Immunochemical Relationships of Chicken Pepsinogens and Pepsins*

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ABSTRACT: Antisera, prepared by immunizing rabbits with chicken pepsinogens A, D, and C, were characterized by gel diffusion, immunoelectrophoresis, and complement fixation.

By immunochemical analyses, pepsinogens A and D are similar and readily distinguishable from pepsinogen C. Pepsins A and D are similar to each other and show a great resemblance to their precursors. Pepsin C also cross-reacts with antibodies to its precursor, indicating that it possesses structural areas in common with pepsinogen C. Pepsin C (but not pepsinogen C from which it was derived) can inhibit the pepsinogen A(D)–antipepsinogen A(D) and pepsin A(D)–antipepsinogen A(D) cross-reacting immune systems; thus the antigenic structure(s) common to these proteins are either masked in pepsinogen C or are created as a result of a conformational change during the conversion process. The thermal denaturation profiles (determined by serological and enzymatic assays) are similar for pepsinogens A and D and different for pepsinogen C. Pepsins A, D, and C react similarly on heating at neutral pH.

Three pepsinogens, A, D, and C, have been isolated from the gastric mucosas of chickens. Pepsinogens A and D are closely related in many of their properties; *e.g.*, amino acid compositions, stabilities, and electrophoretic mobilities. Chicken pepsins A and D resemble their precursors to a much greater degree than do the pepsins of other species, *i.e.*, their molecular weights and amino acid compositions indicate that they contain only 10–15 fewer residues than their precursors. All three pepsins are also unique in their increased stabilities at neutral or alkaline pH when compared with pepsins isolated from other species (Donta and Van Vunakis, 1970). The preparation of chicken pepsinogens and pepsins has been reported in other laboratories (Herriott *et al.*, 1938; Levchuk and Orekhovich, 1963; Bohak, 1969).

The relationships among the chicken pepsinogens and pepsins have been further investigated by immunochemical techniques. Single pepsinogen systems have been studied immunochemically (Van Vunakis *et al.*, 1963; Arnon and Perlmann, 1963; Schlamowitz *et al.*, 1963), and the serological relationships among the several pepsinogen systems have been investigated in the dogfish (Merrett *et al.*, 1970) and human systems (Rapp *et al.*, 1964; Kushner *et al.*, 1964).

Materials and Methods

Purification of the pepsinogens and pepsins and the methods used to assay for enzymic activity and potential enzymic activity have been described previously (Donta and Van Vunakis, 1970). Antisera were obtained from New Zealand Albino rabbits, immunized with 1–3 mg of purified pepsinogen as previously described (Merrett *et al.*, 1970). The immunochemical methods used were those of Levine (1967) for complement (C′) fixation, Ouchterlony (1949) for double diffusion in agar gels, and Grabar and Williams (1953) for immunoelectrophoresis.

Results

Precipitating antibodies, as measured by gel diffusion, were obtained with each of the pepsinogen antisera as early as the first bleeding, although the titers were higher for antipepsinogens A and D as compared with antipepsinogen C. On double diffusion in agar each antiserum gave only one precipitin line with either the pure homologous antigen or the crude stomach extract. The antibodies were also able to inhibit the enzymic activities of the pepsins derived from the homologous pepsinogens, *i.e.*, the ability to clot milk at pH 5.5 when incubated and assayed as described (Van Vunakis *et al.*, 1963). Under these conditions, 50% inhibition of peptic activity was obtained with 0.06 ml of antipepsinogen A, 0.06 ml of antipepsinogen D, and 0.25 ml of antipepsinogen C.

Gel diffusion patterns of the three pepsinogens and their pepsins with antisera directed toward each pepsinogen are shown in Figure 1. With either antipepsinogen A or D, lines of complete identity were observed with pepsinogens A and D, and with pepsins A and D. No reaction was observed between these four proteins and antipepsinogen C. While pepsinogen C failed to precipitate with antipepsinogen A or D, pepsin C (derived from the same preparation) consistently gave a weak band of precipitation with antipepsinogen A. Antipepsinogen C formed precipitin bands with only the homologous pepsinogen C and its pepsin, these two antigens forming a pattern of partial identity.

Since pepsinogens A, C, and D were purified from a pool of many stomachs (Donta and Van Vunakis, 1970), each of thirteen stomach extracts was examined for the presence of pepsinogen C and pepsinogen A (and/or D). Each individual stomach extract formed single bands of precipitation with both antipepsinogen A and antipepsinogen C.

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TABLE I: Inhibition of C′ Fixation in Pepsinogen and Pepsin Immune Systems.

Immune System	Inhibitor	μg of Protein Needed for 50% Inhibn
Pepsinogen A–anti-pepsinogen A (Ra 335C-2 1/2600)	Pepsin A	0.35
	Pepsin D	0.50
	Pepsin C	2.4
	Pepsinogen C	>10[a]
Pepsinogen D–anti-pepsinogen D (Ra 336C-2 1/2000)	Pepsin A	0.24
	Pepsin D	0.35
	Pepsin C	1.9
	Pepsinogen C	>10[b]
Pepsinogen C–anti-pepsinogen C (Ra 334C-2 1/700)	Pepsin C	0.30
	Pepsinogens A and D	No inhibition at 10-μg levels
	Pepsins A and D	
Pepsin A–anti-pepsinogen A (Ra 206C-4 1/4600)	Pepsin C	1.85
	Pepsinogen C	No inhibition at 10-μg levels
Pepsin D–antipep-sinogen A (Ra 206C-4 1/4600)	Pepsin C	1.95
	Pepsinogen C	No inhibition at 10-μg levels

[a] 20% inhibition observed with 10 μg. [b] 30% inhibition observed with 10 μg.

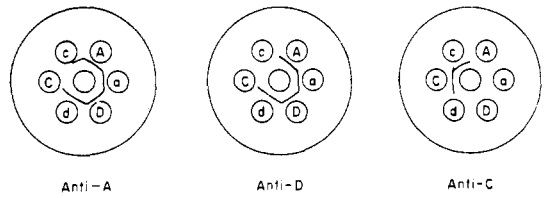

FIGURE 1: Double diffusion of antipepsinogens with pepsinogens (A, D, C) and pepsins (a, d, c) in agar gels, antibodies used undiluted (center wells), antigens all at 100 μg/ml.

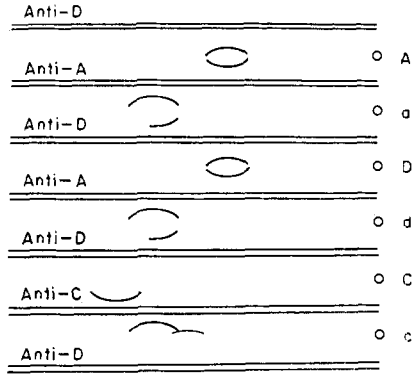

FIGURE 2: Immunoelectrophoresis of pepsinogens (A, D, C) and pepsins (a, d, c) in barbital buffer, pH 8.2. Antigen concentrations 1 mg/ml, antibodies (troughs) undiluted.

Upon immunoelectrophoresis at pH 8.2 (Figure 2), the three pepsins migrated faster toward the anode than pepsinogens A or D, while pepsinogen C moved well in front of the other five proteins. Purified pepsin C formed two bands of partial identity with antipepsinogen C. This heterogeneity of pepsin C was not observed with either pepsin A or D.

Complement fixation was carried out with the six proteins and the antisera to each of the precursors (Figure 3). There were no significant differences between the complement fixation properties of pepsinogens A and D with either antipepsinogen A or antipepsinogen D. Using the same dilutions of antisera, pepsins A and D also reacted with antipepsinogens A and D, but the maximum C′ fixation was decreased. When antipepsinogens A and D were used at higher concentrations (1/2100 and 1/1600, respectively), the peak of C′ fixation with pepsin A or D was increased to 60 and 40%, respectively. Purified pepsin C fixed up to 50% of the C′ with antipepsinogen A or antipepsinogen D sera of stronger titers (*e.g.*, 1/1200). However, compared with pepsinogens A and D or pepsins A and D, 10-fold more pepsin C was required to fix an equivalent amount of C′ in this cross-reaction. Pepsinogen C also reacted with antipepsinogens A and D under these experimental conditions, but about 50–100-fold greater concentrations were required to give 50% fixation. Interpretation of this latter cross-reaction with pepsinogen C is difficult since contamination with as little as 1% of pepsin A or D could account for the observed results. Pepsinogens A and D and their pepsins did not react with antipepsinogen C (Figure 3) even at antigen concentrations 40-fold greater than that of the homologous antigen or with twice the antiserum concentration used to obtain C′ fixation with the homologous system. C′ fixation of pepsin C with antipepsinogen C did occur but, again, the concentration of pepsin C required for this C′ fixation was increased and a complete curve could not be obtained.

Gel diffusion and direct C′ fixation experiments show that pepsins A, D, and C, but not pepsinogen C, are serologically related to the proenzymes, pepsinogens A and D. The data from the inhibition experiments (Figure 4, Table I) show these relationships more directly. By use of concentrations of antisera in which the direct cross-reactions with the homologous pepsins were kept at a minimum, the homologous pepsinogen A, pepsinogen D, and pepsinogen C immune systems were inhibited by the three pepsins and by pepsinogens A and D as well for the pepsinogen C immune system. Pepsins A and D are both effective inhibitors of the pepsinogen A (D) immune system (Figure 4). Pepsin C also inhibits both of these systems at an 8–10-fold higher concentration, whereas more than a 100-fold greater concentration of pepsinogen C is required for a weak inhibition. It cannot be ruled out that this observed inhibition by pepsinogen C is due to a 1–2% contamination with pepsin C. On the other hand, the pepsinogen C immune system is inhibited by pepsin C only, not by pepsin A or D or their precursors, at least at the levels tested. The amount of inhibitor which causes 50% inhibition with five different immune systems is shown in Table I.

Pepsinogens A, D, and C and pepsins A, D, and C were

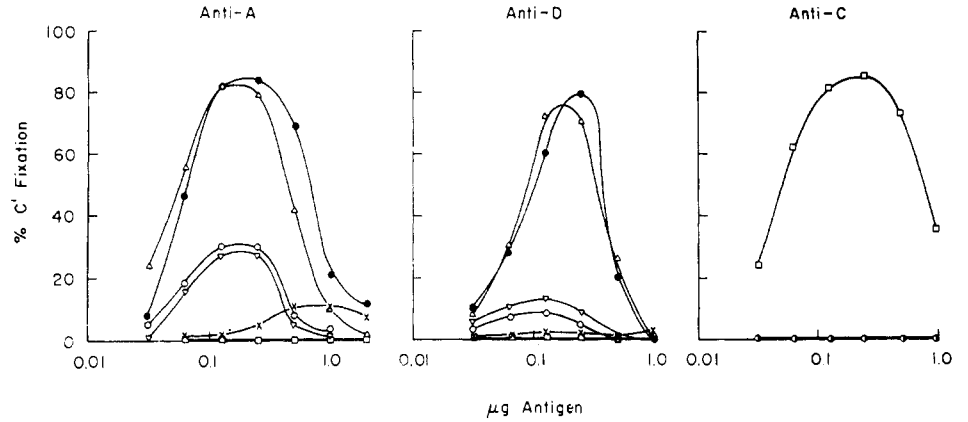

FIGURE 3: Complement fixation of the three antipepsinogens with the pepsinogens and pepsins. (●) Pepsinogen A, (○) pepsin A, (△) pepsinogen D, (▽) pepsin D, (□) pepsinogen C, (×) pepsin C, (◑) pepsinogens A and D and pepsins A, D, and C, antipepsinogen A (Ra 335 C-2), $^{1}/_{2700}$ dilution, antipepsinogen D (Ra 336C-2), $^{1}/_{2100}$ dilution, and antipepsinogen C (Ra 334C-2), $^{1}/_{700}$ dilution.

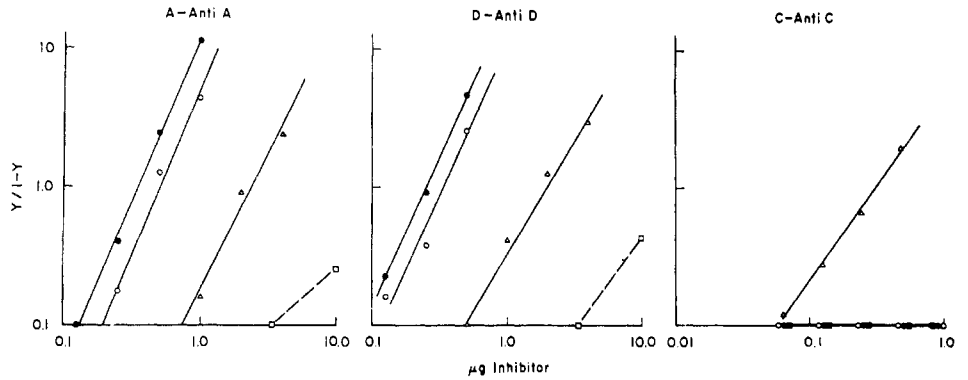

FIGURE 4: Inhibition of complement fixation of the homologous pepsinogen–antipepsinogen systems by the heterologous proteins. Homologous pepsinogen concentrations 0.1 μg/ml, antipepsinogens A, D, and C at $^{1}/_{2600}$, $^{1}/_{2000}$, and $^{1}/_{700}$ dilutions, respectively. Per cent inhibition is expressed as $y/(1 - y)$ values and plotted logarithmically *vs.* inhibitor concentration. (●) Pepsin A, (○) pepsin D, (△) pepsin C, (□) pepsinogen C, and (■) pepsinogen A or D.

exposed to various temperatures and assayed for residual C′ fixing and enzymic activities using the same procedure employed for swine pepsinogens (Gerstein *et al.*, 1963). The thermal stabilities of the pepsinogens are shown in Figure 5 and the temperatures at which 50% of the serologic and catalytic activities are lost are presented in Table II.

Discussion

The immunological data support the idea that two main types of pepsinogen exist in the chick gastric mucosas: pepsinogen C, and the type represented by pepsinogens A and D. These two types of pepsinogen were detected in each of 13 individual stomachs tested. Pepsinogen C differs from the other two pepsinogens in its amino acid composition, its decreased stability on exposure to alkali, and in its electrophoretic mobility (Donta and Van Vunakis, 1970). Almost identical immunological properties have been found for pepsinogens A and D. Their reactions to the heterologous antibody systems and their rates of thermal denaturation (assessed by loss of potential enzymic activity and changes in serological properties) are similar. Their mobilities on immunoelectrophoresis cannot be differentiated. Factors which may be responsible for the slight differences observed on acrylamide electrophoresis, in amino acid compositions, and chromatographic behavior have already been considered (Donta and Van Vunakis, 1970).

Pepsins A and D generated from the purified precursors are also similar to each other. Compared with pepsins of other species, chick pepsins A and D possess a greater similarity to their precursors and are more stable at neutral and alkaline pH's. They are indistinguishable from pepsinogen A and D on gel immuno diffusion. With the sensitive complement fixation technique, only small differences can be demonstrated, these being the decreased maximal fixation points for the pepsins as compared with that of their precursors.

Pepsin C resembles pepsins A and D in thermal stabilities, but, in contrast to the other two pepsins, shows only partial identity with its precursor in the serological assays. On conversion from the precursor, a larger peptide fragment is removed from pepsinogen C as compared with that in the pepsinogen A and D systems. The ability of pepsin C to inhibit the pepsinogen A (D)–antipepsinogen A (D) and pepsin A (D)–antipepsinogen A (D) systems indicates some similarity in tertiary structure of pepsin C to pepsinogens

TABLE II: Thermal Denaturation of Chicken Pepsinogens and Pepsins Assayed Immunologically and Enzymatically.[a]

T_m[b]	T_m[b]
Pepsinogen A, 60°	Pepsin A, 49–52°
Pepsinogen D, 60°	Pepsin D, 49–52°
Pepsinogen C, 53–54°	Pepsin C, 47°[c]

[a] All proteins heated at various temperatures for 10 min in 0.02 M phosphate–0.15 M NaCl (pH 6.9) at concentrations of 10 μg/ml, then diluted with Tris buffer for C′ fixation or aliquots taken for hemoglobin assay. [b] T_m = temperature at which one-half of the serological or enzymatic activity is lost. [c] Only from hemoglobin assay.

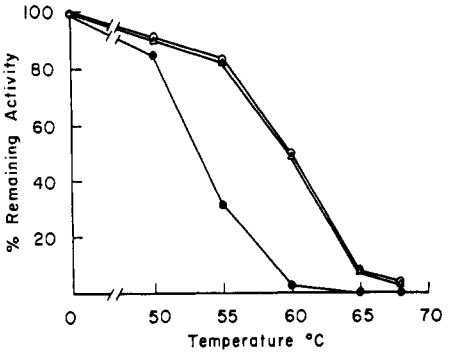

FIGURE 5: Thermal denaturation of the pepsinogens. Each pepsinogen heated for 10 min at various temperatures at concentration of 10 μg/ml in 0.02 M phosphate–0.15 M NaCl (pH 6.9) then diluted with Tris buffer for complement fixation, or assayed with 2% hemoglobin for potential proteolytic activity. (○) Pepsinogen A, (△) pepsinogen D, and (●) pepsinogen C.

A and D and pepsins A and D, and predicts that areas of homology must exist in the primary sequences of these proteins. This area of homology in sequence must also exist in pepsinogen C, but since this precursor (which served as the source of pepsin C) did not inhibit the pepsinogen A(D)–antipepsinogen A(D) and pepsin A(D)–antipepsinogen A (D) systems, the structural area which comprises the antigenic site is either masked in pepsinogen C or is created by a conformational change in the pepsin moiety during the conversion process. Since only pepsin C shows serological reactions with the pepsinogen C immune system, there must also be conformational areas unique to these two proteins which are not markedly altered during the conversion process.

Inhibition of the ability of chicken pepsin C to clot milk was found when this enzyme was incubated with antisera directed against chicken pepsinogen A or D prior to assay. Dogfish pepsin C also appears to have a structural area in common with dogfish pepsinogens A and D since the antiserum to dogfish pepsinogen A was found to neutralize the enzymic activity of dogfish pepsin C, but relatively high concentrations of antisera were required (Merrett *et al.*, 1970).

Previous studies by Gerstein *et al.* (1963) have shown that changes in the serological activities of swine pepsinogen and pepsin resulting from thermal treatment reflect conformational changes in the structure of these proteins just as optical rotatory dispersion and fluorescence can reflect alterations in secondary and tertiary structure. A comparison of the rates of thermal denaturation of the three pepsinogens adds further weight to the structural differences that must exist between pepsinogens A–D and pepsinogen C. The amino acid analyses show fewer basic residues present in pepsinogen C, as well as a reversal of the aspartic acid: glutamic acid ratios as compared with pepsinogens A and D (Donta and Van Vunakis, 1970). Physical–chemical studies similar to the ones carried out by Perlmann (1963, 1967) and Grizzuti and Perlmann (1969) on swine pepsinogen and pepsin are needed for the chicken proteins, but from our chemical and immunological results we would predict that the structures of pepsinogens A and D and pepsins A and D are similar. A greater structural difference should exist between pepsinogen C and pepsin C, since more of the precursor molecule is lost in the conversion and pepsin C appears less related to pepsinogen C in the immunochemical experiments. A comparison of the rates of migration of the proteins on immunoelectrophoresis shows that the most acidic precursor, *i.e.*, pepsinogen C, generates a pepsin(s) which is more basic than itself, while pepsinogens A and D generate pepsins which are more acidic than their respective precursors. The mobilities of pepsins A, D, and the main band of pepsin C are similar. These results are consistent with the differences which exist in the amino acid compositions of these proteins. Pepsins A and D contain fewer basic residues than their precursors and have essentially the same number of acidic residues. The fragment which is removed during the conversion of pepsinogen C, however, contains a predominance of acidic over basic residues rendering pepsin C more positively charged than its precursor.

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Structural and Functional Factors in the Lyotropic Activity of Amides and Alkyl-Substituted Amides on Acid-Soluble Collagen*

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ABSTRACT: A systematic study of the lyotropic activity of formamide, urea, and alkyl-substituted derivatives on acid-soluble calfskin collagen has been undertaken. These perturbants reduced the rate of mutarotation recovery following heat denaturation and cooling and lowered the transition temperature of the native protein. Activity increased with perturbant concentration, functional group content, and number of potential donor hydrogen atoms, and with increasing linear hydrocarbon chain structure in the molecule. As in the case of polar organic solvents previously examined, these factors are all considered to promote perturbant hydrogen bonding, the hydrocarbon structure serving to exert local "hydrophobic shielding" which stabilizes polar interaction at an adjacent functional group. Further evidence for a largely nonspecific mechanism of perturbant action, consistent with hydrogen-bonding interactions, is apparent from general conformity of the data from the present and preceeding solvent studies with Flory–Weaver reversion kinetics. Thus, renaturation rates were largely determined by the degree of "undercooling" irrespective of the particular perturbant present, although perturbants with hydrocarbon structure deviated progressively from the linear trend found for formamide and urea. The present data support the previous proposal that a direct relationship exists between lyotropic activity and perturbant hydrogen bonding to collagen peptide bonds which, in terms of current structural concepts, results in destabilization due to increased rotational freedom in main-chain bonds and competitive disruption of internal structural hydrogen bonds between peptide links.

In recent studies, we have adopted an approach to the elucidation of lyotropic mechanism in which the effects of related perturbants on a standard collagen–buffer system have been compared systematically in order to correlate activity with perturbant structural and functional factors. (Russell and Cooper, 1969a,b). Compared with globular proteins, the rodlike collagen molecule provides an interesting reference system since structural features are fairly regular, resulting in comparatively isotropic behavior over the extended molecular length and a narrow melting range approximating a single-phase transition. Stabilization of the native conformation appears to be largely due to cooperative interchain hydrogen bonding and chain rigidity conferred by the pyrrolidine residues and rotational restrictions at peptide links (reviewed by Ramachandran, 1967). Since side-chain structures are located externally, interpretation of perturbant effects on collagen is not complicated by considerations relating to the exposure of buried hydrophobic structures to the environment in denaturation. In spite of structural distinctions, however, the relative magnitude of lyotropic effects for a variety of perturbants on collagen is the same as for globular proteins (Von Hippel, 1967; Von Hippel and Schleich, 1969; Von Hippel and Wong, 1962, 1963, 1964, 1965) suggesting that a general interaction mechanism, independent of conformational and compositional details, is operative.

Examination of the effects of aliphatic alcohols, ethers, ketones, and nitriles on collagen renaturation kinetics and thermal stability (Russell and Cooper, 1969a) has shown that concentration, polarity, and hydrocarbon structure are the main factors influencing lyotropic activity. It was concluded that lyotropic activity could be directly related to perturbant hydrogen bonding capacity. The effect of hydrocarbon structure was reconciled with this predominantly polar interaction mechanism by proposing that the pendent hydrocarbon chain exerted local hydrophobic shielding which stabilized interaction at adjacent functional groups in the perturbant. Preliminary examination of urea

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