41,200
	0.06	40,350		0.04	41,450
C	0.09	46,900	C	0.08	39,050
	0.05	41,850		0.06	38,750
	0.03	42,500		0.03	37,250

[a] Least-square averages-molecular weights of the chick pepsinogens and pepsins determined by high-speed sedimentation to equilibrium at 31,410 rpm and 5° in either 0.1 M Tris, pH 7.5, or 0.02 M phosphate–0.15 M NaCl, pH 6.9.

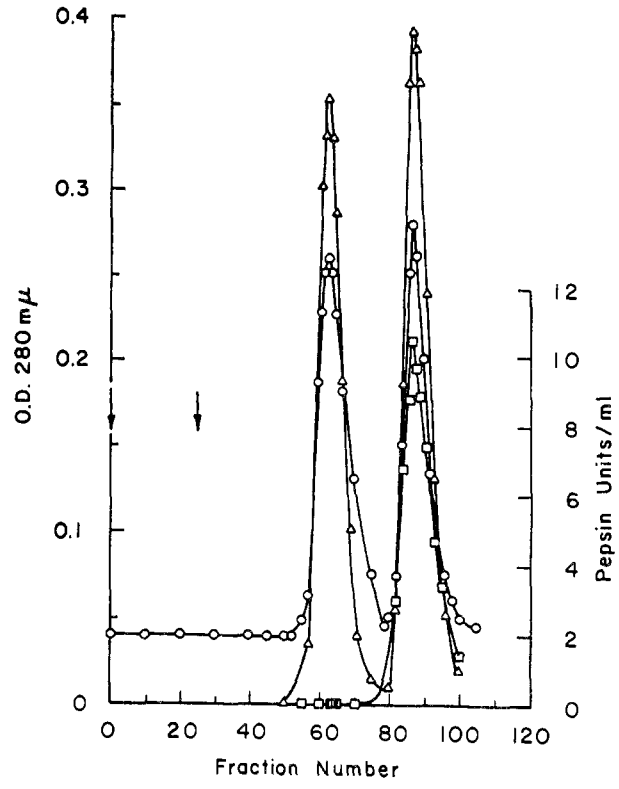

FIGURE 4: Elution of chick pepsinogen A and pepsin A from a Sephadex G-100 column (110 × 1.2 cm) equilibrated with 0.1 M Tris, pH 7.5. Pepsinogen A was placed on column at first arrow; pepsin A was placed on column at point indicated by second arrow. Constant flow rate was maintained throughout and 2.2-ml fractions were collected: (○) $OD_{280\ m\mu}$; (△) proteolytic activity using hemoglobin at pH 2.0 as substrate; (□) proteolytic activity using milk-clot test at pH 5.4 as substrate, expressed as pepsin units per milliliter.

2.3, and 1.7, respectively, and 1.0, 0.29, 0.33, and 0.18 toward milk. The greater reactivity of swine pepsin, as compared with chick pepsin in the milk-clot assay, has already been noted (Levchuk and Orekhovich, 1963). Even at concentrations of 1 mg/ml and incubation times of 1 hr, the chick pepsins A, C, and D did not hydrolyze Cbz-L-Glu-L-Tyr, either at pH 2 or pH 4, the latter being the pH optimum of the chick peptidase (pepsinogen B) (Donta and Van Vunakis, 1970b). Conversely, on exposure to acid pH, the protein corresponding to pepsinogen B showed no enzymatic activity in the hemoglobin or milk-clot assays, even at concentrations (100-fold) and incubation times (10-fold) greater than those used for the other pepsins.

The stabilities of the zymogens and enzymes on exposure to different temperatures and hydrogen ion concentrations were determined with the use of various buffers. The bicarbonate–carbonate buffer systems proved to be the most useful in the pH range where the clearest differences could be demonstrated. At pH 9.5 and at 37°, the inactivation process for the pepsinogens followed first-order kinetics. One-half of the potential enzymatic activity of pepsinogens A and D was lost at 27 min, compared with less than 1 min for pepsinogen C. The similarity of pepsinogens A and D was further demonstrated by their thermal denaturation curves, assayed by immunological methods (Donta and Van Vunakis, 1970b). At pH 8.4 and at 30°, one-half of the enzymatic activity of either pepsin A or D was lost in 4.6–4.7 mins, whereas less than 0.5 min was sufficient, under identical conditions, to inactivate one-half of the activity of pepsin C. All three pepsins are stable for many months at neutral pH in the cold.

Purified pepsinogen samples stored in 0.03–0.1 M Tris, pH 7.5, either refrigerated or frozen, are stable for several weeks, but pepsinogens A, D, and C are activated to pepsins on freezing in 0.02 M sodium phosphate buffer, pH 6.95, as measured by the milk-clot test. Although each pepsinogen migrates as a single band on acrylamide gel electrophoresis prior to freezing, multiple bands with a pattern identical with that observed for pepsins that had been activated at pH 2.0 and passed through Sephadex (Figure 3) were observed after thawing. Several other experiments were carried out to test the stability of pepsinogens on storage under various conditions, *e.g.*, freezing or refrigeration in phosphate buffers, with or without NaCl, or Tris buffers at the same pH and ionic strength. Our findings suggest that the activation of pepsinogen may occur because of a decrease in pH which occurs upon freezing sodium phosphate buffers, with an even further lowering of pH when NaCl is present (van den Berg, 1959).

Discussion

In the chicken, three pepsinogens have been found that correspond to pepsinogens A, D, and C of the swine and dogfish systems and are active on protein substrates (Ryle, 1965; Merrett *et al.*, 1969). In several pooled chicken stomach extracts, the relative concentrations of pepsinogens A, D, and C have been found to be approximately 3:2:1. Their order of elution from the DEAE ion-exchange resin is pepsinogen D, A, and C, with pepsinogens D and A being closely associated and separable only by the new microgranular DE-52 ion-exchange cellulose. There is a good correlation between this pattern of elution and the mobilities of the pepsinogens on acrylamide gel electrophoresis; pepsinogen C migrates fastest

toward the anode, followed by pepsinogens A and D, whose mobilities are very similar. The amino acid compositions of the pepsinogens provide a basis for understanding these patterns of ion-exchange elution and electrophoretic mobilities, and further confirm the similarities of pepsinogens A and D. Whereas the number of acidic residues for pepsinogens A and D is 45 and 44, respectively, pepsinogen C contains 78 such residues. Pepsinogen C also has fewer basic residues, and its ratio of aspartic acid to glutamic acid is reversed. A comparison of the rate of loss of potential enzymatic activities with increasing temperature or pH shows that pepsinogens A and D behave identically and are more stable than pepsinogen C. Pepsinogens A and D of the swine and dogfish are also similar. Swine pepsinogen D is thought to represent a dephosphorylated form of A (Lee and Ryle, 1967), but it has not yet been determined whether pepsinogens D from the dogfish and chicken represent dephosphorylated forms of pepsinogen A.

Sugar residues have been found in swine (Neumann *et al.*, 1969) and chicken pepsinogen (Bohak, 1969). (We have not yet looked for carbohydrates in our preparations.) Thus, another possible explanation for the differences that do exist between pepsinogens A and D might be the presence of carbohydrate. With RNase A and B, for example, chromatographic separation was due to differences in carbohydrate content, although amino acid compositions and immunological properties were almost identical (Plummer and Hirs, 1964).

Still another possibility must be considered; the separation of pepsinogens A and D may be an artifact of the preparative procedure (*e.g.*, deamidation). We cannot exclude this possibility at present, but it would imply that similar changes occur with different preparative schemes in three different species. We have no evidence that pepsinogen D is generated from purified pepsinogen A (or *vice versa*) with prolonged handling.

Herriott *et al.* (1938) prepared chicken pepsinogen (before the availability of chromatographic procedures) by the fractionation of an extract of stomach mucosae with $(NH_4)_2SO_4$ and found that the pepsin(s) that was derived was stable at neutral and alkaline pH. Levchuk and Orekhovich (1963) isolated three pepsinogens from chicken mucosae by chromatography on DEAE-cellulose at pH 5.6, and also noticed the greater stability of chick pepsins as compared to swine. Although they did not report molecular weights, amino acid compositions, and relationships of pepsinogens among each other, it appears likely that they were dealing with a fraction composed of pepsinogens A and D, a fraction composed of pepsinogen C, and a pepsin fraction, since their third peak had proteolytic activity prior to activation. Bohak (1969) has isolated a chicken pepsinogen and the pepsin derived by activating this precursor. Although chromatography of his crude chicken pepsinogen preparations yielded at least two fractions containing potential peptic activity, he concluded that the peaks contained the same zymogen that had been contaminated to a varying degree with impurities which tend to cochromatograph with it. Our data do not support this conclusion. Although the possibility that the differences between A and D result from the preparative procedure has not been definitely excluded, pepsinogen C exists and is a protein distinct from pepsinogens A and D. Based on comparative amino acid analyses, we have concluded that the pepsinogen isolated by Bohak (1969) probably corresponds to pepsinogen A or to a pepsinogen A–D fraction.

When chicken pepsinogens A and D were activated to yield their respective pepsins, enzymes were obtained that had a greater stability than swine pepsin at neutral or higher pH. They were, in fact, isolated by molecular sieve chromatography at neutral pH and were stored at pH 7.0 for months without losing their activities. The molecular weights of pepsins A and D were indistinguishable from those of their precursors. To assure the most valid comparisons of molecular weights, sedimentation of the pepsins and pepsinogens was performed simultaneously under the same conditions in a six-channel centerpiece. Under the same experimental conditions, a difference was found between the dogfish pepsinogen A and pepsin A (42,000 and 35,000, respectively) (Merrett *et al.*, 1969).

The molecular weight of the pepsin that was isolated by Bohak (1969) is stated to be about 35,000, a value that is considerably less than the one we obtained for pepsin A or D. Our pepsin A or D preparations have approximately twice the specific activity toward hemoglobin (using swine pepsin as a standard). Barring inherent differences in the strains of chicken used, the discrepancies in the molecular weights may be due to differences in the isolation and activation procedures or to the methods employed for the determinations of molecular weights. We have already stressed that caution must be exercised in handling pepsinogens of different species if the pepsins that are generated are stable at neutral pH and/or the autocatalytic conversion of the precursor can take place at elevated pH (Merrett *et al.*, 1969). The chicken pepsinogen systems require frequent monitoring (by the milk-clot test), since the pepsinogens can be activated (and autolyzed) during storage.

There is ample documentation that about 40 amino acid residues are released during the activation of swine pepsinogen to pepsin (Rajagopalan *et al.*, 1966; Ong and Perlmann, 1968). During activation of chicken pepsinogens A and D (not C), about 15 amino acids were released. The released residues include five or six lysines, two histidines, and one arginine. Although swine (Rajagopalan *et al.*, 1966; Arnon and Perlmann, 1963) and human pepsin (Tang *et al.*, 1967) contain a total of four basic residues, chick pepsins A and D have 21 or 22 such residues. The disparity in amino acid composition between a highly basic N-terminal peptide portion and an acidic pepsin moiety, which exists in the swine pepsinogen system, is not present here. Chicken pepsins A and D possess more basic residues than swine pepsinogen. This greater number of basic residues may contribute to the increased stability of these chick pepsins at neutral and basic pH as compared with swine pepsin.

The fragment that is removed on activation of pepsinogen C is larger (the molecular weight of pepsin C was found to be 38,500) and would not be expected to contain more than five basic residues in the approximately 38 residues split from the precursor. Chick pepsin C, which is less stable than the other two chick pepsins at elevated pH, contains only six of the eleven basic residues originally present in the precursor.

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