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PURIFICATION AND CHARACTERIZATION OF A PEPSINOGEN AND ITS PEPSIN FROM PROVENTRICULUS OF THE JAPANESE QUAIL

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Summary

A crude extract of the proventriculus of the Japanese quail gave at least five bands of peptic activity at pH 2.2 on polyacrylamide gel electrophoresis. The main component, constituting about 40% of the total acid protease activity, was purified to homogeneity by hydroxyapatite and DEAE-Sepharose column chromatographies. At below pH 4.0, the pepsinogen was converted to a pepsin, which had the same electrophoretic mobility as one of the five bands of peptic activity present in the crude extract. The molecular weights of the pepsinogen and the pepsin were 40 000 and 36 000, respectively. Quail pepsin was stable in alkali up to pH 8.5. The optimal pH of the pepsin on hemoglobin was pH 3.0. The pepsin had about half the milk-clotting activitiy of purified porcine pepsin, but the pepsinogen itself had no activity. The hydrolytic activity of quail pepsin on *N*-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine was about 1% of that of porcine pepsin. Among the various protease inhibitors tested, only pepstatin inhibited the proteolytic activity of the pepsin. The amino acid composition of quail pepsinogen was found to be rather similar to that of chick pepsinogen C, and these two pepsinogens possessed common antigenicity.

Introduction

The allantoic endoderm of the Japanese quail or chick embryo produced pepsinogen when cultivated with homo- or heterospecific mesenchyme of the proventriculus [1,2]. Recently, we found that chick pepsinogens undergo great changes in molecular species and activities during development before and after birth [3]. The Japanese quail showed similar developmental changes in pepsinogen (unpublished results).

Chick pepsinogen has been purified and characterized by several workers and shown to be heterogeneous [4—6]. Comparative studies on the pepsinogens of the Japanese quail and chick would be of great help in understanding of phylogeny of pepsinogen molecules, because the Japanese quail and chick are both members of the familiy Phasianidae. However, pepsinogens of the Japanese quail have not yet been characterized on a molecular basis.

The present paper reports a simple method for purification of the main pepsinogen of the Japanese quail and comparative biochemical and immunological studies on pepsinogens of the quail and chick.

Material and Methods

Materials. Proventriculi were removed from Japanese quails, *Coturnix coturnix japonica*, immediately after killing the birds.

Bovine hemoglobin (type I), crystallized porcine pepsin, and *N*-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Pepstatin, leupeptin, antipain, and chymostatin were obtained through the Resources Program under Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan. DEAE-Sepharose CL-6B was a product of Pharmacia Fine Chemicals, Uppsala, Sweden, and hydroxyapatite (Bio-Gel HTP) was from Bio-Rad Laboratories, Richmond, CA, U.S.A. Other reagents were standard analytical grade products.

Enzyme assay. Protease activity was determined at pH 2.2 by the method of Anson [7] with the slight modification of Furihata et al. [8]. One unit of enzyme activity was defined as the amount causing an increase in absorbance at 280 nm of 1.0 per min. Hydrolytic activity against *N*-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine was measured by the method of Tang [9]. Milk-clotting activity was assayed by the method of Seijffers et al. [10]. Protein was determined by the method of Lowry et al. [11] with bovine serum albumin as standard.

Polyacrylamide gel electrophoresis. Potential peptic activity separated on 7.5% polyacrylamide gel in 0.05 M Tris/acetate buffer (pH 8.2) was located by the method of Samloff and Townes [12]. Protein in the gel was stained with 1% Amido black 10B. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed by the method of Laemmli [13] and protein was located with 0.2% Coomassie brilliant blue R-250. Molecular weight was determined by SDS-polyacrylamide gel electrophoresis. Sperm whale myoglobin, chymotrypsinogen, ovalbumin and bovine serum albumin were used as standard markers.

Amino acid analysis. Amino acids were analyzed in a Durrum amino acid analyzer Model D-500 (Palo Alto, CA, U.S.A.). Before analysis protein was hydrolyzed in 6 N hydrochloric acid at 110°C for 20 h. For determination of cysteine and methionine, samples were first oxidized with performic acid [14]. For determination of tryptophan, hydrolysis was achieved with 6 N hydrochloric acid containing 4% thioglycolic acid [15]. The yield of tryptophan from egg lysozyme was used as a reference. The amount of serine was obtained by extrapolation to zero time of hydrolysis.

Immunological tests. Rabbits were immunized with purified quail or chick

(see Results) pepsinogen. The cross-reactivity of quail pepsinogen with chick pepsinogen C, and vice versa, was tested by the double-diffusion method [16].

Results

Polyacrylamide gel electrophoresis of crude extract

Proventriculi were homogenized in a polytron homogenizer with 9 vols. of ice-cold 0.01 M sodium phosphate buffer (pH 7.0) containing 50 μg/ml each of antipain and leupeptin and then the homogenate was centrifuged at 105 000 × *g* for 60 min. More than 97% of the total proteolytic activity was recovered in the supernatant and polyacrylamide gel electrophoresis of this supernatant (Fig. 1a) gave at least five distinct bands of proteolytic activity at pH 2.2 (bands I—V). Band I disappeared almost completely when the supernatant was adjusted to pH 9.5 before electrophoresis. On the other hand, when the supernatant was incubated at pH 2.2, all the pepsinogens were activated and band I increased, band II became very faint, and three other bands (bands III—V) changed in electrophoretic mobility.

Purification of the main pepsinogen from the crude extract

A typical purification is summarized in Table I. The supernatant obtained by centrifugation at 105 000 × *g* as described above was applied to a column (3 × 6 cm) of hydroxyapatite equilibrated with 0.01 M sodium phosphate buffer. When the column was washed with the same buffer, about 60% of total peptic activity passed through the column (fraction 1), and the remainder was eluted with 0.2 M sodium phosphate buffer (pH 7.0) (fraction 2). A zymogram after polyacrylamide gel electrophoresis showed that fraction 1 contained bands I and II. For separation of the two enzymes, fraction 1 was applied to a DEAE-

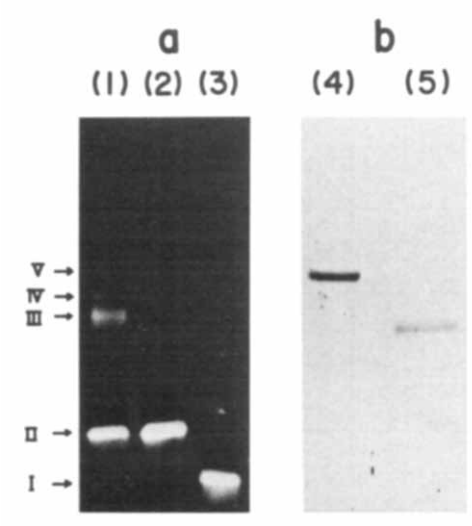


Fig. 1. Zymograms on polyacrylamide gel electrophoresis and SDS gel electrophoresis. (a) Zymogram, enzyme activities were located as described in the text. 1, Crude extract; 2, purified pepsinogen; 3, purified pepsin. (b) SDS-polyacrylamide gel electrophoresis. Protein was stained with Coomassie brilliant blue. 4, purified pepsinogen; 5, purified pepsin.

TABLE I

PURIFICATION OF JAPANESE QUAIL PEPSINOGEN

20 proventriculi were used as starting material. HTP, hydroxyapatite column.

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Recovery (%)
105 000 × *g* supernatant	2028	397	5.1	100
HTP				
Fraction 1	1230	42	29.4	60.7
Fraction 2	733	117	6.3	36.1
DEAE-Sepharose CL-6B fraction 1	797	19	41.2	39.3

Sepharose CL-6B column (1.5 × 28 cm) equilibrated with 0.01 M sodium phosphate buffer (pH 7.0). The column was washed with the same buffer and then material was eluted with a linear gradient of 0.25—0.4 M NaCl in the same buffer. Two clearly separated peaks of potential peptic activity were obtained (Fig. 2). Polyacrylamide gel electrophoresis showed that the first peak of pepsinogen corresponded to band II and the second, usually a small peak, to band I. The material in the first peak gave a single band of protein and activity on polyacrylamide gel electrophoresis (Fig. 1b).

Activation of pepsinogen to pepsin and purification of pepsin

The activation of the purified quail pepsinogen was examined by keeping the preparation in buffers of the various pH values at 4°C. At below pH 4.0, pepsinogen was rapidly activated to a pepsin, whereas even after 16 h treatment at pH 5.0 it remained inactive. Purified pepsinogen was activated by incubation at pH 2.2 for 60 min at room temperature, and then mixed with 4 vols. of 0.02 M sodium phosphate buffer (pH 6.0) and applied to a DEAE-Sepharose

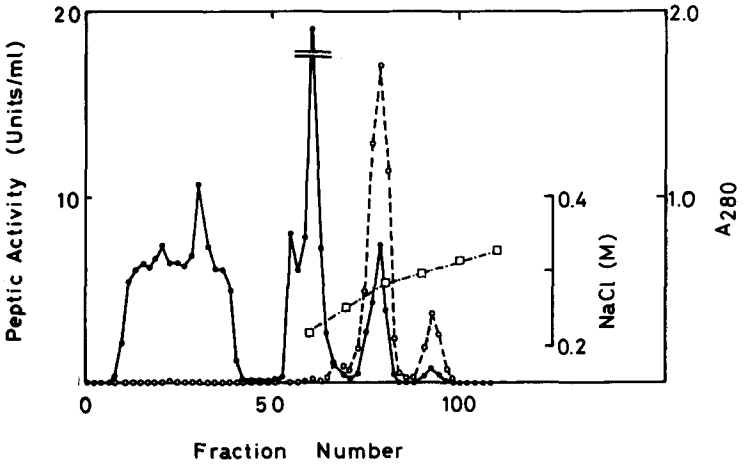


Fig. 2 Chromatogram on a DEAE-Sepharose CL-6B column of fraction 1 from hydroxyapatite. Details of the chromatographic procedure are described in the text. ○- - - - -○, proteolytic acitivity; ●—— —●, absorbance at 280 nm, and □— · — · —□, molarity of NaCl.

CL-6B column. Pepsin was eluted with a linear gradient of 0—0.4 M NaCl in the same buffer. The purified pepsin gave a single band of activity coinciding with band I (Fig. 1a), and a single band on SDS-polyacrylamide gel electrophoresis (Fig. 1b). The pepsin was characterized further using this purified pepsin preparation.

Molecular weight

The molecular weights of the quail pepsinogen and pepsin derived from it were determined by SDS-polyacrylamide gel electrophoresis as 40 000 and 36 000, respectively.

pH optimum and substrate specificity

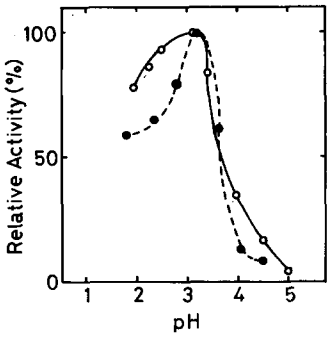
The proteolytic activities of quail and chicken pepsins on hemoglobin at various pH values are shown in Fig. 3. The optimal pH values of quail and chicken pepsins were about 3.0 and the peptic activities decreased rapidly at above pH 3.0.

Quail pepsin hydrolyzed the synthetic substrate *N*-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine very slowly. The specific activity, expressed as described by Tang [9], was $1.6 \cdot 10^{-6}$ unit/μg protein. The specific acitivity of porcine pepsin on the same substrate measured under the same conditions was $1.7 \cdot 10^{-4}$ unit/μg protein.

After activation at pH 2.2, quail pepsinogen showed milk-clotting activity at pH 5.3, but pepsinogen itself showed no activity. 25 μg of quail pepsin clotted milk in 60 s at 37°C. This activity corresponded to half that of porcine pepsin.

pH stability

Quail and chick pepsin solutions were kept for 20 min at room temperature (26°C) in buffers of pH 6.5—10.5, and then residual peptic activity on hemo-



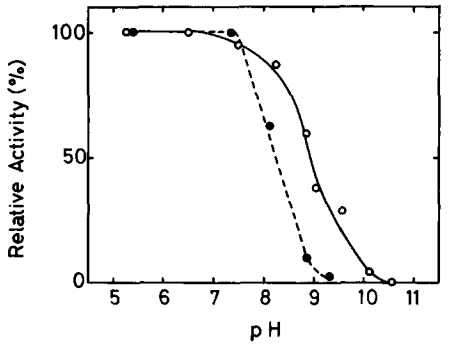


Fig. 3. pH vs. activity profiles of purified quail and chicken pepsins. The reaction mixtures were adjusted to various pH values by addition of 1 N HCl or 1 N NaOH to the standard assay mixture. Activities are shown as percentages of the maximum activity. ○———○, purified quail pepsin; ●- - - - - -●, purified chicken pepsin.

Fig. 4. pH vs. stability curves of purified quail and chicken pepsins. Solutions were adjusted to various pH values with 0.05 M borate buffer (pH 7.6—9.2) or 0.2 M carbonate buffer (pH 9.0—10.5). The solutions were stood for 20 min at room temperature (26°C) and then assayed at pH 2.2. Activities are shown as percentages of the maximum activity. ○———○, purified quail pepsin; ●- - - - -●, purified chicken pepsin.

globin was measured by the standard assay method. Results showed that quail pepsin was stable at pH values of up to pH 8.5, and was rapidly inactivated at above pH 8.8 (Fig. 4). Chicken pepsin showed almost similar stability, although it was less stable under alkaline condition.

Effects of inhibitors

Effects of inhibitors were examined using 0.9 μg of purified quail pepsin in the standard assay mixture. Pepstatin inhibited the activity of quail pepsin, the ID_{50} being 0.017 μg/ml. On the other hand, leupeptin, antipain and chymostatin (each 100 μg/ml) showed no inhibitory effect. Divalent cations (Ca^{2+}, Mg^{2+}, or Mn^{2+}, each 10 mM), which react with sulfhydryl groups, had little inhibitory effect on quail pepsin.

Amino acid analysis

The results of allamino acid analysis, after 20 h hydrolysis, are given in Table II. The average number of amino acid residues per molecule was calculated taking the molecular weight of an amino acid as 110. Amide nitrogen was not determined. The amino acid compositions of chick pepsinogen C and chick pepsin C [5] are also included in Table II for comparison. Quail pepsinogen and pepsin contain fairly large amounts of glutamic acid residues. The ratio of glutamic to aspartic acid and of glutamic acid to serine are especially characteristic in gastricsin [17,18]. The results suggest that quail pepsinogen and pepsin

TABLE II

AMINO ACID COMPOSITION OF QUAIL PEPSINOGEN AND PEPSIN

Numbers of residues in chick pepsinogen C and pepsin C was cited from Donta and Van Vunakis [5]. Integral numbers are expressed as residues/molecule.

	Quail pepsinogen			Chick pepsinogen C number	Quail pepsin			Chick pepsinogen C number
	mol%	Average number	Integral number		mol%	Average number	Integral number	
Lys	0.39	1.4	1	5	0.26	0.8	1	3
His	0.84	3.0	3	3	0.74	2.4	2	1
Arg	0.67	2.4	2	3	0.56	1.8	2	2
Asp	9.28	33.6	34	41	9.41	30.7	31	35
Thr	8.72	31.6	32	33	8.59	28.0	28	32
Ser	11.68	42.3	42	40	11.65	38.0	38	39
Glu	12.99	47.1	47	48	13.04	42.6	43	43
Pro	2.63	9.5	10	22	2.65	8.6	9	17
Gly	11.30	41.0	41	40	11.30	36.9	37	39
Ala	5.36	19.4	19	22	5.35	17.4	17	18
Cys	1.75	6.3	6	6	1.86	6.0	6	6
Val	5.14	18.6	19	22	5.35	17.4	17	21
Met	2.21	8.0	8	9	2.19	7.1	7	10
Ile	5.42	19.6	20	24	5.48	17.9	18	23
Leu	8.70	31.5	32	30	8.47	27.6	28	28
Tyr	5.01	18.1	18	24	4.86	15.8	16	20
Phe	6.69	24.2	24	27	6.72	21.9	22	24
Trp	1.13	4.1	4		1.34	4.3	4	
Total			362	399			326	361

are more closely related to pig and human gastricsins [17,18] than to pig and human pepsin. Although the activation peptide was not isolated in this work, it was suggested from the results of amino acid analyses of quail pepsinogen and pepsin that during activation of quail pepsinogen to pepsin about 36 amino acids were released, but these did not include lysine, arginine, cysteine and tryptophan. There are six cysteines in pepsinogen and pepsin, and also in chicken pepsinogen A, D and C and their pepsins, porcine pepsin, human pepsin and human gastricsin. Thus these six cysteine residues may be important for maintaining the conformation required for proteolytic activity.

Immunological tests on quail and chicken pepsinogens

Using DEAE-Sepharose CL-6B and hydroxyapatite column chromatographies, we have purified a pepsinogen from the chicken proventriculus which seems to be identical with pepsinogen C of Donta and Van Vunakis [5]. Antibody prepared by injection of purified chicken pepsinogen C with Freund's complete adjuvant did not cross-react with chicken pepsinogens A or D (data not shown). This is consistent with the results of Donta and Van Vunakis [5].

Antiserum against quail pepsinogen gave only one precipitin line with quail pepsinogen, which showed spur formation with the line of chicken pepsinogen C. Antiserum against chicken pepsinogen C gave a precipitin line with chicken pepsinogen C, which formed a spur with the precipitin line of quail pepsinogen. These data indicate that chicken pepsinogen C shares some antigenicity with quail pepsinogen, but not with other chicken pepsinogens.

Discussion

In the present study we found that on polyacrylamide gel electrophoresis a crude extract of the proventriculus of the Japanese quail, gave at least five bands of material with peptic activity on acid-denatured hemoglobin. One of these (band I) was concluded to be a pepsin for the following reasons: (1) when the crude extract was treated with alkali (pH 9.5), band I disappeared. (2) When the crude extract was activated with acid (pH 2.2), band I increased. (3) When the purified pepsinogen was activated to pepsin at pH 2.2, the mobility of the restulting pepsin coincided with that of band I and was stable at pH 8.2. On the other hand, band II was purified and characterized as pepsinogen in this work, and bands III—V of the crude extract were also concluded to be pepsinogens, because they changed in electrophoretic mobility on acid treatment.

Bohak [4] reported the existence of only one pepsinogen in chicken proventriculus, whereas Donta and Van Vunakis [5] and Green and Llewellin [6] purified and characterized several pepsinogens from this tissue. Our present results indicate that there are also several pepsinogens in a crude extract of the proventriculus of the Japanese quail.

The pepsinogen purified in this study seems to be very similar to chicken pepsinogen C of Donta and Van Vunakis [5], because the two have similar amino acid compositions and both cross-reacted with antiserum to pepsinogen of the other species. However, chicken pepsinogens A and D did not cross-react

with either chicken pepsinogen C or quail pepsinogen. Other characteristics of the quail pepsinogen, such as its molecular weight, pH vs. activity profile, substrate specificity, pH vs. stability, and milk-clotting activity, are in general similar to those of chicken pepsinogens. Bohak [4] and Green and Llewellin [6] reported the existence of one sulfhydryl group per molecule of chicken pepsinogen and pepsin, but found that this was not essential for the proteolytic activity of the enzyme. Since quail pepsin contains six half-cysteines, and its proteolytic activity was not affected by 10 mM $HgCl_2$ or 10 mM iodoacetamide, it probably does not contain a free sulfhydryl group.

The characteristics of quail and chicken pepsinogens and pepsins differ considerably from those of mammalian pepsinogens and pepsins in several respects. Porcine and human pepsins are unstable at alkaline pH values, whereas quail and chicken pepsins are stable up to pH 8.5. The optimal pH for the peptic activity of porcine pepsin is about pH 2.0, whereas that of quail and chicken pepsins is about pH 3.0. The hydrolytic activity of the quail pepsin against *N*-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine was only 1% of that of porcine pepsin. Avian pepsinogens contain more glutamic acid than aspartic acid, whereas porcine and human pepsinogens contain more aspartic acid than glutamic acid. Except for alkaline stability, these characters suggest a close similarity of avain pepsinogens to gastricsin [17]. Amongst mammalian pepsinogens, mouse pepsinogens (PgI and PgII) [19] have very similar characters to those of avian pepsinogens (unpublished results). These findings on pepsinogens are interesting with respect to the evolution of pepsinogens.

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