Genetic and Biochemical Studies on Chymotrypsin of Japanese Quail (*Coturnix coturnix japonica*)

(日本ウズラのキモトリプシンに関する遺伝生化学的研究)

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Contents

Chapter 1 Introduction

Chymotrypsin (EC 3.4.4.5) is an important enzyme for protein digestion that catalyzes the hydrolysis of proteins in the small intestine. Its inactive precursor, chymotrypsinogen, is synthesized in the acinar cells of the pancreas and stored in lipid-protein membrane in pancreas. When needed for digestive purpose, chymotrypsinogen with other pancreatic zymogens are secreted into a duct leading into the duodenum (Armstrong, 1989).

In the duodenum, chymotrypsinogen is activated by the

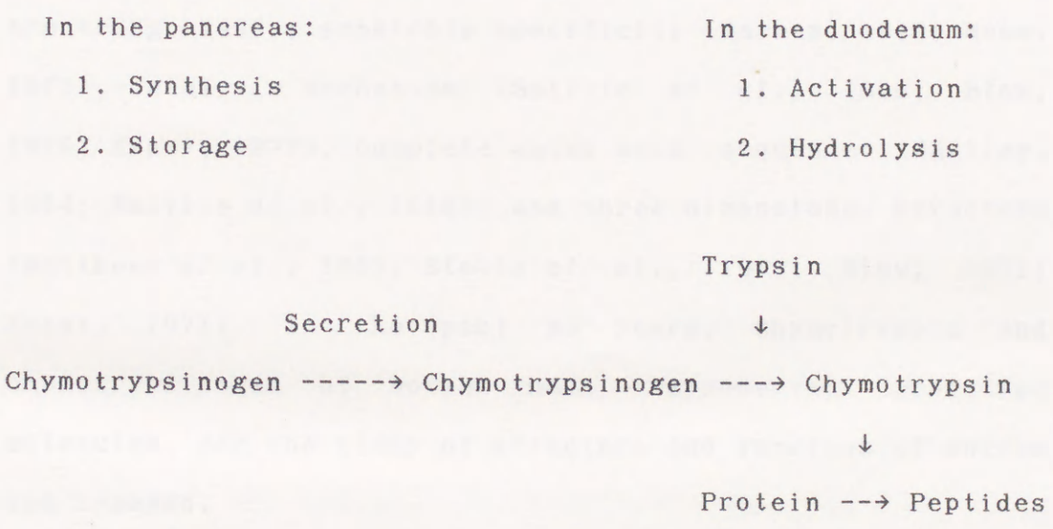


Fig. 1-1. Synthesis, activation of chymotrypsinogen and catalysis of chymotrypsin.

trypsin and converted into chymotrypsin as active enzyme. Then, chymotrypsin catalyzes protein hydrolysis into smaller peptides by cleaving peptide bonds on the carboxyl side of amino acid with aromatic (phenylalanine, tyrosine, and tryptophan) or bulky hydrophobic (e.g., methionine) R groups. These smaller peptides may then be hydrolyzed to amino acid by peptidase enzymes or absorbed as peptides (Armstrong, 1989). These processes can be summarized in Fig. 1-1.

Kunitz and Northrop (1936) obtained chymotrypsin in crystalline form from bovine pancreas. Since then, the properties of chymotrypsin and chymotrypsinogen have been extensively studied in the preparations from bovines, including the activation processing (Stroud *et al.*, 1977; Armstrong, 1989), substrate specificity (Barman, 1969; Hess, 1971), catalytic mechanism (Smillie *et al.*, 1966; Blow, 1976; Kraut, 1977), complete amino acid sequence (Hartley, 1964; Smillie *et al.*, 1968), and three dimensional structure (Matthews *et al.*, 1967; Steitz *et al.*, 1969; Blow, 1971; Kraut, 1971). In the past 30 years, chymotrypsin and chymotrypsinogen of bovine have represented prototype molecules for the study of structure and function of enzyme and zymogen.

The later studies indicate that chymotrypsin is widely distributed in various species, from vertebrates to invertebrates (Zendzian and Barnard, 1967a, b; Wilcox, 1970;

Birk *et al.*, 1983). Furthermore, chymotrypsins and chymotrypsinogens have been purified and characterized from a number of species, including bovine (Wilcox *et al.*, 1957; Guy *et al.*, 1966; Uren and Neurath, 1972), human (Coan and Travis, 1972; Caro *et al.*, 1975), porcine (Charles *et al.*, 1967; Gratecos *et al.*, 1969; Folk and Schirmer, 1965; Folk and Cole, 1965), ovine (Koide *et al.*,1969), rat (Vandermeers and Christophe, 1969), mouse (Watanabe and Ogasawara, 1982), chicken (Ryan, 1965; Ryan *et al.*, 1965), ostrich (Westhuizen *et al.*, 1989), dogfish (Prahl and Neurath, 1966), fin whale (Matsuoka and Koide, 1966; Koide and Matsuoka, 1970), carp (Jónás *et al.*, 1983), sheatfish (Jónás *et al.*, 1983), turtle (Möckel and Barnard, 1969a, b), locust (Sakal *et al.*, 1988). Since the data of structure and function of chymotrypsins and chymotrypsinogens from various species were accumulated, the phylogenetic tree and molecular evolution relation have been discussed (Neurath *et al.*, 1967; Bhargava and Barnard, 1973; Haën *et al.*, 1975; Neurath, 1984, 1985). With recent advance of molecular biology, cDNAs for chymotrypsinogens have been cloned, and their nucleotide sequences and deduced amino acid sequences have been determined in some mammalian species, including canine (Pinsky *et al.*, 1983), rat (Bell *et al.*, 1984) and human (Tomita *et al.*, 1989). The amino acid sequence homologies of chymotrypsinogens between these mammalian

species were very large, over 80% (Tomita *et al.*, 1989). More recently, redesignings of substrate specificity of trypsin which has the same active structure as chymotrypsin have been carried out by using the method of site-directed mutagenesis (Craik *et al.*, 1985, 1987), and mutant trypsins have been expressed in a mammalian tissue culture system (Craik *et al.*, 1985, 1987) and a prokaryotic expression system (Vasquez *et al.*, 1989).

On the other hand, very little is known about the genetic aspect of multiple forms of chymotrypsin because the attentions of past studies on chymotrypsin have been focused on the aspect of biochemical properties. It seems that genetic studies on chymotrypsin have been carried out in detail only in mouse. Watanabe and co-workers have found that genetic variation of mouse chymotrypsin was shown in difference in electrophoretic mobility of chymotrypsin, and this variation is controlled by two codominant allelic genes on the chromosome 8 (Watanabe and Tomita, 1974; Watanabe *et al.*, 1976a, b; Watanabe, 1983; Watanabe *et al.*, 1987, 1989).

In avian, a chymotrypsin from chicken (Ryan, 1965; Ryan *et al.*,1965) and a chymotrypsinogen from ostrich (Westhuizen *et al.*, 1989) have been isolated and characterized. However, no information of genetic variation is known for pancreatic chymotrypsin of avian.

The Japanese quail, *Coturnix coturnix japonica*, is an

excellent laboratory animal for studies on avian genetics, physiology and so on because of its hardiness, ease of handling, precociousness, great laying ability and amount mutations (Padgett and Ivey, 1959; Wilson *et al.*, 1961; Woodard *et al.*, 1973; Cheng and Kimura, 1990).

In the present study, the attention was focused on genetic and biochemical aspects of pancreatic chymotrypsin and chymotrypsinogen of Japanese quail in order to give some basic informations of genetics and biochemistry for chymotrypsin and chymotrypsinogen of avian.

In Chapter 1, the history of studies on chymotrypsin and chymotrypsinogen was introduced, and the objectives of the present study were described.

In Chapter 2, the variation of pancreatic proteinase of Japanese quail was investigated by using agarose gel electrophoresis, and the zymograms of pancreatic proteinase were identified as either chymotrypsin or trypsin. Moreover, the mode of inheritance of chymotrypsin variation was determined by mating experiments, and their gene frequencies were estimated in three lines of quail selected for body weight.

Furthermore, in Chapter 3, the relationship between chymotrypsin variants and chymotrypsinogen was researched by comparing their zymograms. The results suggest that the chymotrypsin variants might be derived from the activated

products of chymotrypsinogens.

Therefore, in Chapter 4, the two chymotrypsinogens showing chymotrypsin variants were purified from quail pancreas by H_2SO_4 extraction, $(NH_4)_2SO_4$ fractionation and chromatography separation on a CM-cellulose column and a Sephadex G-100 column.

In Chapter 5, the structural difference of the purified two chymotrypsinogens was investigated by peptide mapping, and the formation of chymotrypsin variants was clarified to be due to the difference in molecular structure of chymotrypsinogens.

Chapter 6 was conducted to analyzed the expression of chymotrypsin variants in embryo development and localization of chymotrypsin variants in pancreas of quail.

Finally, quail chymotrypsinogen was characterized, and its biochemical and physical properties were compared with those of bovine chymotrypsinogen A in Chapter 7.

Chapter 2 Genetic Variation of Quail Chymotrypsin

Introduction

The pancreas has a number of tissue-specific enzymes, two of which are chymotrypsin (EC 3.4.4.5) and trypsin (EC 3.4.4.4). The two enzymes catalyze the hydrolysis of protein, therefore, they are also called proteinase. The two enzymes have similarity both in mode of action and in various molecular properties. On the other hand, the two enzymes differ markedly in their patterns of specificity, chymotrypsin being specific to bonds involving aromatic amino acids and some others with aliphatic side chains whilst trypsin is specific to those involving the carboxyl groups of lysine and arginine (Inagami, 1971). Therefore, the two enzymes can be identified by specific substrates. For example, the specific activity of chymotrypsin can be detected by the substrate of benzoyl-L-tyrosine ethyl ester (BTEE) whereas the specific activity of trypsin can be detected by the substrate of *p*-tosyl-L-arginine methyl ester hydrochloride (TAME) (Rick, 1974).

The detection methods of pancreatic proteinase described above provides an easy approach to study the genetic

variation of pancreatic proteinase. It is known that the genetic variation of pancreatic proteinase have been reported in mice (Watanabe and Tomita, 1974; Watanabe *et al.*, 1976a, b, 1987, 1989; Watanabe, 1981, 1983) and pigs (Takahashi *et al.*, 1974). However, no genetic variation is known for pancreatic proteinase of avian.

This chapter was undertaken 1) to detect the variation of the pancreatic proteinase of Japanese quail by using the agarose gel electrophoresis, 2) to identify the variation of proteinase as either chymotrypsin variation or trypsin variation, 3) to determine the mode of inheritance of chymotrypsin isozyme, and 4) to estimate the gene frequencies of chymotrypsin locus in three lines of quail selected for body weight.

Materials and Methods

Quail

The Japanese quail (*Coturnix coturnix japonica*) used in this study were obtained from a random-bred population (RR) and two lines selected for either large (LL) or small (SS) body weight for 50 generations maintained at the Laboratory of Animal Breeding in Kagoshima University. All birds were reared in wire-floored individual cages with 14 hr of

artificial light per day. Both feed (Toyohashi New Quail Diet, Japan) and water were available *ad libitum*.

Preparation of samples

The fresh pancrei were obtained from experimental quail After being kept on ice within 3 hr, and then stored at -30°C until use. The pancreatic tissues were homogenized at 4°C in 5 volume of 50 mM Tris-HCl buffer, pH 8.0 (Buffer A). The homogenates were centrifuged at 16,300*g* at 0~4°C for 30 min, and the supernatant fluids were used as experimental samples.

Activation of chymotrypsinogen and trypsinogen

Activation of chymotrypsinogen and trypsinogen were activated by the method of author described previously (Hou *et al.*, 1988a, b). The chymotrypsinogen was activated at 4°C for 1 hr by addition of equal volume of 50 μg/ml bovine trypsin (Type III, Sigma Chemical Company, U.S.A.) in 100 mM Tris-HCl buffer (pH 8.0) containing 50 mM $CaCl_2$ (Buffer B). The trypsinogen was activated at 30°C for 1 hr by addition of 50 μg/ml porcine enteropeptidase (Sigma Chemical Company, U.S.A.) in Buffer B.

Agarose gel electrophoresis

Agarose gel electrophoresis was carried out by the

modified procedure of Ogita (1964). The gel buffer (pH 8.6, μ=0.10) contained 20.6 g of sodium diethylbarbiturate and 3.68 g of diethylbarbituric acid in 1 liter of deionized water. A onefold dilution of the gel buffer with deionized water was used as the chamber buffer (pH 8.6, μ=0.05). The agarose gel stock consisted of 0.7 g of agarose GP-36 (Nacalai Tesque, Japan), 0.2 g of hydroxyethyl cellulose (Nacalai Tesque, Japan), 30 ml of the gel buffer, and 70 ml of deionized water.

The 20 ml of agarose stock was melted and poured on warmed glass plate of size 12 x 16 cm. The sample was absorbed on cotton thread about 0.7 cm in length, and placed on the agarose gel layer. The electrophoresis was carried out at 4°C with a constant current of 1.5 mA/cm gel width for about 2 hr. In order to detect the activities due to pancreatic proteinase, 1% casein (E. Merk Ag, Darmstadt, Germany) solution (pH 8.0) was poured on the plate, following incubation at 37°C for 1 hr. Then, the plate was stained with 0.5% amino black 10B in the solution of methanol : acetic acid : distilled water = 4:1:5, and destained with the above solution containing no amino black 10B. The sites with proteinase activity were revealed as colorless bands on a blue background. The procedures of of proteinase detection from quail pancreas by agarose gel electrophoresis are represented as Flow chart 2-1.

Supernatant from pancreatic extract

↓

Activated by enteropeptidase (50 μg/ml) for 1 hr

↓

Samples were loaded on agarose gel plate

↓

Run at 4°C with a current of 1.5 mA/cm gel for 2 hr

↓

1% casein was poured into agarose gel plate

↓

Incubated at 37°C for 1 hr

↓

Stained, then destained

↓

Proteinase band

Flow chart 2-1. The procedures of proteinase detection from quail pancreas by agarose gel electrophoresis.

Measurements of activities of chymotrypsin and trypsin

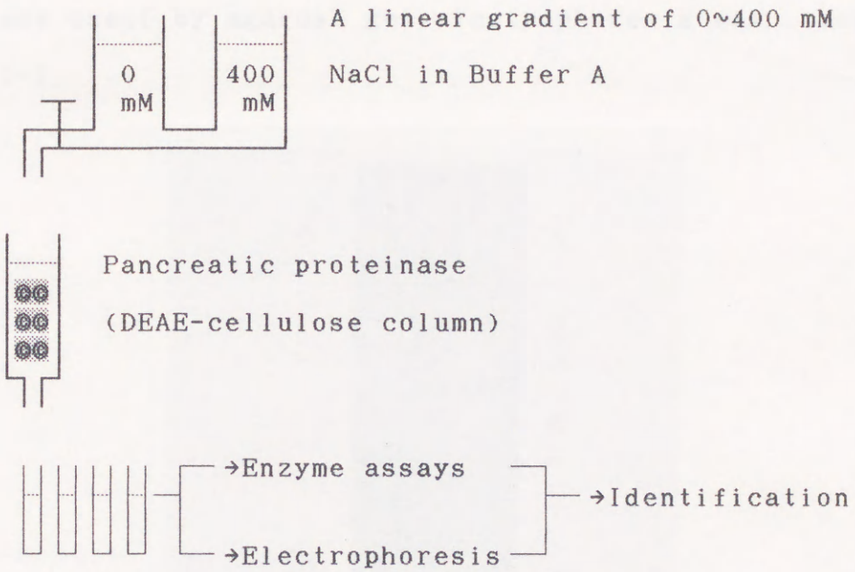
Chymotryptic and tryptic activities were measured at 256 nm and 247 nm by the spectrophotometric method of Hummel (1959), using benzoyl-L-tyrosine ethyl ester (BTEE, Nacalai Tesque, Japan) and p-tosyl-L-arginine methyl ester hydro-

chloride (TAME, Nacalai Tesque, Japan) as substrates, respectively. For chymotrypsin assay, 0.1 ml of chymotrypsin solution was added to 3 ml of 0.5 mM BTEE substrate solution (pH 8.0) containing 50 mM Tris, 10 mM $CaCl_2$ and 15% methanol. For trypsin assay, 0.1 ml of trypsin solution was added to 3 ml of 0.5 mM TAME substrate solution (pH 8.0) containing 50 mM Tris and 10 mM $CaCl_2$. The change value of absorbance was recorded per min, and one unit of chymotrypsin or of trypsin was the amount required to hydrolyze 1 μmol of BTEE or TAME per min at 25°C, respectively. Protein concentration was determined by the method of Miller (1959).

Separation and identification of chymotrypsin and trypsin

A DEAE-cellulose column chromatography was used to separate the chymotrypsin and trypsin from the activated supernatant of pancreatic extract. 4 ml of supernatants were placed on a DEAE-cellulose column (1 x 10 cm) equilibrated with Buffer A, and the column was eluted at 25 ml/hr flow rate with a linear gradient from 0 to 400 mM NaCl in Buffer A. At the end of the gradient, the column was washed with 800 mM NaCl in Buffer A. Fractions of 5 ml were collected, and the chymotryptic and tryptic activities were measured at 256 nm and 247 nm, respectively. The fractions with high enzyme activity were precipitated by adding solid $(NH_4)_2SO_4$ to 70% saturation, respectively. The precipitates were

suspended in 0.5 ml of deionized water and dialyzed against deionized water. The dialysates were used as the samples for agarose gel electrophoresis. The procedures of separation and identification of chymotrypsin and trypsin are represented as Flow chart 2-2.



Flow chart 2-2. The procedures of separation and identification of chymotrypsin and trypsin from pancreatic extract of quail.

RESULTS

Description of pancreatic proteinase zymogram in Japanese quail

The result of detection of pancreatic proteinase of Japanese quail by agarose gel electrophoresis was shown in Fig. 2-1.

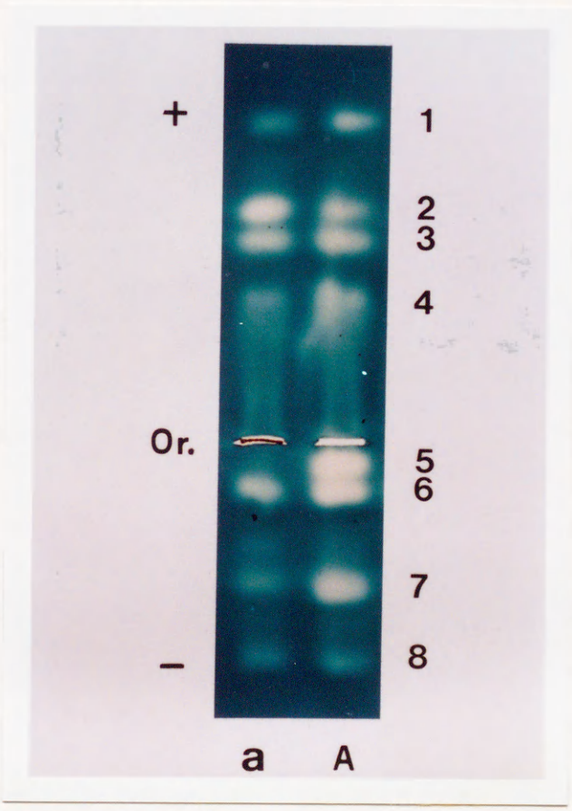


Fig. 2-1. Zymogram of pancreatic proteinase of quail. An individual variation was found in the presence (lane A) and absence (lane a) of band 5.

Four bands in the anode and three or four bands in the cathode were observed, and they were designated bands 1, 2, 3, 4, 5, 6, 7, and 8 from the most rapid anodal band. An individual variation was found in the presence (lane A) or absence (lane a) of band 5, designated *Prt-5*, but no variation was detected at other bands in all materials used in this study.

In order to examine whether proteinase activity is present in the activated pancreatic extract, inactive extracts of pancreas and enteropeptidase used as activator were also applied to agarose gel electrophoresis. No zymogram was detectable in the inactive extract of pancreas or enteropeptidase alone. These facts indicate that eight bands of pancreatic proteinase in Japanese quail were formed after activation of the pancreatic proteinase zymogen by enteropeptidase.

Identification of chymotrypsin and trypsin

Pancreatic proteinase includes trypsin (EC 3.4.4.4) and chymotrypsin (EC 3.4.4.5). To distinguish between these, an activated extract of pancreas was applied to a DEAE-cellulose column (1 x 10 cm) equilibrated with Buffer A. The column was eluted with a linear gradient from 0 to 400 mM NaCl in Buffer A. As shown in Fig. 2-2, two peaks of chymotrypsin and a single peak of trypsin were observed, and

they were designated Chy-I, Chy-II, and Try-I, respectively. No further peptidase activity was detected when the column was washed with 800 mM NaCl in Buffer A.

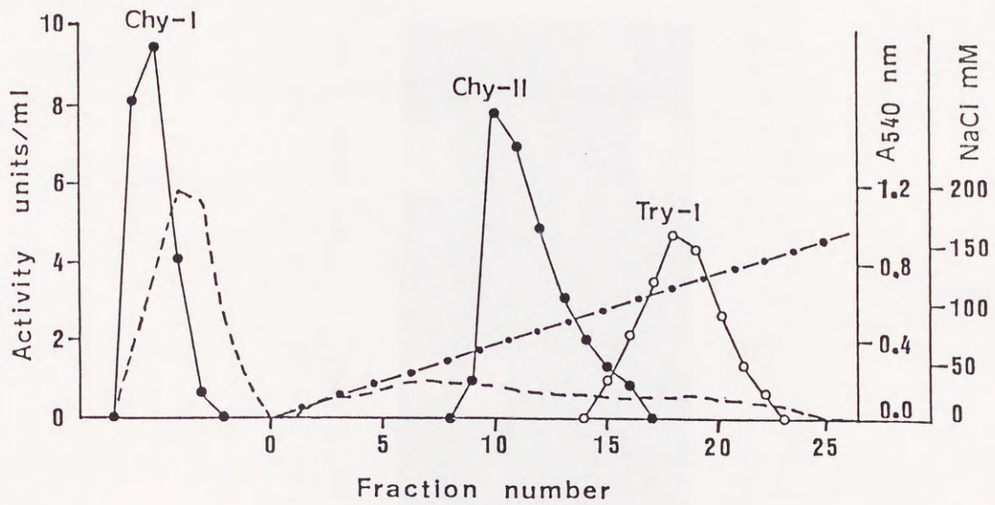


Fig. 2-2. Chromatography profile of the activated pancreatic extract from quail on a column (1 x 10 cm) of DEAE-cellulose. The column was eluted by a linear gradient from 0 to 400 mM NaCl (—●—) in 50 mM Tris-HCl buffer (pH 8.0) (each 100 ml). Fractions of 5 ml were collected, and the chymotryptic (●—●) and tryptic (○—○) activities were measured. Absorbance at 540 nm (·····).

The Chy-I fraction was not absorbed by DEAE-cellulose. Chy-II was eluted at 60~100 mM NaCl, and Try-I at 98~110 mM NaCl. The fractions with higher enzyme activity were concentrated and applied to agarose gel electrophoresis,

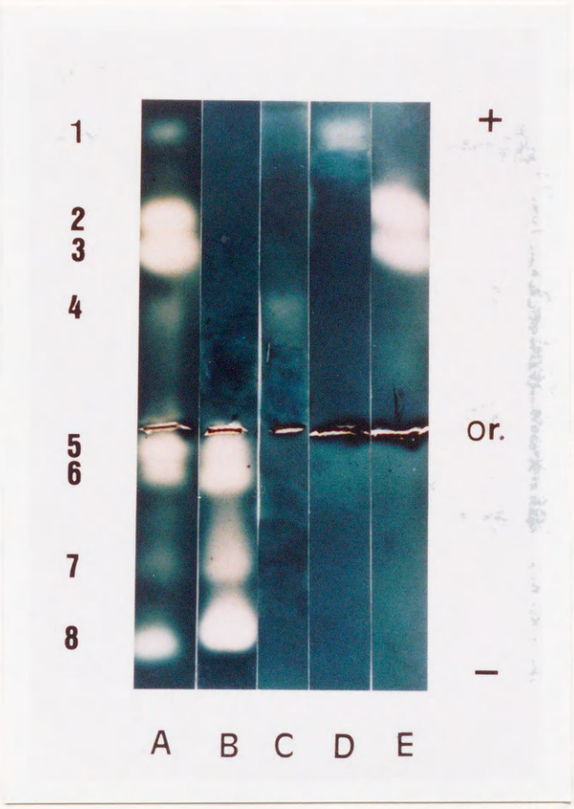


Fig. 2-3. Zymogram of pancreatic proteinase from the fractionated samples. Lane A is pancreatic proteinase without fraction as control, lane B is Chy-I (bands 5, 6, 7, and 8), lane C is Chy-II (band 4), and lanes D and E are Try-I (bands 1, 2, and 3), respectively.

respectively. The result of identification of proteinase zymogram was shown in Fig. 2-3. Lane A is a zymogram of the activated extract of pancreas without fractionation, and lanes B~E are the zymograms of the fractionated samples. The Chy-I, Chy-II, and Try-I fractions separated into bands 5~8 (lane B), band 4 (lane C), and bands 1~3 (lanes D and E), respectively. Therefore, band 5, showing individual variation, is due to a chymotrypsin.

Genetic analysis at the *Prt-5* locus

To study the mode of inheritance of band 5, various matings were carried out. The presence and absence of band 5 were designated phenotypes *A-* and *aa*, respectively. As shown in Table 2-1, phenotype distribution of progeny from each mating are in good agreement with the expected numbers from simple Mendelian law, without exception. All progenies of *aa* x *aa* cross showed the *aa* phenotype. In the case of *aa* x *Aa* cross, the *Aa* phenotype and the *aa* phenotype were distributed at the ratio of about 1:1, and in the case of *Aa* x *Aa* cross, progenies were segregated to the phenotypes *A-* and *aa* at the ratio of about 3:1. These data of cross experiments indicate that the presence or absence of band 5 is controlled by a pair of allelic genes on an autosomal locus, designated the *Prt-5* locus; the allele, $Prt\text{-}5^{A}$, expressing the presence of band 5, is dominant to $Prt\text{-}5^{a}$,

not expressing band 5. Therefore, the genotypes corresponding to the phenotypes *AA*, *Aa*, and *aa* are *Prt-5^A/Prt-5^A*, *Prt-5^A/Prt-5^a*, and *Prt-5^a/Prt-5^a*, respectively.

Table 2-1. Phenotype distribution of pancreatic chymotrypsin isozyme (*Prt-5*) in progeny from quail various matings.

Parents	No. of	No. of	Progeny		
Male x Female	matings	progeny	*A-*	*aa*	*P* of X^2
aa x *aa*	5	112	-	112	
aa x *Aa*	3	50	26/25*	24/25	>0.75
Aa x *Aa*	2	41	30/30.75	11/10.25	>0.75
A- x *A-*	2	32	32	-	

* Observed/expected numbers.

Distribution of phenotypes and gene frequencies of *Prt-5* locus in three lines of quail selected for body weight

The phenotypes and gene frequencies of *Prt-5* locus in a random-bred population (RR) and in two lines selected for either large (LL) or small (SS) body weight for 50 generations were estimated, and the results are shown in Table 2-2. The gene frequencies of *Prt-5^A* (0.21~0.29) were lower than those of *Prt-5^a* (0.71~0.79) in all of three lines

of quail. On the other hand, the gene frequencies of *Prt-5*^{A} or *Prt-5*^{a} were no significant difference among three lines of quail selected for body weight.

Table 2-2. Phenotypes and gene frequencies of pancreatic chymotrypsin isozyme (*Prt-5*) in three lines of quail selected for body weight.

Line	No. of birds	Phenotype		Gene frequency	
		A-	*aa*	*Prt-5*^{A}	*Prt-5*^{a}
SS	43	16	27	0.21^{a}*	0.79^{a}
RR	122	53	69	0.25^{a}	0.75^{a}
LL	57	28	29	0.29^{a}	0.71^{a}

* Column values with the same superscript letter are not statistically significant ($P>0.05$).

Discussion

Although pancreatic proteinase play very important role in protein digestion, genetic study on pancreatic proteinase was carried out in limited mammalian animal. Probably, the proteinase detection from pancreas is more difficult than

other enzymes from blood and other tissues. Watanabe *et al.* (1974, 1976a,b, 1981, 1983, 1987) have reported that the variation of pancreatic proteinase in mice show the modes of inheritance of allelic genes (*Prt-1* and *Prt-3* loci) and codominant allelic genes (*Prt-2* locus). Furthermore, they have clarified that *Prt-2* locus is involved with chymotrypsin, and is located on chromosome 8 while the *Prt-1* and *Prt-3* loci are related to trypsin. Takahashi *et al.* (1974) have reported that the variation of pancreatic proteinase in pigs show the mode of inheritance of codominant allelic genes (*PPr-1* and *PPr-2* loci) but the loci of pancreatic proteinase in pigs have been not identified as chymotrypsin loci or/and trypsin loci. On the other hand, no genetic variation is known for pancreatic proteinase of avian.

In this chapter, a genetic variation in pancreatic proteinase of Japanese quail was found in the presence and absence of band 5, using agarose gel electrophoresis. Furthermore, the band 5, showing individual variation, was identified as a chymotrypsin. The results of various matings showed that the presence or absence of band 5 is controlled by a pair of allelic genes on an autosomal locus (*Prt-5*^{A} and *Prt-5*^{a}), and *Prt-5*^{A}, expressing the presence of band 5, is dominant to *Prt-5*^{a}, not expressing band 5. The gene frequencies of *Prt-5*^{A} or *Prt-5*^{a} were no significant

difference among three lines of quail selected for body weight.

The variation of chymotrypsin suggests a possibility of presence of chymotrypsinogen variation, since chymotrypsin is formed by activation of chymotrypsinogen. In the next chapter, the relationship between chymotrypsin variants and chymotrypsinogens will be investigated.

Summary

Genetic studies on pancreatic chymotrypsin in Japanese quail was undertaken in this chapter. The results can be summarized as follows:

1. By means of agarose gel electrophoresis, four bands in the anode and three or four bands in the cathode were detected in the zymogram of pancreatic proteinase, and they were designated bands 1~8 from the most rapid anodal band. An individual variation was found in the presence or absence of band 5.

2. The bands 5~8 were identified as chymotrypsin by the methods of chromatography separation, substrate specificity analysis and electrophoretic detection.

3. The results of various matings indicate that the presence or absence of band 5 is controlled by a pair of allelic genes on an autosomal locus, designated the *Prt-5* locus; the gene $Prt\text{-}5^{A}$, causing expression of band 5, is dominant to gene $Prt\text{-}5^{a}$, causing absence of band 5 gene $Prt\text{-}5^{A}$, expressing the presence of band 5, is dominant to gene $Prt\text{-}5^{a}$.

4. The gene frequencies of $Prt\text{-}5^{A}$ and $Prt\text{-}5^{a}$ were estimated in a random-bred population (RR) and two lines selected for either large (LL) or small (SS) body weight of

quail. The results indicate that the gene frequencies of *Prt-5*^{A} (0.21~0.29) were lower than those of *Prt-5*^{a} (0.71~0.79) in all of three lines of quail. On the other hand, the gene frequencies of *Prt-5*^{A} or *Prt-5*^{a} were no significant difference among three lines of quail.

Chapter 3 Relationship Between Quail Chymotrypsin Variants and Chymotrypsinogens

Introduction

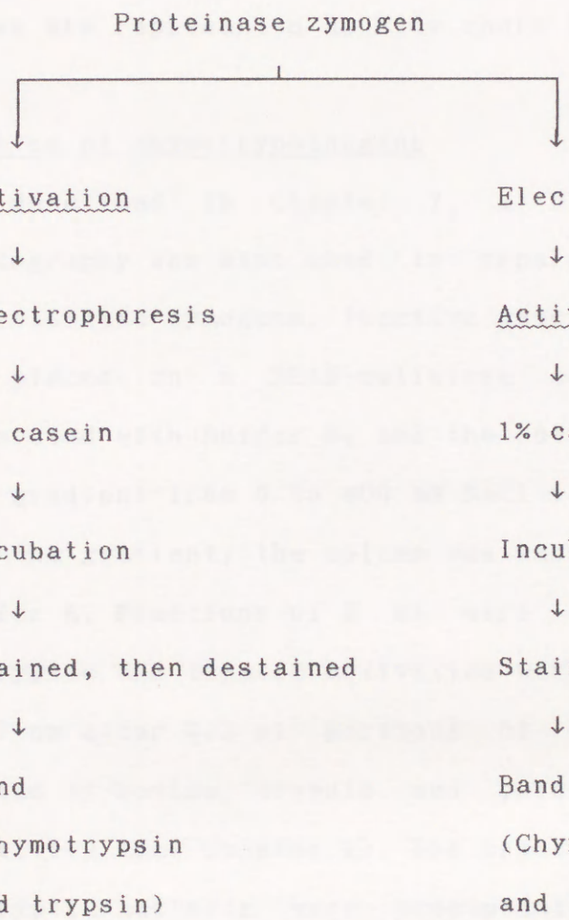
It is known that chymotrypsin is formed by activation of chymotrypsinogen. The different forms of bovine chymotrypsin can be formed from chymotrypsinogen by activation with trypsin and autocatalytic interconversions (Wright *et al.*, 1968; Blow, 1971). In Japanese quail, the chymotrypsin variants were found in the presence or absence of band 5 of chymotrypsin, and this variation is controlled by a pair of allelic genes (see Chapter 2). The chymotrypsin variation of quail suggests a possibility of the presence of chymotrypsinogen variation, since quail chymotrypsin is also formed by activation of quail chymotrypsinogen (Hou *et al.*, 1988a).

Therefore, this chapter was conducted to investigate whether the chymotrypsin variants is detected also in chymotrypsinogen by comparing the zymograms of chymotrypsin with that of chymotrypsinogen, and to clarify the relationship between chymotrypsin variants and chymotrypsinogens.

Materials and Methods

Detection of proteinase and proteinase zymogens

Agarose gel electrophoresis described in Chapter 2 was



Flow chart 3-1. Procedures of electrophoretic detection of proteinase and proteinase zymogen from quail pancreas.

used to detect proteinase and proteinase zymogens. For detection of proteinase, the proteinase zymogen was activated before electrophoresis while in order to detect proteinase zymogen, the activation was performed after electrophoresis. The procedures of electrophoresis detection of proteinase and proteinase zymogen from Japanese quail pancreas are represented as Flow chart 3-1.

Separation of chymotrypsinogens

As described in Chapter 2, a DEAE-cellulose column chromatography was also used to separate chymotrypsinogen from proteinase zymogens. Inactive pancreatic extract of 4 ml was placed on a DEAE-cellulose column (1 x 10 cm) equilibrated with Buffer A, and the column was eluted with a linear gradient from 0 to 400 mM NaCl in Buffer A. At the end of the gradient, the column was washed with 800 mM NaCl in Buffer A. Fractions of 5 ml were collected, and the chymotryptic and tryptic activities were measured at 256 nm and 247 nm after 0.2 ml portions of each fractions were activated by bovine trypsin and porcine enteropeptidase, respectively (see Chapter 2). The fractions containing only chymotryptic activity were precipitated by adding solid $(NH_4)_2SO_4$ to 70% saturation. The precipitates were suspended in 0.5 ml of deionized water, and dialyzed against deionized water. One-half of dialysates was converted into chymo-

trypsin by activation, and then applied to agarose gel electrophoresis with separated chymotrypsinogens.

Other methods

See Chapter 2.

Results

As shown in Fig. 3-1, eight (lane A_2) or seven bands (lane a_2) were observed in the activated proteinases whereas only four bands (bands I, II, III in the anode, and band IV in the cathode) were detected in proteinase zymogens (lanes A_1 and a_1). Moreover, no difference in electrophoretic mobility was found between the zymograms of proteinase zymogens (lane A_1 and a_1) although they showed the presence (lane A_2) or absence (a_2) of band 5 after activation.

Furthermore, the separation of quail chymotrypsinogens from proteinase zymogens was performed to determine which band of the proteinase zymogens shows band 5 after activation. The zymograms of the separated chymotrypsinogens and their chymotrypsins were shown in Fig. 3-2. The results indicate that bands 5~8 of chymotrypsin were derived from activated products of chymotrypsinogen (band IV). However, one chymotrypsinogen (lane A_1) produced band 5 of chymo-

trypsin (lane A_2) whereas another chymotrypsinogen (lane a_1) did not produce band 5 of chymotrypsin (lane a_2) although the two chymotrypsinogens showed the same electrophoretic band (lanes A_1 and a_1) without activation.

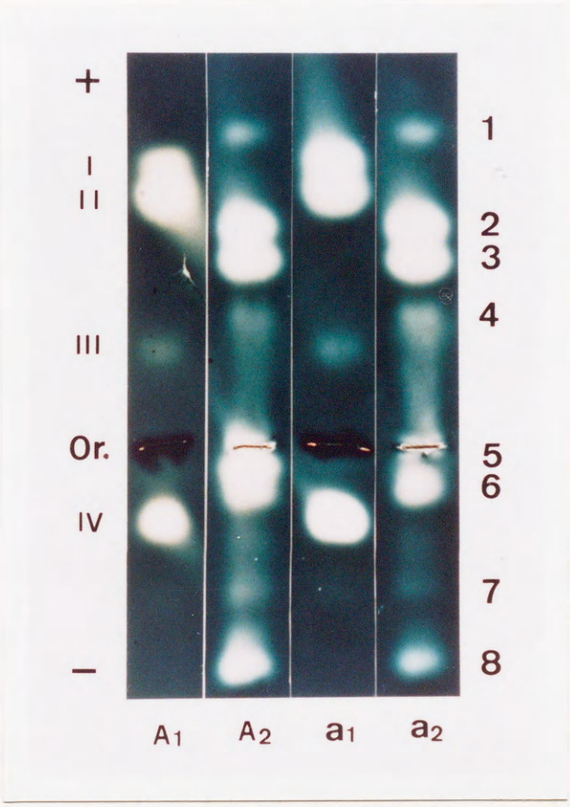


Fig. 3-1. Zymogram comparison of proteinases and their zymogens. Lanes A_1 and A_2 are the electrophoretic patterns of proteinase zymogen and proteinase showing band 5, respectively; lanes a_1 and a_2 are the electrophoretic patterns of proteinase zymogen and proteinase lacking band 5, respectively.

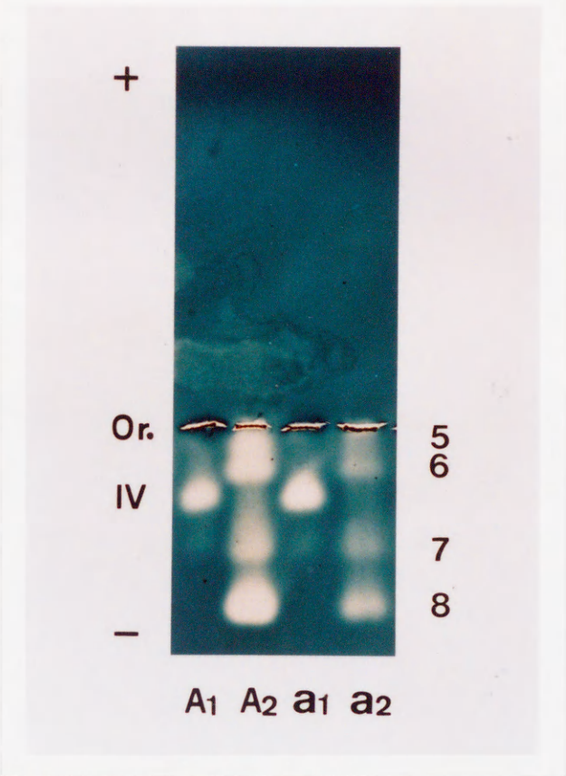


Fig.3-2. Zymogram comparison of chymotrypsinogen and chymotrypsin. Lanes A_1 and A_2 are the electrophoretic patterns of chymotrypsinogen and chymotrypsin showing band 5, respectively; lanes a_1 and a_2 are the electrophoretic patterns of chymotrypsinogen and chymotrypsin lacking band 5, respectively.

In order to investigate the activation process of quail chymotrypsinogen, the quail chymotrypsinogen was activated at 25~30° C by using different concentrations of entero-

peptidase for 30 min. As shown in Fig. 3-3, the appearance order of chymotrypsin from chymotrypsinogen (band IV) is as follows: band 7 (lane C)---→ bands 5 and 6 (lane E)---→ band 8 (lane F). No detectable band appeared without the addition of enteropeptidase (lane B), and all bands of chymotrypsin were revealed when chymotrypsinogen was activated with 50 μg

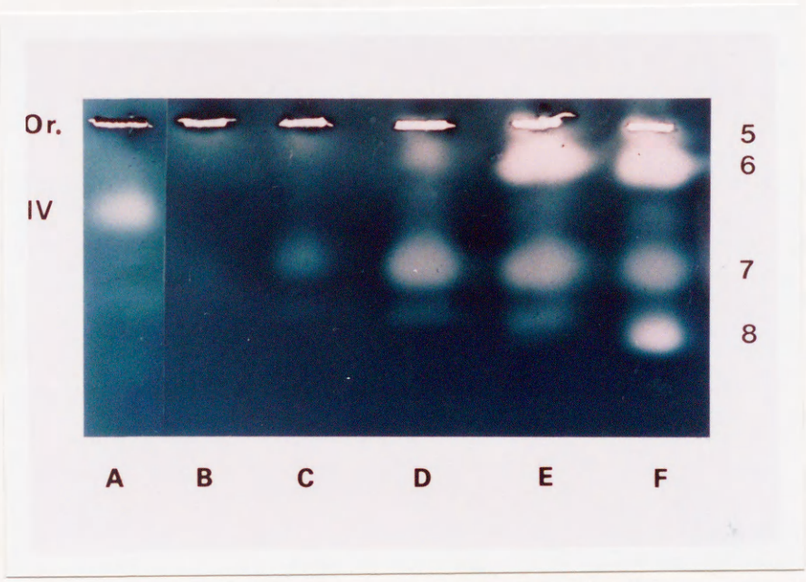


Fig. 3-3. Activation process of chymotrypsinogen using different concentrations of enteropeptidase at 25~30° C for 30 min. Lane A is the chymotrypsinogen as control; lanes B~F are the electrophoretic patterns of chymotrypsin after activation with 0, 3.1, 6.3, 12.5, and 50 μg porcine enteropeptidase/ml Buffer A (pH 8.0), respectively.

porcine enteropeptidase/ml Buffer A (lane F). It is recognized that quail chymotrypsinogen also has an activation process, and the activator concentrations used in this experiment (50 μg/ml) is enough to activate quail chymotrypsinogen, therefore, the formation of chymotrypsin variants is not due to activator concentration.

Discussion

In Chapter 2, a variation of chymotrypsin was found in Japanese quail, and this variation is controlled by a pair of genes (*Prt-5*^{A} and *Prt-5*^{a}) on an autosomal locus. The variation of chymotrypsin suggests that variation of chymotrypsinogen will exist, since chymotrypsin is formed by activation of chymotrypsinogen. In this chapter, however, no variant chymotrypsinogen corresponding to band 5 of chymotrypsin was detected by comparing the zymogram of chymotrypsinogen with that of chymotrypsin. It is known that bovine chymotrypsinogen can produce different forms of chymotrypsin after activation with trypsin and other autocatalytic interconversions, e.g., bovine α-chymotrypsin is formed by splitting off the dipeptide Ser^{14}-Arg^{15} only, and π-chymotrypsin simply by hydrolysis of the Arg^{15}-Ile^{16} peptide bond of bovine chymotrypsinogen A (Wright *et al.*,

1968; Blow, 1971). Moreover, these different forms of bovine chymotrypsins can be distinguished by their electrophoretic mobility and their chromatographic behaviour (Rick, 1974). From these facts, it is suggested that the chymotrypsin variants of quail might be formed during activation of chymotrypsinogen. The chymotrypsinogens which have different structure each other would exist and cause the formation of band 5. In order to demonstrate whether the chymotrypsin variants are derived from different structural chymotrypsinogens, it is necessary to purify the chymotrypsinogens showing chymotrypsin variants, and to clarify the structural difference of the chymotrypsinogens.

Summary

1. By means of agarose gel electrophoresis, three bands (bands I, II, and III) in the anode and one band (band IV) in the cathode were detected in the zymogram of proteinase zymogen from quail pancreas. Moreover, band IV on zymogram of proteinase zymogen was identified as a chymotrypsinogen, and it produced bands 5~8 of chymotrypsin after activation.

2. The comparison of zymograms of chymotrypsin and chymotrypsinogen indicate that no variant chymotrypsinogen corresponding to band 5 of chymotrypsin was detected. Furthermore, the activation process of quail chymotrypsinogen suggests that chymotrypsin variants might be derived from activated products of different structural chymotrypsinogens.

Chapter 4 Purification of Quail Chymotrypsinogens Showing Chymotrypsin Variants

Introduction

In Chapter 2, a genetic variation of chymotrypsin in Japanese quail was found by using agarose gel electrophoresis. The phenotype *AA* or *Aα* of chymotrypsin variants showed band 5 of chymotrypsin while the phenotype *αα* lacked band 5. The presence or absence of band 5 of chymotrypsin is known to be controlled by a pair of allelic genes (*Prt-5*^{A} and *Prt-5*^{a}). The variation of chymotrypsin suggests that variation of chymotrypsinogen would exist, since chymotrypsins are formed by activation of chymotrypsinogen. However, no electrophoretic variation of chymotrypsinogen corresponding to band 5 of chymotrypsin was found. Therefore, it is proposed that variants of chymotrypsin could result from activated products of chymotrypsinogens which would have different molecular structures (see Chapter 3).

In order to clarify the structural difference of chymotrypsinogens showing chymotrypsin variants, the purifications of the two chymotrypsinogens from the preparations of

the *AA* and *aa* phenotypes were undertaken in this chapter.

The purification of chymotrypsinogen have been performed in a number of species, including bovine (Wilcox *et al.*, 1957; Guy *et al.*, 1966; Uren and Neurath, 1972), human (Caro *et al.*, 1975), porcine (Folk and Schirmer, 1965; Folk and Cole, 1965; Charles *et al.*, 1967; Gratecos *et al.*, 1969), rat (Vandermeers and Christophe, 1969), mouse (Watanabe and Ogasawara, 1982), ostrich (Westhuizen *et al.*, 1989), dogfish (Prahl and Neurath, 1966), fin whale (Koide and Matsuoka, 1970), turtle (Möckel and Barnard, 1969a, b). The results indicate that extraction of pancreatic enzymes from avian with acid differed from mammals. For example, acid extraction of turkey pancreas preferentially yield trypsin, but almost completely excluded chymotrypsin and *vice versa* for chicken (Ryan, 1965). On the other hand, acid extraction of ostrich pancreas can obtained chymotrypsinogen and trypsin (Westhuizen *et al.*, 1989). Therefore, a modified method of purification of ostrich chymotrypsinogen was used for purification of quail chymotrypsinogen.

Materials and Methods

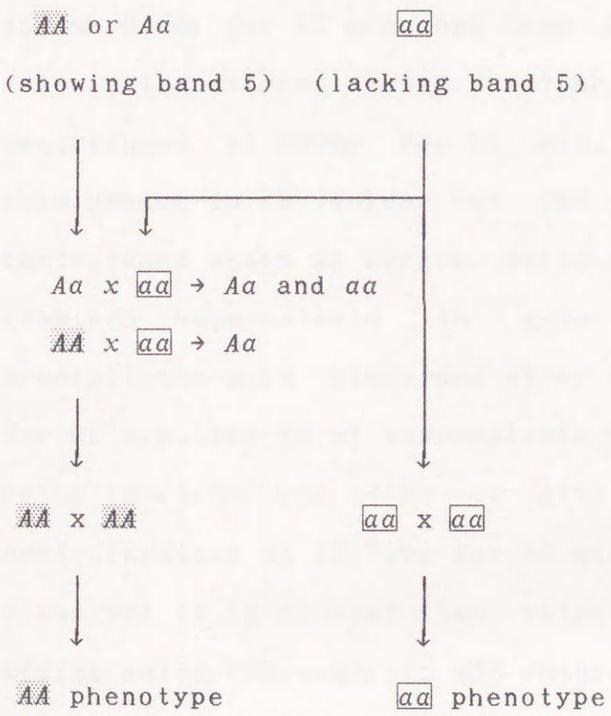
Pancreatic biopsy

In order to know the phenotypes of chymotrypsin isozyme prior to mating, the pancreatic biopsy of quail was performed by modified liver biopsy technique of quail (Maeda *et al.*, 1973). The quail of 90 days of age was used as pancreatic biopsy. The bird was stretched and fastened on the table on its right side, and the skin was cut under the seventh rib about 2 cm in length, after the quail was picked from seventh rib region to the thigh region and disinfected with 70% ethyl alcohol. The *Mm. abdominis* (*M.obliquus abdominis internus* and *M.rectus abdominis*) and *peritoneum* were then incised by a scalpel. After the duodenum was found, the pancreas was lobectomized with a small scissor, and pancreatic tissue of approximate 0.5 mg was obtained and used for electrophoresis. Finally, the *Mm. abdominis* and the skin were sutured and disinfected with 70% ethyl alcohol. The pancreatic biopsy of quail was demonstrated to be very safe because the viability and growth of quail after operation were not affected (Hou, unpublished data).

Selection of the AA and aa phenotypes

The *A-* (*AA* or *Aa*) and *aa* phenotypes were detected by a

pancreatic biopsy of Japanese quail. The quail showing the *aa* phenotype was directly kept to obtain its progenies. However, The *A-* phenotype includes the *AA* and *Aa* phenotypes, therefore, the *A-* phenotype was backcrossed with the *aa* phenotype in order to identify the *AA* phenotype. Then, the quail whose progenies did not show the *aa* phenotype was kept to get progenies showing the *AA* phenotype. The selections of the *AA* and *aa* phenotypes are represented in Flow chart 4-1.



1. Detection of the *A-* and *aa* phenotypes (pancreatic biopsy)

2. Progeny test (backcross mating)

3. To get progenies of the *AA* and *aa* phenotypes

Flow chart 4-1. Selections of the *AA* and *aa* phenotypes.

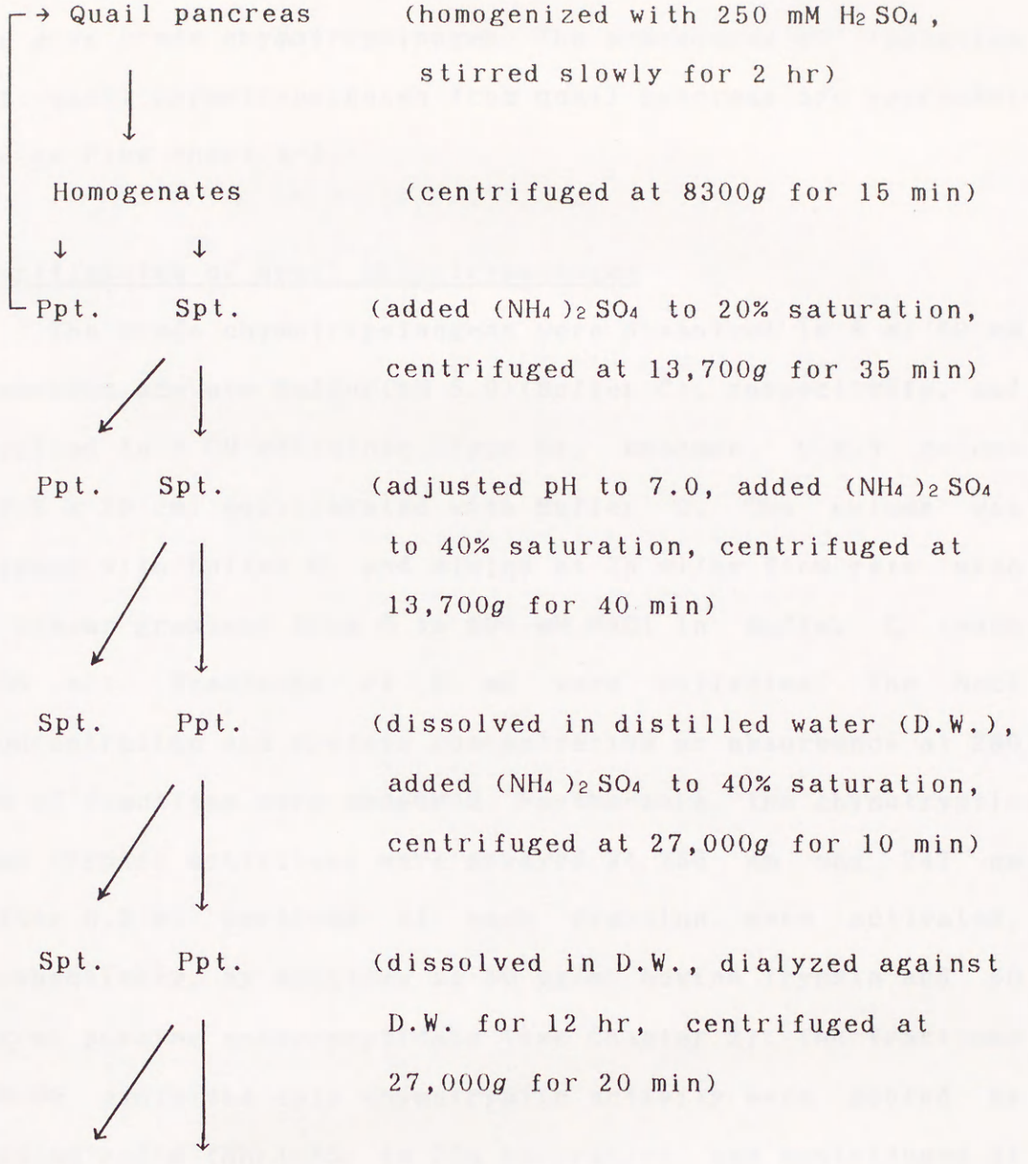
Zymogen activation and enzyme assay

See Chapter 2.

Isolation of crude chymotrypsinogen

The pancreatic tissues were immediately frozen in liquid nitrogen after killing, and then stored at -30° C until use.

All procedures of isolation were carried out at 0~4° C. The frozen pancreatic tissues were thawed in 2 volume of 250 mM H_2SO_4 for 30 min, and then homogenized for 1 min. After being stirred slowly for 2 hr, the homogenates were centrifuged at 8300*g* for 15 min. The precipitates were resuspended in 1 volume of 250 mM H_2SO_4, stirred and centrifuged again as before. Solid $(NH_4)_2SO_4$ was added to combined supernatants to give 20% saturation. The precipitates were discarded after centrifugation at 13,700*g* for 35 min. The pH of supernatants were adjusted to 7.0, and solid $(NH_4)_2SO_4$ was added to give 40% saturation. After centrifugation at 13,700*g* for 40 min, the precipitates were dissolved in 10 ml distilled water and reprecipitated by adding solid $(NH_4)_2SO_4$ to 40% saturation. The precipitates were collected by centrifugation at 27,000*g* for 10 min, and dissolved in 8 ml distilled water. After dialysis against distilled water for 12 hr, the dialysates were centrifuged at 27,000*g* for 20 min and the supernatants were lyophilized

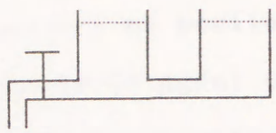


Flow chart 4-2. Procedures of isolation of chymotrypsinogen from quail pancreas (Spt.:supernatant; Ppt.: precipitate).

to give crude chymotrypsinogen. The procedures of isolation of quail chymotrypsinogen from quail pancreas are represented as Flow chart 4-2.

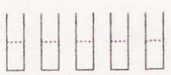
Purification of quail chymotrypsinogen

The crude chymotrypsinogens were dissolved in 8 ml 50 mM ammonium acetate buffer(pH 5.0)(Buffer C), respectively, and applied to a CM-cellulose (Type 52, Whatman, U.K.) column (2.5 x 25 cm) equilibrated with Buffer C. The column was washed with Buffer C, and eluted at 25 ml/hr flow rate with a linear gradient from 0 to 600 mM NaCl in Buffer C (each 200 ml). Fractions of 5 ml were collected. The NaCl concentration and protein concentration or absorbance at 280 nm of fractions were measured. Furthermore, the chymotryptic and tryptic activities were assayed at 256 nm and 247 nm after 0.2 ml portions of each fraction were activated, respectively, by addition of 50 μg/ml bovine trypsin and 50 μg/ml porcine enteropeptidase (see Chapter 2). The fractions which exhibited only chymotryptic activity were pooled by adding solid $(NH_4)_2SO_4$ to 70% saturation, and centrifuged at 27,000*g* for 10 min. The precipitates were dialyzed against distilled water for 12 hr. The dialysates were then subjected to gel filtration on Sephadex G-100 (Pharmacia Fine Chemicals, Sweden) column (2.5 x 65 cm), using Buffer C as eluant. The chymotryptic activities were assayed at 256



A linear gradient of 0~600 mM NaCl in Buffer C

Crude chymotrypsinogen (CM-cellulose column)



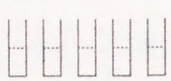
Assays ─ chymotrypsin and trypsin
Assays ─ NaCl and protein

↓

Fractions containing chymotryptic activity were pooled and dialyzed against distilled water

↓

Dialysates (Sephadex G-100 column)



Assays ─ chymotrypsin
Assays ─ protein

↓

Fractions containing chymotryptic activity were pooled and lyophilized

↓

Pure chymotrypsinogen

Flow chart 4-3. Procedures of purification of chymotrypsinogen from pancreatic extract of quail.

nm after 0.2 ml portions of each fraction were activated by addition of 50 μg/ml bovine trypsin. The fractions which exhibited chymotryptic activity were pooled by adding solid $(NH_4)_2SO_4$ to 70% saturation, and centrifuged at 27,000*g* for 10 min. The precipitates were dialyzed against distilled water for 6 hr. The dialysates were then lyophilized two times to remove ammonium acetate. The procedures of purification of chymotrypsinogen from pancreatic extract of Japanese quail are represented in Flow chart 4-3.

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli(1970), using Slab-Gel Electrophoresis System (160 x 160 mm, ATTO, Japan). The samples were treated for 3 min in a boiling-water bath with 10 mM Tris-HCl buffer (pH 6.8), 1% SDS, 1% 2-mercaptoethanol and 20% glycerol to reduce protein. Electrophoresis was conducted at 20 mA constant current in 15% acrylamide gel with 25 mM Tris-192 mM glycine buffer (pH 8.3) containing 0.1% SDS. Proteins were stained with 0.25% Coomassie Brilliant Blue R-250 in the solution of methanol : acetic acid : distilled water = 4:1:5, and destained with the above solution containing no Coomassie Brilliant Blue R-250. An electrophoresis calibration kit (Pharmacia Fine Chamicals, Sweden) was used for determining molecular weight of protein: phosphory-

lase b (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20100), and α-lactalbumin (14400).

Results

Purification of chymotrypsinogens

A typical result of CM-cellulose column chromatography from the preparation of the *aa* phenotype is shown in Fig.4-1a. Low amounts of two trypsinogens were first eluted (Tg-I in void volume, Tg-II at 53-105 mM NaCl), then one chymotrypsinogen (Chtg a) was eluted at 110-190 mM NaCl. The chromatography profile from the preparation of the *AA* phenotype was very similar to that from the preparation of the *aa* phenotype, one chymotrypsinogen (Chtg A at 130-200 mM NaCl) and two trypsinogens (Tg-I in void volume, Tg-II at 72-130 mM NaCl) were separated (Fig. 4-1A). The fractions of each sample which exhibited only chymotryptic activity were pooled and dialyzed against distilled water, respectively. The dialysates of each sample were, respectively, subjected to gel filtration on Sephadex G-100 column, using Buffer C as eluant. The results indicate that chymotrypsinogens from the preparations of both phenotypes *aa* and *AA* were eluted as a single peak (Fig. 4-2a and 4-2A).

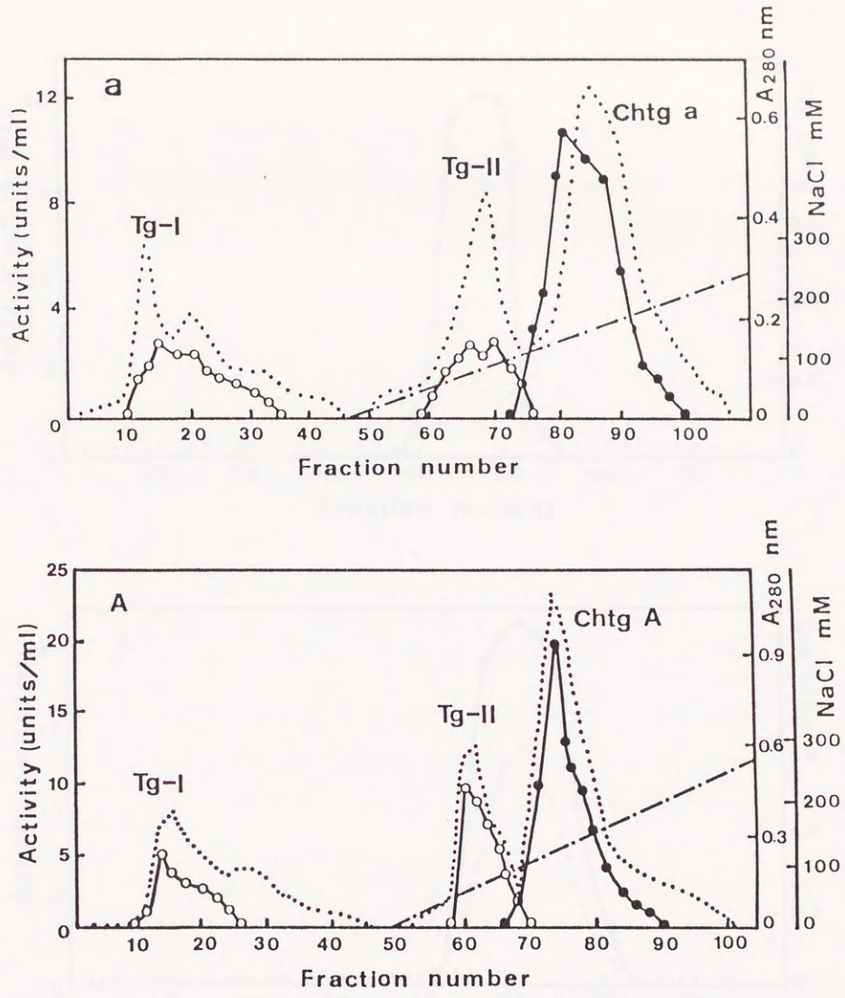


Fig. 4-1. Chromatography profiles of pancreatic extracts from the phenotypes *aa* (a) and *AA* (A) on a column (2.5 x 25 cm) of CM-cellulose. The column was eluted by a linear gradient from 0 to 600 mM NaCl (— • —) in 50 mM ammonium acetate buffer (pH 5.0). Fractions of 5 ml were collected and the chymotryptic (●—●) and tryptic activities(○—○) were assayed after activation. Absorbance at 280 nm(•••••).

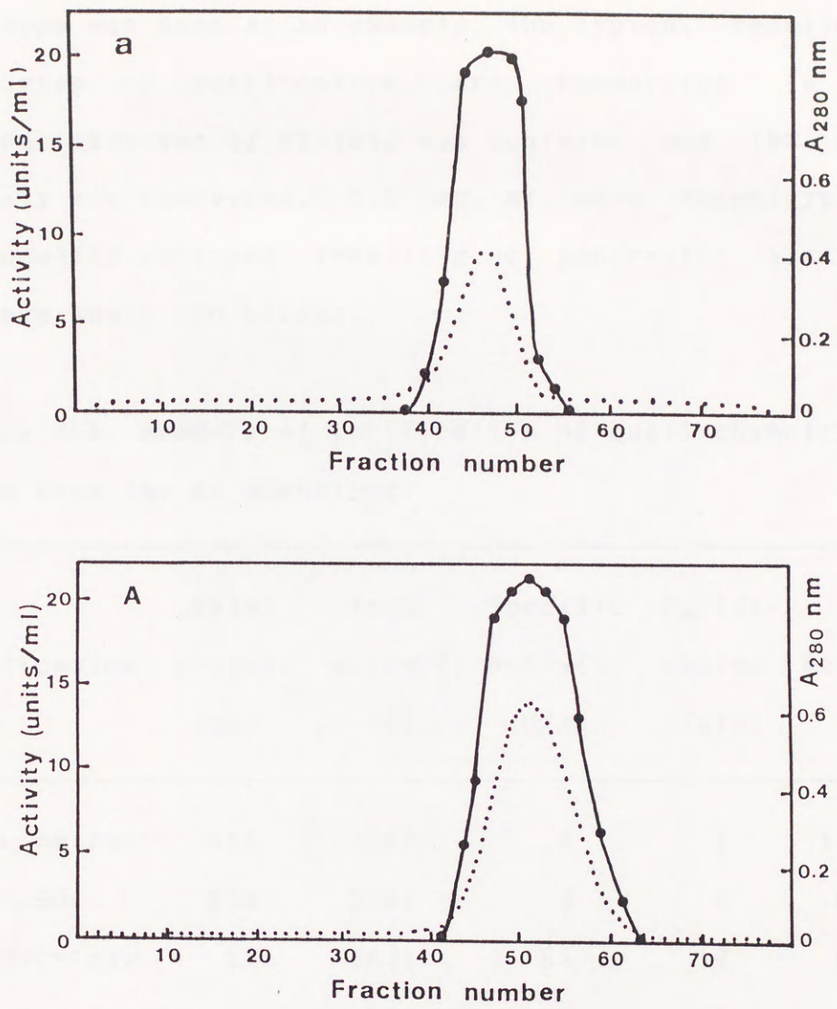


Fig. 4-2. Gel filtration of quail chymotrypsinogens from the phenotypes *aa* (a) and *AA* (A) on a column (2.5 x 65 cm) of Sephadex G-100. The column was eluted by 50 mM ammonium acetate buffer (pH 5.0). Fractions of 5 ml were collected. The chymotryptic activities (●—●) were assayed after activation. Absorbance at 280 nm (·····).

The purification of chymotrypsinogen from the *aa* phenotype was used as an example, the typical results from each step of purification are summarized in Table 4-1. Purification of 22-fold was achieved and 19% of the activity was recovered. 6.0 mg of pure chymotrypsinogen were usually obtained from 11 g of pancreatic tissues of Japanese quail (30 birds).

Table 4-1. Summary of purification of quail chymotrypsinogen from the *aa* phenotype.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
H_2SO_4 extract	681	2723	4	1	100
$(NH_4)_2SO_4$	296	2334	8	2	85
CM-cellulose	27	1841	68	17	67
Sephadex G-100	6	516	86	22	19

Molecular weight

In order to examine the purity of chymotrypsinogens purified from quail pancreas and to determine their molecular weights. The two chymotrypsinogens were loaded on

a SDS-PAGE. The results indicate that both chymotrypsinogens have a monomeric structure and the same electrophoretic mobility (Fig. 4-3). Their molecular weights were estimated as 28500 (Fig. 4-4).

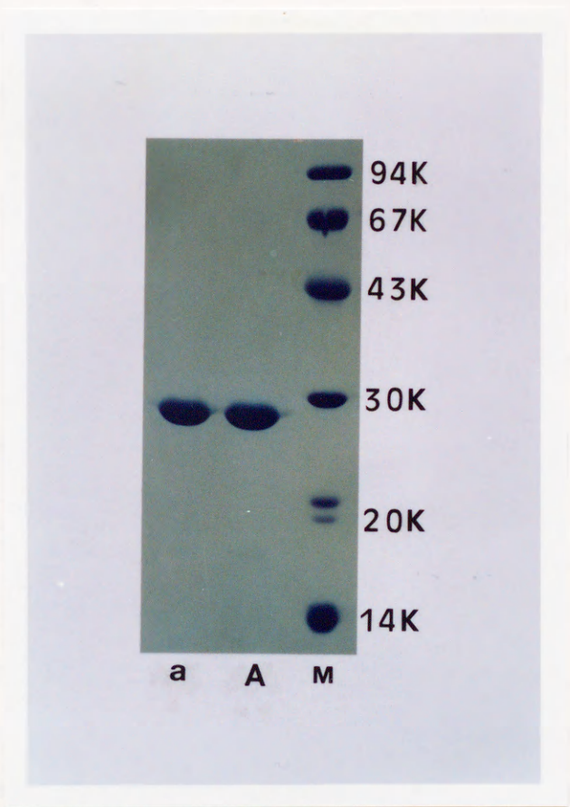


Fig. 4-3. SDS-polyacrylamide gel electrophoresis of purified chymotrypsinogens of quail. Lane a is the chymotrypsinogen from the *aa* phenotype; lane A is the chymotrypsinogen from the *AA* phenotype; lane M is the marker proteins.

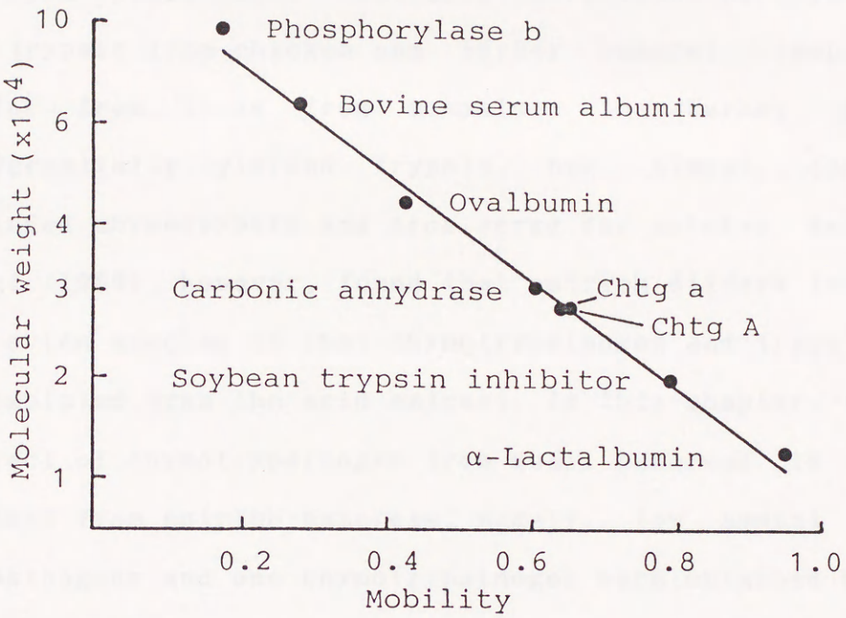


Fig. 4-4. Molecular weight determination of quail chymotrypsinogens by SDS-polyacrylamide gel electrophoresis. The logarithms of the molecular weights of the marker proteins are plotted vs the observed electrophoretic migration. Chtg a is the chymotrypsinogen from the *aa* phenotype; Chtg A is the chymotrypsinogen from the *AA* phenotype.

Discussion

Ryan (1965) found that acid extractions of chymotrypsin and trypsin from chicken and turkey pancrei respectively differ from those from mammals, the turkey pancreas preferentially yielded trypsin, but almost completely excluded chymotrypsin and *vice versa* for chicken. Westhuizen *et al.*(1989), however, found that ostrich differs from these two avian species in that chymotrypsinogen and trypsin could be isolated from the acid extract. In this chapter, the acid extract of chymotrypsinogen from quail pancreas is similar to that from ostrich pancreas, namely, low amount of two trypsinogens and one chymotrypsinogen were obtained by acid extract. However, the purification of quail chymotrypsinogen differs from that of ostrich chymotrypsinogen, quail chymotrypsinogen can be purified with a single ion exchange chromatography step on a CM-cellulose column with a final clean-up step on a Sephadex G-100 column whereas ostrich chymotrypsinogen is purified by a SP-Sephadex C-50 column because its purification could not be accomplished on a CM-cellulose column (Westhuizen *et al.* 1989). The results indicate that purification of quail chymotrypsinogens by this method is more simple and practical.

The two chymotrypsinogens, one from the *aa* phenotype

and another one from the *AA* phenotype, showed the same chromatographic behaviour and the same molecular weight although they show chymotrypsin variants after activation. These results suggest that the two chymotrypsinogens would differ in molecular structure. In next chapter, the difference in molecular structure between the two chymotrypsinogens will be clarified by peptide mapping.

Summary

The *AA* and *aa* phenotypes of chymotrypsin variants were obtained by using a pancreatic biopsy and backcross matings. The quail pancrei from the *AA* and *aa* phenotypes were used as the materials for purification of chymotrypsinogens.

The two chymotrypsinogens from above two phenotypes were purified by 50 mM H_2SO_4 extraction, 0.2~0.4 saturated $(NH_4)_2SO_4$ fractionation, and chromatographic separation on a CM-cellulose column and a Sephadex G-100 column.

The two chymotrypsinogens, one from the *aa* phenotype and another one from the *AA* phenotype, showed the same chromatographic behaviour and the same molecular weight estimated as 28500 by SDS-polyacrylamide gel electrophoresis.

Chapter 5 Structural Difference of Quail Chymotrypsinogens Forming Chymotrypsin Variants

Introduction

A genetic variation of quail chymotrypsin was found in Chapter 2, and zymogram comparison of chymotrypsin variants and chymotrypsinogens indicate that chymotrypsin variants could be derived from activated products of different chymotrypsinogens (see Chapter 3). Therefore, the two chymotrypsinogens showing chymotrypsin variants were purified from quail pancreas in Chapter 4. However, the two chymotrypsinogens showed the same chromatographic behaviour and the same molecular weight. These results furthermore suggest that the two chymotrypsinogens would differ in molecular structure, so they produce chymotrypsin variants after activation.

This chapter was conducted to clarify the difference in molecular structures between the two chymotrypsinogens from the phenotypes *AA* and *aa*, using peptide mapping. Furthermore, the formation process of chymotrypsin variants in Japanese quail was summarized.

Materials and Methods

Samples

The two chymotrypsinogens purified from the phenotypes *AA* and *aa* (see Chapter 3) were used as samples for agarose gel electrophoresis and peptide mapping.

Agarose gel electrophoresis

See Chapter 2

Protein concentration

Chymotrypsinogen concentrations for peptide mapping were determined by the method of Smith *et al.*(1985). The BCA (bicinchoninic acid) protein assay reagent (Pierce Chemical Company, U.S.A.) was used to assay the protein concentration. The absorbances of chymotrypsinogens at 562 nm were then measured, and the protein concentrations were calculated from the standard curve of bovine serum albumin.

Peptide mapping

Peptide mapping of quail chymotrypsinogens was carried out by the method of Cleveland *et al.*(1977). The two chymotrypsinogens were dissolved at 1 mg/ml in sample buffer (pH 6.8) containing 125 mM Tris, 0.5% SDS and 10% glycerol,

respectively. The samples were then heated to 100°C for 2 min. Proteolytic digestions were carried out at 37°C for 30

Quail chymotrypsinogen

↓

Dissolved in the sample buffer (1 mg/ml)

↓

Heated to 100°C for 2 min, added V8 protease (77 μg/ml)

↓

Incubated at 37°C for 30 min

↓

Added 2-mercaptoethanol and SDS (10% and 2%)

↓

Boiled for 2 min

↓

Loaded on 15% acrylamide gel (160mm x 160mm)

↓

Run at 25 mA constant current for 2 hr

↓

Stained, then destained

↓

Peptide bands

Flow chart 5-1. Procedures of peptide mapping of quail chymotrypsinogen.

min by addition of a final concentration of 77 μg/ml of V8 protease (EC 3.4.21.19) from *Staphylococcus aureus* V8 (Wako Pure Chemical Industries, Japan). Following addition of 2-mercaptoethanol and SDS to final concentrations of 10% and 2%, respectively, proteolysis was stopped by boiling the samples for 2 min. Then about 30 μl of each sample were loaded into a sample well of the 15% acrylamide gel, and the gel was run in SDS-polyacrylamide gel electrophoresis described in Chapter 3. The peptide bands were detected by 0.25% Coomassie Brilliant Blue R-250 staining. The procedures of peptide mapping of quail chymotrypsinogens are represented as Flow chart 5-1.

Results

The two chymotrypsinogens and their chymotrypsins were subjected to agarose gel electrophoresis. As shown in Fig. 5-1, both chymotrypsinogens showed a single band and the same electrophoretic mobility (lane a_1 and A_1). However, the activated chymotrypsinogen from the preparation from the *AA* phenotype showed four bands (lane A_2) whereas another activated chymotrypsinogen from the preparation from the *aa* phenotype showed only three bands, lacking band 5 (lane a_2).

In order to determine if the two chymotrypsinogens

possess different molecular structures, both were proteolyzed by V8 protease and the peptides were separated in SDS-polyacrylamide gel electrophoresis. As shown in Fig. 5-2, the peptide map of chymotrypsinogen from the *aa* phenotype had more two bands of peptide (lane a) than that from the *AA* phenotype (lane A). This result demonstrates that the

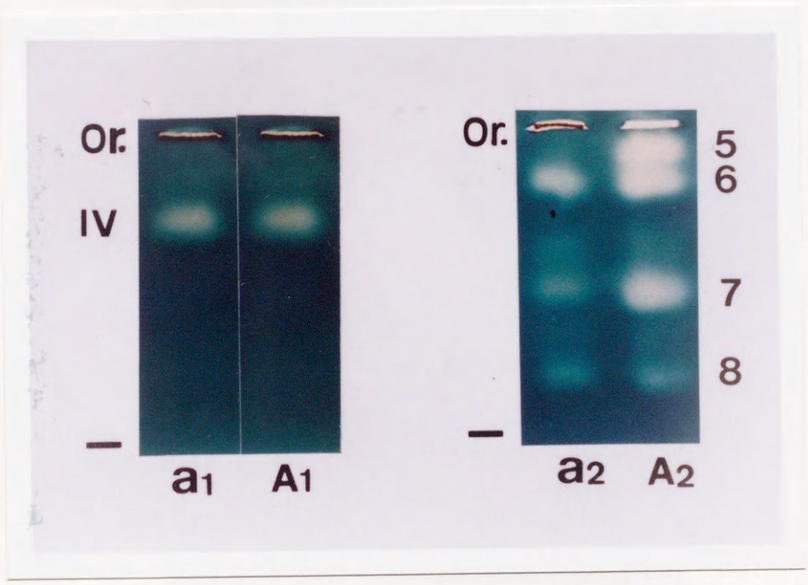


Fig. 5-1. Zymogram comparison of purified chymotrypsinogens (left) and their chymotrypsins (right) of quail. Lanes a_1 and a_2 are the chymotrypsinogen and its chymotrypsin from the *aa* phenotype; lanes A_1 and A_2 are the chymotrypsinogen and its chymotrypsin from the *AA* phenotype, respectively.

molecular structures of the two chymotrypsinogens are different, although they showed almost the same chromatographic behaviour, molecular weight and electrophoretic mobility (see Chapter 3).

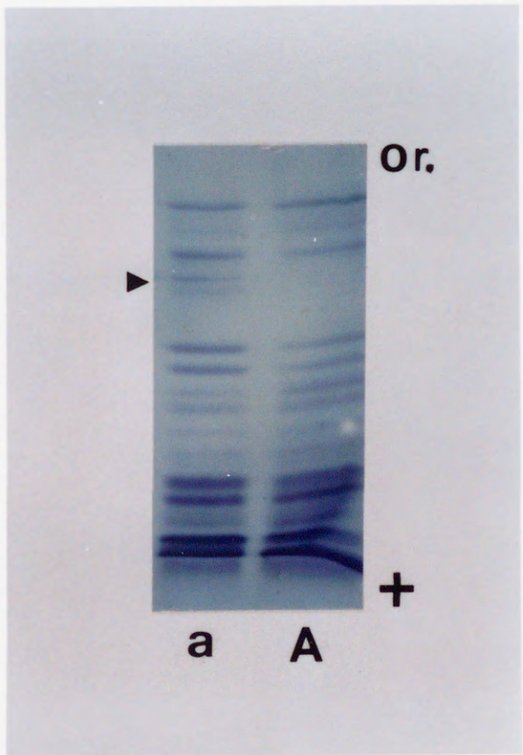


Fig. 5-2. Peptide maps of two chymotrypsinogens from quail pancreas. Lane a is the peptide map of chymotrypsinogen from the *aa* phenotype; lane A is the peptide map of chymotrypsinogen from the *AA* phenotype.

Discussion

Chymotrypsinogen is a precursor of chymotrypsin, and its activation process has been studied extensively in bovine chymotrypsinogen. The π-, δ-, γ- and α-chymotrypsins can be formed by splitting off or hydrolyzing different peptide bonds of bovine chymotrypsinogen when it is activated by trypsin and chymotrypsin (Wright *et al.*,1968; Blow, 1971). It is known that quail chymotrypsinogens also have an activation process, and produce the variants of chymotrypsin after activation (see Chapter 2). In Chapter 3, the two chymotrypsinogens showing chymotrypsin variants were purified from quail pancreas, and no difference was found in their chromatographic behaviour, molecular weights. In this chapter, however, peptide mapping demonstrated that the structures of the two chymotrypsinogens differed, and this difference would determine the formation of chymotrypsin variants because band 5 of chymotrypsin was either detected or was absent after their activation (lane A_2 and a_2 in Fig. 5-1). From these results, it is recognized that the chymotrypsin variants are derived from the activated products of different structures of chymotrypsinogens in Japanese quail. The formation process of chymotrypsin variants can be summarized as Fig. 5-3. The allelic genes, *Prt-5*^{a} and

Prt-5^{A}, control the expression of chymotrypsinogen *a* and chymotrypsinogen *A*. The two chymotrypsinogens have the same molecular weight but possess different molecular structure, therefore, they show the presence and absence of band 5 of chymotrypsin, respectively, after their activation.

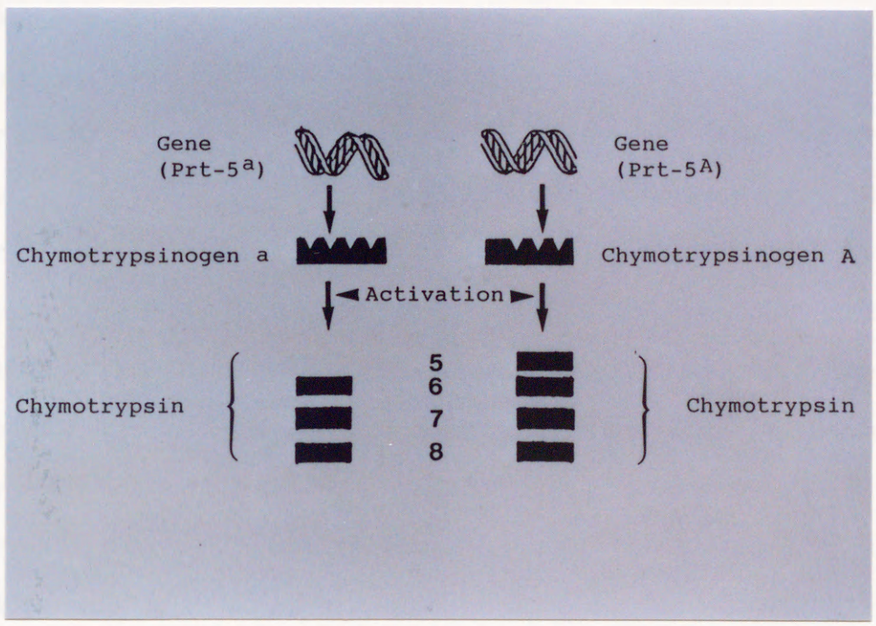


Fig. 5-3. A schematic representation of the formation of chymotrypsin variants in quail.

Summary

The two chymotrypsinogens purified from quail pancreas were activated and subjected to agarose gel electrophoresis. The activated chymotrypsinogen from the *AA* phenotype showed four bands whereas the activated chymotrypsinogen from the *aa* phenotype showed only three bands, lacking band 5.

Furthermore, the two chymotrypsinogens were proteolyzed by V8 protease, and their peptides were separated in SDS-polyacrylamide gel electrophoresis. The peptide map of the chymotrypsinogen from the *aa* phenotype showed more two bands of peptide than that from the *AA* phenotype. This difference of molecular structure of chymotrypsinogens would determine the formation of chymotrypsin variants because band 5 of chymotrypsin was either detected or was absent after their activation.

Chapter 6 Development and Expression of Quail Chymotrypsin Variants

Introduction

In Chapter 2, four bands (5, 6, 7, and 8) in cathode of proteinase zymogram were identified as chymotrypsin, and a genetic variation was found in the presence or absence of band 5. Further study demonstrate that the chymotrypsin variants were derived from activated products of different molecular structure of chymotrypsinogens (see Chapter 5). However, the expression of quail chymotrypsin variants in embryonic development are not yet known.

From the ontogenic point of view, the structural gene loci contained in the genome can be divided into two separate groups: 1) those which engage in transcriptional activity in most or all tissue of the body, and 2) those which become active only in specific tissues (Ohno *et al.*, 1969). A lot of isozyme loci belong to first group (Ohno *et al.*, 1968; Engel *et al.*, 1975; Maeda *et al.*, 1976; Felder *et al.*, 1983). The chymotrypsin locus, on the contrary, belongs to the second group because the chymotrypsin precursor,

chymotrypsinogen, is synthesized only in pancreas.

On the other hand, quail pancreas consists of the dorsal, ventral and third lobes. It is necessary to determine whether the variation at *Prt-5* of chymotrypsin is expressed in all of three lobes of pancreas.

Therefore, this chapter was undertaken 1) to investigate the ontogeny of quail chymotrypsinogen and expression of chymotrypsin variants in embryonic development, 2) to determine the expression of chymotrypsin locus in three lobes of pancreas, and 3) to determine the distribution of activity of chymotrypsin variants on zymograms.

Materials and Methods

Preparation of samples

Eggs of Japanese quail were collected daily and stored at 15°C within two weeks. They were incubated at 38°C with 65~75 percent relative humidity and turned at 6 hr intervals throughout incubation. Embryos at various incubation times were carefully removed and the pancreatic tissues were used as the materials. Before the 13th day of incubation, the pancreas could not be obtained alone, the contents of pancreas and intestines were used as the materials. The pancreatic tissues were weighed and homogenized in 5 volume

of distilled water. The supernatant fluids were stored at -30° C until use.

In order to analyze the expressing relationship of the genes controlling chymotrypsin variants between in adult and in embryo, the birds which show genotypes, *Prt-5*^{A}/*Prt-5*^{A} expressing band 5 of chymotrypsin and *Prt-5*^{a}/*Prt-5*^{a} expressing absence of band 5, were obtained by a pancreatic biopsy and following backcross matings (see Chapter 4). The pancreatic extracts of their progeny in embryos were used as chymotrypsinogen samples, one-half of which were converted into chymotrypsin by activation and used as chymotrypsin samples.

To investigate the expression of chymotrypsin locus in three lobes of pancreas, the pancreatic tissues were carefully separated into the dorsal, ventral and third lobes, and then homogenized. Their homogenates were used as the samples for electrophoresis, respectively.

Agarose gel electrophoresis

see Chapter 2

Densitometric assay

The thin agarose gel was dried in the glass plate at room temperature. The densitometric assay of activity band of chymotrypsin was performed with a Shimadzu Dual-

Wavelength TLC Scanner equipped with a computer system for automatic integration. In order to scan the sites of chymotrypsin activity, the agarose gel glass was loaded on a plate with uniform fluorescence, and was scanned at 313 nm. The integration counts of activity bands of chymotrypsin were obtained by densitometry, and the activity ratio of each band was represented as percentage to total activity of chymotrypsin.

Results

Ontogeny of chymotrypsinogen and trypsinogen in Japanese quail

The ontogeny of chymotrypsinogen and trypsinogen were investigated from the 11th day of incubation to adult quail, using agarose gel electrophoresis. The extracts of pancreas and intestines from the 11th day to the 13th day (-4 day) of incubation showed no detectable band of proteinase, and the pancreatic extracts from the 3rd day after hatching to adult quail showed constant four bands of proteinase. The developmental changes of chymotrypsinogen and trypsinogen were in the period from the 14th day of incubation (-3 days) to the 2nd day after hatching (Fig. 6-1). Bands II and I of trypsinogen, identified in Chapter 3, were observed from

the 14th day and the 15th day of incubation (-3 days and -2 days), respectively. Bands IV and III of chymotrypsinogen, identified in Chapter 3, were detected from the 16th day of incubation (-1 day) and the hatching day (day 0), respectively. At the hatching day (day 0), additional bands except bands I~IV were also observed, and they disappeared at the 2nd day after hatching. The appearance difference of chymotrypsinogen or trypsinogen was observed between

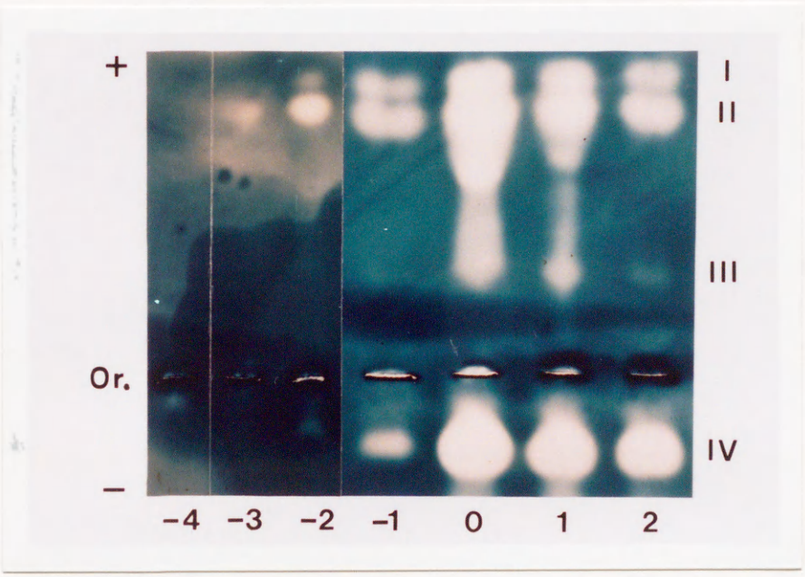


Fig. 6-1. Developmental changes of pancreatic chymotrypsinogen and trypsinogen of quail. The time is represented as day, and the quail is hatched in the 17th day of incubation (day 0).

individual embryos (Table 6-1). For example, at the 14th day of incubation (-3 days), two embryos showed band II whereas other two embryos showed no band. The similar results were also observed in bands I, and III. These results indicate that pancreatic chymotrypsinogen and trypsinogen of Japanese quail appeared in latter period of embryonic development, and they significantly increased to reach highest level at the hatching day, then decreased to constant level at the 2nd day after hatching.

Table 6-1. Ontogeny of pancreatic chymotrypsinogen and trypsinogen of quail.

Incubation time (day)	No. of birds	Zymogram			
		Trypsinogen		Chymotrypsinogen	
		I	II	III	IV
13 (-4)	5	0	0	0	0
14 (-3)	4	0	2*	0	0
15 (-2)	4	3*	4	0	0
16 (-1)	4	4	4	2*	4
17 (0)	4	4	4	4	4

* : Enzyme activity was weak.

( ): The hatching day is represented as day 0.

Expression of chymotrypsin variants in embryo of quail

Figure 6-2 shows the zymograms of chymotrypsinogen and chymotrypsin at the 16th day of incubation (-1 day). Although the two genotypes expressed the same band of chymotrypsinogen (Lane a_1 and A_1), the chymotrypsin variants were observed between two genotypes after activation. The genotype, *Prt-5*^{A}*/Prt-5*^{A}, expressed band 5 of chymotrypsin

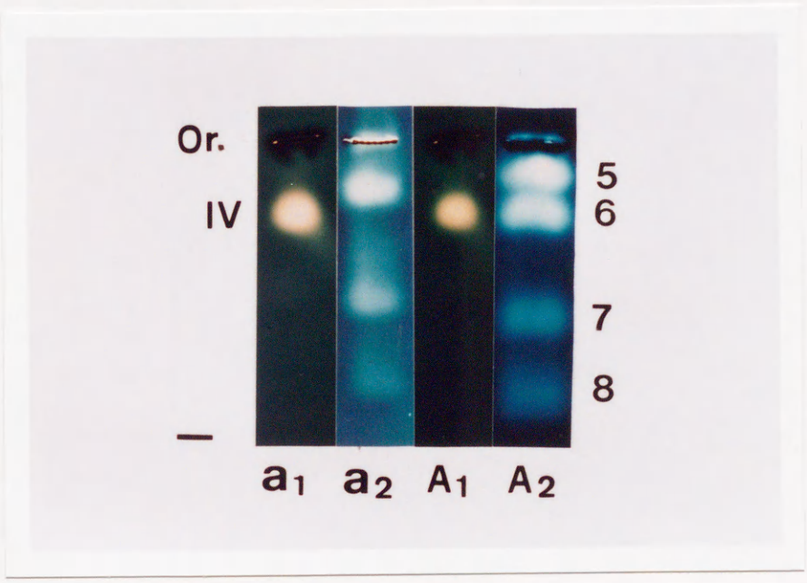


Fig. 6-2. Zymograms of pancreatic chymotrypsinogens and chymotrypsins at the 16th day of incubation in quail. Lane a_1 and a_2 are the electrophoretic patterns of chymotrypsinogen and chymotrypsin from the genotype, *Prt-5*^{a}*/Prt-5*^{a}; lane A_1 and A_2 are the electrophoretic patterns of chymotrypsinogen and chymotrypsin from the genotype, *Prt-5*^{A}*/Prt-5*^{A}.

(lane A_2) while the genotype, *Prt-5*^{a}*/Prt-5*^{a}, did not express band 5 of chymotrypsin (Lane a_2).

Expression of chymotrypsin variants in three lobes of quail pancreas

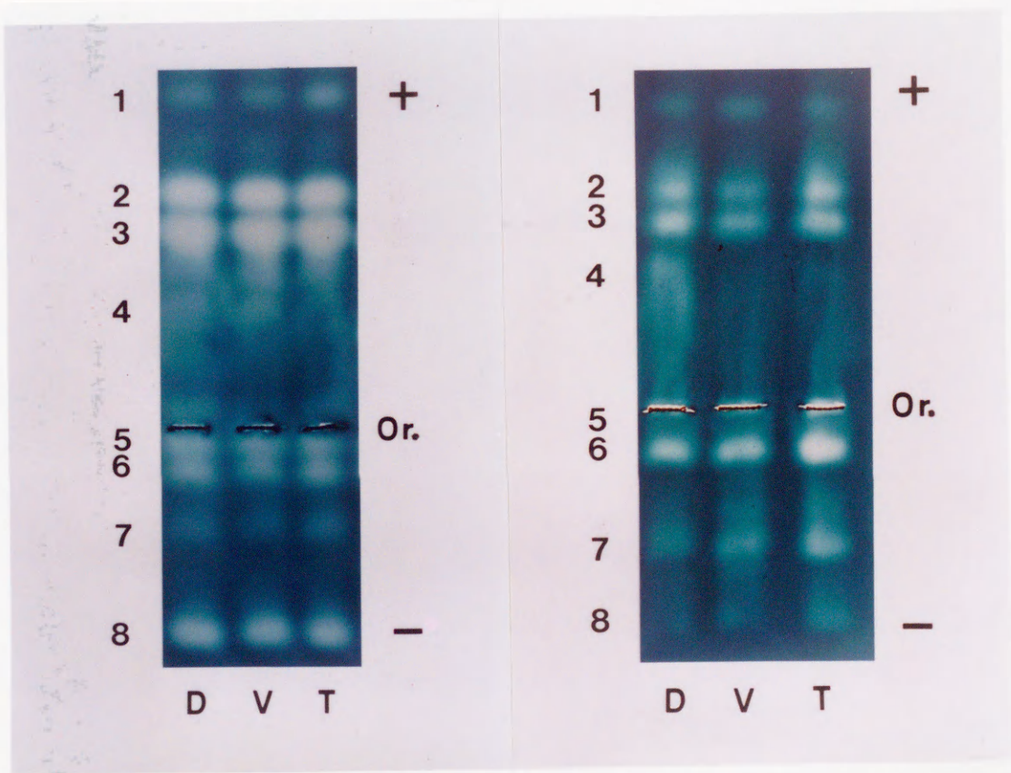


Fig. 6-3. Expression of band 5 in three lobes of quail pancreas. Left is the zymogram of proteinase showing band 5. Right is the zymogram of proteinase lacking band 5. (D) dorsal lobe, (V) ventral lobe, and (T) third lobe of pancreas shown for comparison.

The zymograms of proteinase in three lobes of pancreas were shown in Fig. 6-3, both the presence (left in Fig. 6-3) and the absence (right in Fig. 6-3) of band 5 of chymotrypsin showed no difference in their respective zymograms among the three lobes of pancreas. These results indicate that the allelic genes controlling chymotrypsin variants are expressed in all of three lobes of pancreas.

Distribution of activity of chymotrypsin variants on zymogram

In Chapter 2, chymotrypsin variants were found in the presence or absence of band 5. This experiment was conducted to investigate the distribution of activity of chymotrypsin variants on zymogram, using densitometrical method. As shown in Fig. 6-4, the activities of chymotrypsin variant controlled by genotype, $Prt\text{-}5^{A}/Prt\text{-}5^{A}$, were uniformly distributed in band 5 (31.8 ± 5.5%), band 6 (26.3 ± 0.7%), band 7 (36.1 ± 7.0%), and band 8 (5.8 ± 1.1%) whereas the activities of chymotrypsin variant controlled by genotype, $Prt\text{-}5^{a}/Prt\text{-}5^{a}$ were mainly distributed in band 6 (68.7 ± 6.6%) (a in Fig. 6-4).

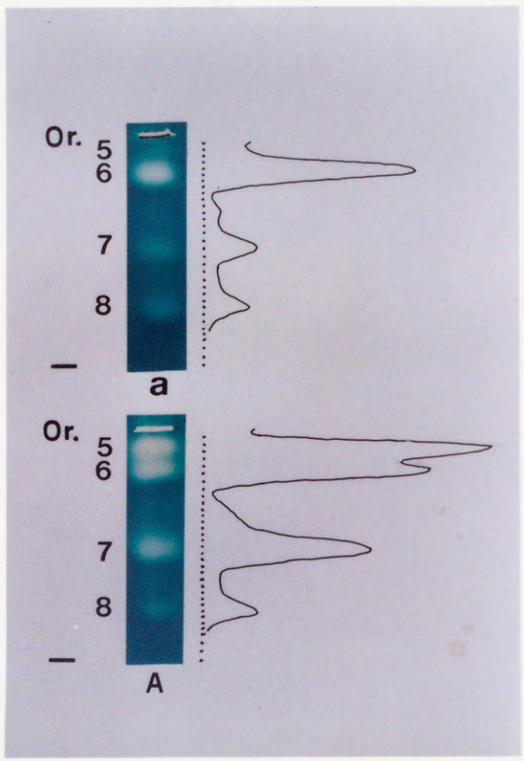


Fig. 6-4. The distribution of activity of quail chymotrypsin variants on zymogram. Left are electrophoretic patterns of chymotrypsin variants, middle are their densitometric patterns, and right are their relative activities. (a) the genotype, *Prt-5*^{a}*/Prt-5*^{a}; (A) the genotype, *Prt-5*^{A}*/Prt-5*^{A}, of chymotrypsin variants.

Discussion

Although the embryology of Japanese quail have been reported (Padgett and Ivey, 1960; Sato *et al.*, 1971), no information is known for chymotrypsinogen development in early embryo. In this chapter, chymotrypsinogen and chymotrypsin variants were detected at the 16th day (-1 day) of incubation which is a latter period of embryonic development. The ontogeny of chymotrypsinogen closely relates to early development of digestive function since chymotrypsinogen is a digestive enzyme which is synthesized in pancreas and secreted into the duodenum for digestive purpose. Expression of quail chymotrypsinogen in latter period of embryonic development would be a ready for digestive function after hatching.

A lot of isozymes showed changes in zymogram patterns with development, and these changes are thought to be indicators of changes in gene expression (Whitt, 1975). However, the patterns of chymotrypsin isozyme in Japanese quail showed no change from first detection at the 16th day of incubation to adult quail. It is recognized that the allelic genes, *Prt-5*^{A} and *Prt-5*^{a}, controlling the chymotrypsin variants are expressed in embryo with the same manner in adult quail.

The pancreas is histologically divided into three parts, i.e., the dorsal lobe, ventral lobe and third lobe. The zymogram did not vary depending on the part. It is recognized that the allelic genes controlling chymotrypsin variants are expressed in all of three lobes of pancreas. The similar result has been reported in pancreatic esterase isozyme in Japanese quail (Hashiguchi *et al.*, 1976).

The difference of activity distribution of chymotrypsin variants on zymograms was observed. The activity of chymotrypsin variant controlled by $Prt\text{-}5^{A}/Prt\text{-}5^{A}$ was uniformly distributed in the four bands whereas the activity of chymotrypsin variant controlled by $Prt\text{-}5^{a}/Prt\text{-}5^{a}$ was mainly distributed in band 6. The author has measured the enzyme activities of two genotypes, $Prt\text{-}5^{A}/Prt\text{-}5^{A}$ and $Prt\text{-}5^{a}/Prt\text{-}5^{a}$, and has found no difference in enzyme activity between them (data not shown). It is suggested that although the genotype, $Prt\text{-}5^{a}/Prt\text{-}5^{a}$, does not possess band 5, the enzyme activity can be compensated by a stronger activity of band 6.

Summary

1. The ontogeny of pancreatic chymotrypsinogen in Japanese quail was determined by using agarose gel electrophoresis. The chymotrypsinogen and chymotrypsin variants of Japanese quail were detected from the 16th day of incubation, and increased to reach highest level at the hatching day, then decreased to constant level at the 2nd day after hatching. It is recognized that the allelic genes, *Prt-5*^{A} and *Prt-5*^{a}, controlling the chymotrypsin variants express in latter period of embryonic development with the same manner in adult quail.

2. Both the presence and absence of band 5 of chymotrypsin showed no difference in their respective zymograms among the three lobes of pancreas. It is recognized that the allelic genes controlling chymotrypsin variants are expressed in all of three lobes of pancreas.

3. The activity of chymotrypsin variant controlled by *Prt-5*^{A}/*Prt-5*^{A} was uniformly distributed in the four bands while the activity of chymotrypsin variant controlled by *Prt-5*^{a}/*Prt-5*^{a}, was mainly distributed in band 6.

Chapter 7 Biochemical Properties of Quail Chymotrypsinogen

Introduction

In the previous chapters, a genetic variation of chymotrypsin was found in Japanese quail, and the modes of inheritance and expression of allelic genes were determined. Furthermore, the relationship between chymotrypsin variants and chymotrypsinogens were analyzed, and it is demonstrated that chymotrypsin variants are derived from the activated products of different molecular structures of chymotrypsinogens. In Chapter 4, the two chymotrypsinogens from the *AA* and *aa* phenotypes were purified, and they showed the same chromatographic behaviour, the same electrophoretic mobility and the same molecular weight. These results suggest that the two chymotrypsinogens have some similarity in the properties. Therefore, the chymotrypsinogen from the *aa* phenotype was used as a represent for characterization of quail chymotrypsinogen in this chapter.

The properties of chymotrypsinogen have been extensively studied in preparations from bovines, including the activation processing (Stroud *et al.*, 1977; Armstrong, 1989), substrate specificity (Barman, 1969; Hess, 1971),

catalytic mechanism (Blow, 1976; Kraut, 1977), complete amino acid sequence (Hartley, 1964; Smillie *et al.*, 1968), and three dimensional structure (Matthews *at al.*, 1967; Blow, 1971; Kraut, 1971). In the past 30 years, bovine chymotrypsinogen has represented prototype molecule for the study of zymogen structure and function. Therefore, some properties of bovine chymotrypsinogen A were analyzed for comparing the properties of quail chymotrypsinogen in this chapter.

Materials and Methods

Materials

Quail chymotrypsinogen from the *aa* phenotype was prepared as described in the Chapter 4. Bovine chymotrypsinogen A (from bovine pancreas) was purchased from Sigma Chemical Company, U.S.A..

SDS-polyacrylamide gel electrophoresis

See Chapter 4.

Isoelectric focusing

Isoelectric focusing was performed using 1% Servalyt (Serva Feinbiochemica GmbH and Company, U.S.A.) covering the

pH range of 3~10 in a 110-ml column of LKB. The dense solution consisted of 2.1 ml of Servalyt and 57.9 ml of 50% sucrose. The light solution consisted of 0.7 ml of Servalyt and 59.3 ml of 50% sucrose. For stepwise gradient formation, 0, 0.2, 0.4, 0.6 ml....4.6 ml of light solution were measured into 24 tubes labeled 1, 2, 3, 4....24, respectively. Then, 4.6, 4.4, 4.2, 4.0 ml.... 0 ml of dense solution were mixed into tubes 1~24, respectively. Moreover, 0.25 ml of quail chymotrypsinogen at a concentration of 0.60 mg/ml was added to tubes 10~14, respectively.

The lower electrode solution consisted of 50 ml of 50% sucrose and 0.4 ml of concentrated phosphoric acid, and upper electrode solution consisted of 50 ml of distilled water and 0.4 ml of ethylenediamine (Nacalai Tesque, Japan). The column was first applied with the lower electrode solution by a pump, then, the density gradient solutions were poured in the order of 1~24 by the pump. Finally, the upper electrode solution was applied as layer over the gradient.

The voltage for electrolysis was maintained at 800 V for the first 4 hr and at 1200 V for the next 18 hr. Fractions of 1.1 ml were collected. The pH and absorbance of fractions at 280 nm were measured.

Examination of pH effect

The effect of pH on the activity of quail chymotrypsin at a concentration of 20 μg/ml was examined at 25°C with 0.5 mM BTEE substrate at different pH using 100 mM citrate-NaOH buffer (pH 2.0~6.0), 100 mM Tris-HCl buffer (pH 7.0~9.0), and 100 mM Sodium tetraborate-NaOH buffer (pH 10.0~11.0). In order to examine the pH stability, the enzyme was treated at 4°C for 10 hr at different pH buffers described as above. Then, their activities were assayed at 256 nm. Relative activity values were based on the maximum activity taken as 100%.

Examination of temperature effect

The effect of temperature on the activity of quail chymotrypsin at a concentration of 20 μg/ml was examined at pH 8.0 with 0.5 mM BTEE substrate at temperatures ranging from 10°C to 70°C. To examine the thermal stability, the enzyme was treated at temperatures ranging from 0°C to 70°C at pH 8.0 for 30 min, and cooled rapidly on ice. Then, their activities were assayed at 256 nm. Relative activity values were based on the maximum activity taken as 100%.

Determination of kinetic constants

The kinetic constants, Michaelis constant (K_m) and molecular activity (k_o), of quail chymotrypsin for a synthetic substrate (BTEE) were determined at a enzyme

concentration of 100 μg/ml. Enzyme assays were conducted at 25°C in 50 mM Tris-HCl buffer (pH 8.0) containing 15% methanol and 25 mM $CaCl_2$ with 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, and 0.8 mM of BTEE substrate, respectively. The ΔA_{256}/min values were recorded and the activities of chymotrypsin were calculated. Then, values for K_m were calculated with Lineweaver-Burk plots, and values for k_o were calculated with the assumption that there is one active site per mol of enzyme.

Protein inhibitor

The effects of soybean trypsin inhibitor (STI) against the chymotrypsins of quail and bovine were tested at an inhibitor to enzyme molar ratio from 0 to 29 in 50 mM Tris-HCl buffer (pH 8.0) containing 15% methanol and 25 mM $CaCl_2$. The chymotryptic activity was measured before and after the addition of inhibitor. Inhibition was expressed as percentage of the remaining chymotryptic activity as a function of the molar ratio of inhibitor to enzyme.

Active site-directed, irreversible inhibitor

Diisopropylfluorophosphate (DFP, Wako Pure Chemical Industries, Japan) was dissolved in propylene glycol, and the final concentration of propylene glycol was 0.4%. L-1-tosylamino-2-phenylethyl chloromethyl ketone (TPCK, Aldrich

Chemical Company, U.S.A.) was dissolved in methanol, and the final concentration of methanol was 3%. The DEP solution and enzyme solution of quail or bovine were mixed in final concentrations of 0.001 mM and 50 μg/ml, respectively. The TPCK solution and enzyme solution of quail and bovine were mixed in final concentrations of 0.15 mM and 50 μg/ml, respectively. Assays of chymotrypsin activities with inhibitor and without inhibitor were performed at the same time intervals in 50 mM Tris-HCl buffer (pH 8.0) containing 15% methanol and 25 mM $CaCl_2$. Inhibition was expressed as percentage of the remaining chymotryptic activity as a function of time.

Amino acid composition analysis

Hydrolysis of quail chymotrypsinogen was performed by the method of Simpson *et al.*(1975). 20 μg of quail chymotrypsinogen and 20 μl of 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole (Pierce Chemical Company, U.S.A.) were added into heavy walled ignition tube which had been washed with a mixture of H_2SO_4/HNO_3 (3/1), rinsed in deionized water, and oven-dried. Then, the tube was closed *in vacuo* under 5×10^{-2} mm Hg. Quail chymotrypsinogen was hydrolyzed at 115°C for 24, 48 or 72 hr.

For the analysis of half-cystine, the hydrolysate was cooled to 4°C, and 6 μl of pyridine was added, followed by

18 μl of 4 N NaOH to adjusted the pH to 6.8. 20 μl of dithiothreitol (DTT, Wako Pure Chemical Industries, Japan) was added to the neutralized hydrolysate. The hydrolysis tube was immediately flushed with nitrogen for 1 min, and sealed with a layer of Parafilm, then incubated at 37°C for 1 hr. After reduction, 1.2 mg of solid sodium tetrathionate (Pierce Chemical Company, U.S.A.) was added to the hydrolysate, and the mixture was allowed to stand at 25°C for a minimum of 5 hr. The mixture was rotary-evaporated to dryness *in vacuo* at 35°C. To ensure complete removal of pyridine, the residue was redissolved in 10 μl of distilled water and redried. The residue was then dissolved in 40 μl of 0.2 N Na-citrate acid buffer (pH 2.2), filtered through a Millipore membrane (0.45 μm), and subjected to amino acid analysis. The sample preparation for amino acid analysis is represented in Flow chart 7-1.

Amino acid analyses were performed on a Shimadsu LC-6A HPLC instrument equipped with a computer system for automatic integration. For automatic integration, a calibration run with a standard amino acid mixture (Wako Pure Chemical Industries, Japan), prepared in the same manner as the unknown sample, was made to obtain color constants and elution times for each amino acid.

Added 20 μg of quail chymotrypsinogen and 20 μl of 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole into heavy walled tube

↓

Closed the tube *in vacuo* under 5 x 10^{-2} mm Hg and hydrolyzed at 115°C for 24, 48 or 72 hr

↓

The tube was cooled to 4°C and opened. Then, 6 μl of pyridine, 18 μl of 4 N NaOH, and 20 μl of 4 μmole/ml DTT were added

↓

Flushed with nitrogen for 1 min, sealed with Parafilm and incubated at 37°C for 1 hr

↓

Added 1.2 mg of Na-tetrathionate, standed at 25°C for 5 hr

↓

Dried *in vacuo* by evaporator at 35°C, then rinsed with 10 μl of distilled water (repeat 2 times)

↓

Dissolved in 40 μl of 0.2 N Na-citrate acid buffer(pH 2.2)

↓

Filtered through Millipore membrane(0.45 μm), and subjected to amino acid composition analysis

Flow chart 7-1. Sample preparation for amino acid analysis.

Activation of chymotrypsinogen and enzyme assay

See Chapter 2

Peptide mapping

See Chapter 5.

Results

Molecular weight

The molecular weights of quail and bovine chymotrypsinogens were estimated by SDS-polyacryamide gel electrophoresis. As shown in (Fig. 7-1), both quail and bovine chymotrypsinogens had a monomeric structure, respectively. Furthermore, molecular weights of quail and bovine chymotrypsinogens were estimated as 28500 and 27000 (Fig. 7-2), respectively.

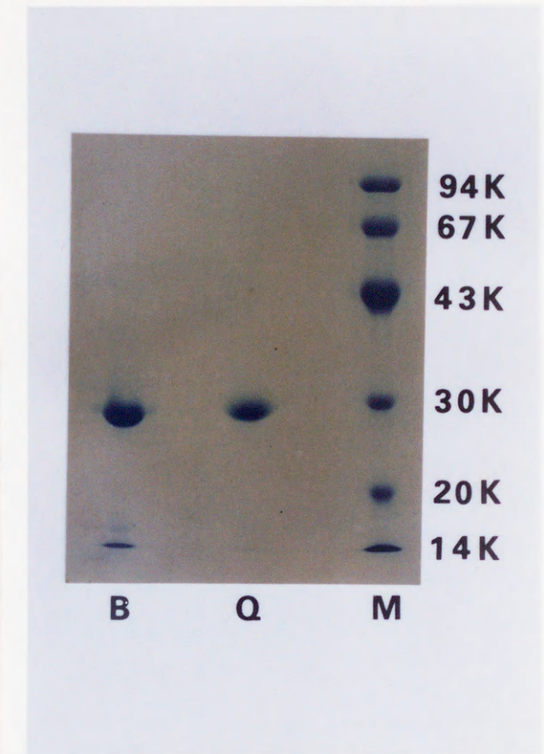


Fig. 7-1. SDS-polyacryamide gel electrophoresis of quail and bovine chymotrypsinogen A. Electrophoresis was performed in 12.5% gel with 25 mM Tris-192 mM glycine buffer (pH 8.3) containing 0.1% SDS at a constant current of 20 mA. Proteins were stained with 0.25% Coomassie Brilliant Blue R-250. lane B is bovine chymotrypsinogen A; lane Q is quail chymotrypsinogen from the *aa* phenotype; lane M is the marker proteins.

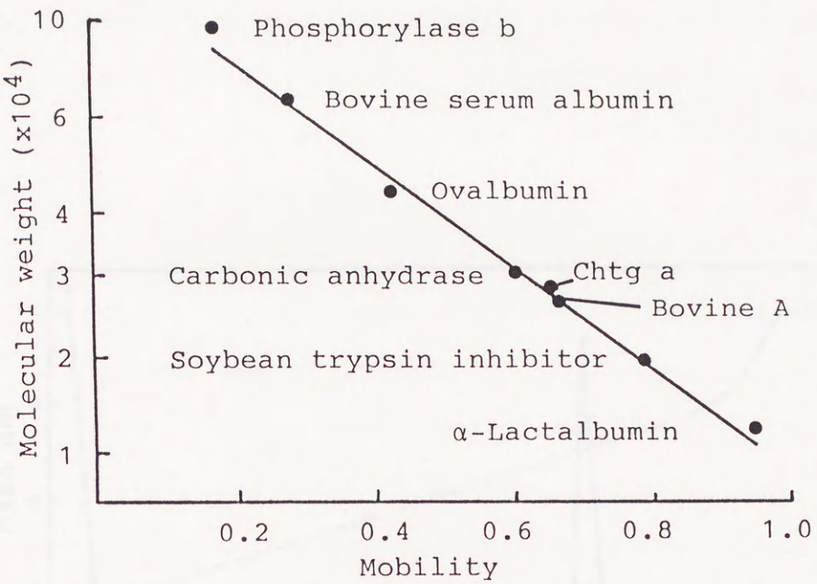


Fig. 7-2. Molecular weight determination of quail and bovine chymotrypsinogen A by SDS-polyacryamide gel electrophoresis. The logarithms of the molecular weights of the marker proteins are plotted vs the observed electrophoretic migration. Chtg a is the chymotrypsinogen from the the *aa* phenotype; Bovine A is bovine chymotrypsinogen A.

Isoelectric point

As illustrated in Fig. 7-3, the quail chymotrypsinogen

appeared as a single peak with an isoelectric point at pH 7.68. On the other hand, the isoelectric point of bovine chymotrypsinogen A has been reported as pH 9.1 (Wilcox, 1970).

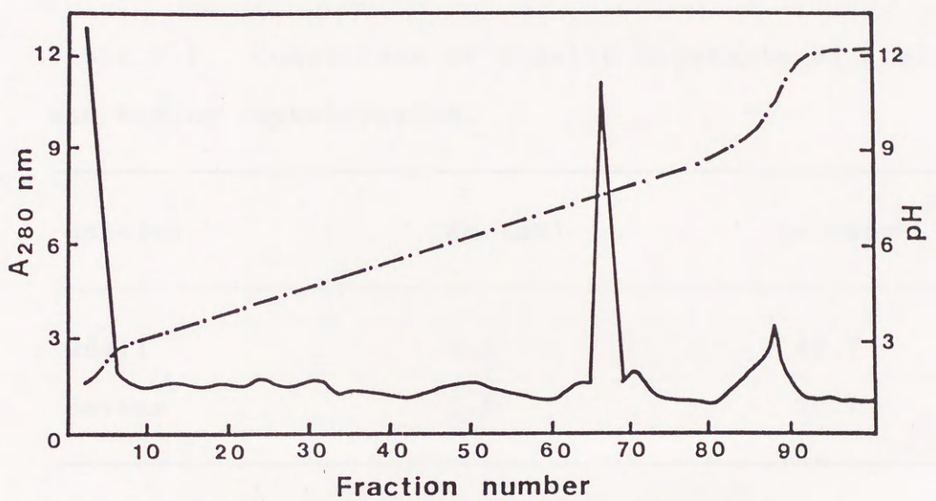


Fig. 7-3. Isoelectric focusing of quail chymotrypsinogen on a sucrose gradient column (vol 110 ml, LKB) with carrier Servalyt of pH 3~10. Electric focusing was performed at 800 V for the first 4 hr and 1200 V for the next 18 hr. Fractions of 1.1 ml were collected, and their pH values(— • —) and absorbance at 280 nm (——) were measured.

Kinetic constants

The kinetic constants, Michaelis constant (K_m) and molecular activity (k_o), of quail and bovine chymotrypsins for a synthetic substrate (BTEE) are compiled in Table 7-1. The K_m and k_o values of quail chymotrypsin were 3.1 mM and 40.7 sec^{-1}, respectively, and these values are very close to those of bovine chymotrypsin.

Table 7-1. Comparison of kinetic constants of quail and bovine chymotrypsins.

Species	*Km* (mM)	*ko* (sec^{-1})
Quail	3.1	40.7
Bovine	2.8	39.1

pH and thermal stability

Figure 7-4 indicates that quail chymotrypsin had maximum activity at pH 7.0~8.0, and was stable at a pH range of 4.0~6.0, however, the activity was lost completely at pH 2.5. It has been reported that bovine chymotrypsin was most stable at pH 3.0~3.5 and had maximum activity at pH 8.0~9.0 (Wilcox, 1970).

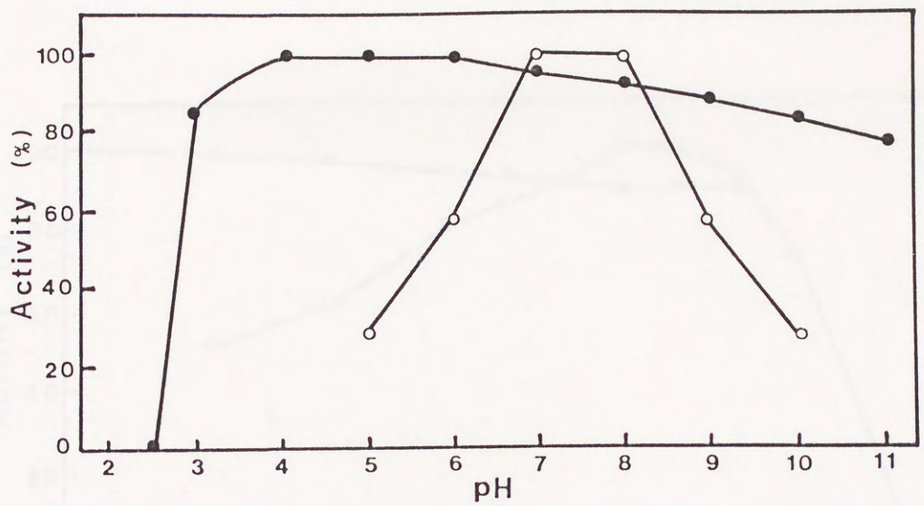


Fig. 7-4. Effect of pH on the activity and stability of quail chymotrypsin. The chymotryptic activity was assayed at the indicated pH. To examine the pH stability, the enzyme was treated for 10 hr at 4°C at the indicated pH. The residual activity was assayed. Optimal pH (○—○); pH stability (●—●).

The effect of temperature on the activity of quail chymotrypsin was shown in Fig. 7-5, quail chymotrypsin had maximum activity at 45°C and was stable below 55°C, however the activity was lost completely at 70°C.

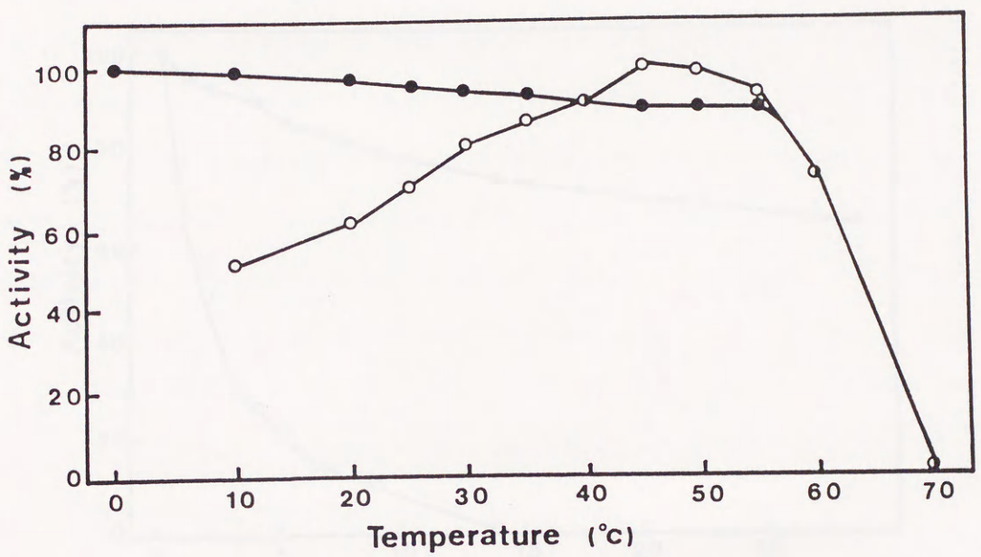


Fig. 7-5. Effect of temperature on the activity and stability of quail chymotrypsin. The chymotryptic activity was assayed at the indicated temperature at pH 8.0. To examine the thermal stability, the enzyme was treated at pH 8.0 at indicated temperature for 30 min. The residual activity was assayed. Optimal temperature(○—○); thermal stability (●—●).

Inhibition of chymotryptic activity by inhibitor

As shown in Fig. 7-6, the soybean trypsin inhibitor(STI) hardly inhibited the activity of quail chymotrypsin. Even as high as 29:1 molar ratio of inhibitor to enzyme, only 35%

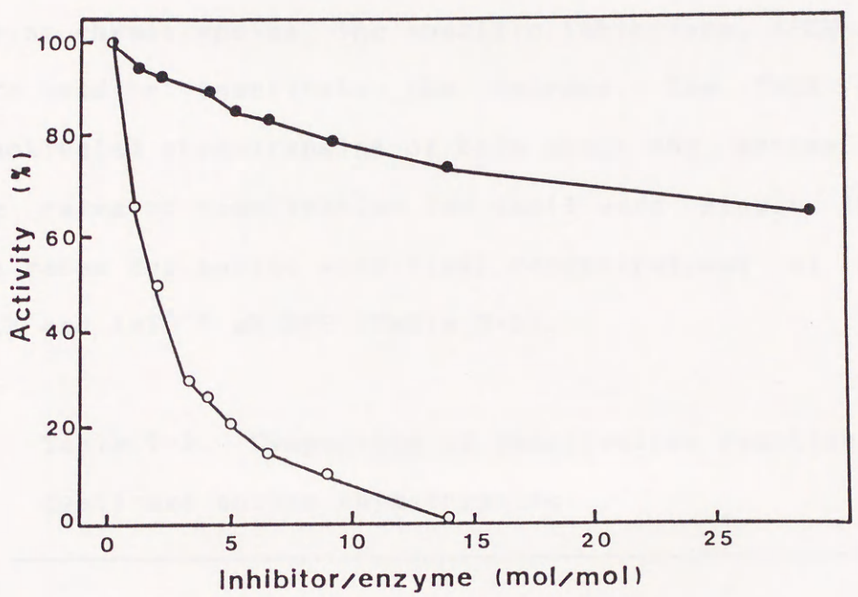


Fig. 7-6. Inhibition of quail(●—●) and bovine (○—○) chymotrypsins by soybean trypsin inhibitor (STI). After the mixture of chymotrypsins and the STI were incubated at 25°C for 6 min, the residual activities of chymotrypsins were assayed and plotted against the molar ratio of inhibitor to enzyme.

inhibition was observed whereas STI easily inhibited the bovine chymotrypsin, and 50% and 100% inhibition were observed at 2:1 and 14:1 ratios of inhibitor to enzyme, respectively.

Examination of active structure

In order to examine the active structure of quail and bovine chymotrypsins, the specific inhibitors, TPCK and DFP, were used to inactivate the enzymes. The TPCK and DFP inactivated chymotrypsins of both quail and bovine. However, the rates of inactivation for quail were slower than the the rates for bovine with final concentrations of 0.15 mM TPCK and 1×10^{-3} mM DFP (Table 7-2).

Table 7-2. Comparison of inactivation reaction of quail and bovine chymotrypsins.

Species	$t_{1/2}$ of inactivation reaction (min)*	
	TPCK	DFP
Quail	300	240
Bovine	95	60

* Half-time of reaction determined from semi-logarithmic first-order plots of the reactions.

Amino acid composition

The amino acid composition of quail chymotrypsinogen was analyzed, and was compared with that of bovine chymotrypsinogen A in Table 7-3. The numbers of amino acid

Table 7-3. Comparison of amino acid composition of quail and bovine chymotrypsinogens.

Amino acid	Quail chymotrypsinogen[1)]	Bovine chymotrypsinogen A[2)]
Lys	10	14
His	6	2
Arg	10	4
Asp	22	23
Thr	14	23
Ser	20	28
Glu	24	15
Pro	14	9
Gly	24	23
Ala	18	22
Val	20	23
Met	2	2
Ile	12	10
Leu	18	19
Tyr	8	4
Phe	4	6
Cyh	10	10
Try	6	8
Total	242	245
Mol.wt.($\times 10^3$)	26.1	25.6

1) Integer of average of 24, 48 and 72 hr hydrolysates.

2) Hartley *et al.* (1964).

residues and minimum molecular weight of quail chymotrypsinogen calculated from these data were 242 and 26100, respectively. Moreover, the quail chymotrypsinogen contained relatively larger amounts of histidine, arginine, glutamic acid and tyrosine; but smaller amounts of threonine and serine than bovine chymotrypsinogen A although they were very similar in total residues of amino acid. The absorbance indexes of quail and bovine chymotrypsinogen A, $E^{1\%}_{1cm}$, were found to be 19.6 and 20.0, respectively, at 280 nm.

Peptide maps

The peptide maps of quail and bovine chymotrypsinogens proteolyzed by V8 protease from *Staphylococcus aureus* V8 are illustrated in Fig.7-7. The number and size of peptides of the two zymogens showed definite differences. It indicates that some differences in the primary structure would exist between quail and bovine chymotrypsinogens, and the quail chymotrypsinogen is more easily to be proteolyzed by V8 protease than bovine chymotrypsinogen A.

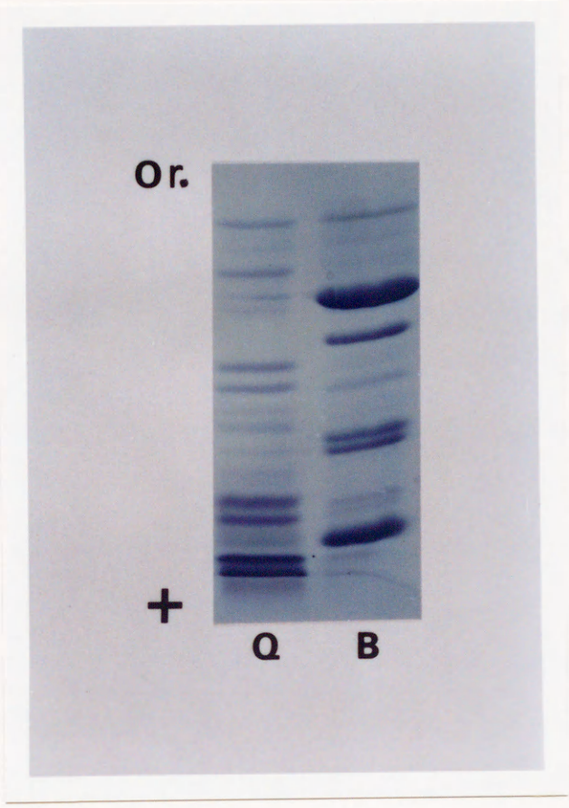


Fig. 7-7. Peptide maps of quail and bovine chymotrypsinogen A. Lane Q is the peptide map of quail chymotrypsinogen from the *aa* phenotype; lane B is the peptide map of bovine chymotrypsinogen A.

Discussion

In this chapter, a quail chymotrypsinogen from the *aa* phenotype was characterized, and the properties were compared with bovine chymotrypsinogen A. The results indicate that quail chymotrypsinogen is very similar to bovine chymotrypsinogen A in active structure of enzyme, specific activity, pH and thermal stability. These facts suggest that this enzyme contributes to the digestion of protein in avians in much the same way as in the mammal, and support our previous results that activation conditions of quail chymotrypsinogen and its activity after activation were more similar to mammalian chymotrypsinogen (Hou *et al.*, 1988a, b)

On the other hand, quail chymotrypsinogen showed definite differences with bovine chymotrypsinogen A in molecular weight, amino acid composition, peptide maps and isoelectric point. These suggest that some portions of the molecular structure are different between quail and bovine chymotrypsinogens. The difference in the isoelectric point may be evidence of differences in the amino acid composition between quail and bovine. The differences in peptide maps proteolyzed by V8 protease demonstrate that there are some differences in the primary structure between quail and

bovine chymotrypsinogens, and supports the finding that quail chymotrypsinogen contains larger amounts of glutamic acid than bovine chymotrypsinogen A (Table 7-3) as V8 protease specifically cleaves peptide bonds of the COOH-terminal side of either aspartic acid or glutamic acid (Drapeau *et al.*, 1972).

Quail chymotrypsin was irreversibly inhibited by the specific inhibitors, DFP and TPCK. It is known that DFP reacts with one active center serine-195 of bovine chymotrypsin (Hartley, 1964), and that TPCK reacts only with another active center histidine-57 of bovine chymotrypsin (Stevenson and Smillie, 1965). Hence, it could be concluded that quail chymotrypsin is present with active structure of the same histidine-serine type as bovine chymotrypsin. On the other hand, the inhibition reaction to quail chymotrypsin was slower than that to bovine, and it is thought that the three-dimensional structure of quail chymotrypsin would differs from bovine chymotrypsin so that access to the active center may be slower.

The comparison of quail and bovine chymotrypsinogens indicates that the activity of this enzyme from quail and bovine is more constant than their physical characteristics.

Summary

A quail chymotrypsinogen from the *aa* phenotype was characterized, and the properties were compared with bovine chymotrypsinogen A.

1. Quail chymotrypsinogen had the amino acid residues of 242 and a molecular weight of 26100 calculated from amino acid composition data. The quail chymotrypsinogen contained larger amounts of histidine, arginine, glutamic acid and tyrosine; and smaller amounts of threonine and serine than bovine chymotrypsinogen A. The isoelectric point was 7.68.

2. Michalis constant (K_m) of quail chymotrypsinogen was 3.10 mM, and molecular activity (k_o) was 40.7 sec^{-1} for tyrosine ester substrate.

3. The quail chymotrypsin had maximum activity at pH 7.0~8.0 and at 45°C, and was stable at pH 4.0~6.0 below 55°C.

4. Quail chymotrypsin had the same active structure of histidine-serine type as bovine chymotrypsin. However, quail chymotrypsin was hardly inhibited than bovine chymotrypsin by protein or specific inhibitors.

5. Comparisons of quail and bovine chymotrypsinogen indicate that they showed similarity in specific activity, optimal pH and temperature, but difference in molecular

weight, amino acid composition, peptide map and isoelectric point. It is recognized that the activity of this enzyme from quail and bovine is more constant than their physical characteristics.

Chapter 8 General Discussion

1. The mode of inheritance of chymotrypsin

Genetic studies on chymotrypsin have been first carried out in mouse. Watanabe *et al.* (1974, 1976a, b, 1987, 1989) have reported that a variation of mouse chymotrypsin showed a difference in electrophoretic mobility of bands II and III on pancreatic proteinase zymogram, and it is controlled by two codominant allelic genes, *Prt-2*^{a} and *Prt-2*^{b}, on chromosome 8.

In the present study, a variation of chymotrypsin in Japanese quail was found in the presence or absence of band 5 on pancreatic proteinase zymogram, and this variation is controlled by a pair of allelic genes, *Prt-5*^{A} and *Prt-5*^{a}, on an autosomal locus.

On the basis of data of isozymes, the modes of inheritance of isozymes controlled by one locus can be divided into two types:

I. Codominant:

A. Heterozygote forms a hybrid enzyme.

Dove 6-phosphogluconate dehydrogenase (*6-PGD*)

(Cooper *et al.*,1969)

Chicken acid phosphatase

(Okada and Hachinohe, 1968)

B. Heterozygote forms no hybrid enzyme.

Fowl serum amylase (*Amy-1*)

(Hashiguchi *et al.*, 1970)

Quail acid phosphatase (*Acp-2*)

(Maeda *et al.*, 1976)

Mouse chymotrypsin (*Prt-2*)

(Watanabe *et al.*, 1974, 1976a, b)

II. Dominant:

A. One band is dominant to other band

Chicken serum alkaline phosphatase

(Wilcox, 1966)

Chicken leucine aminopeptidase

(Law, 1967)

B. Presence of band is dominant to absence of band.

Cattle acid phosphatase

(Gahne, 1963)

Quail serum esterase (*Es-5*, *Es-6*, and *Es-7*)

(Hashiguchi *et al.*, 1981)

According to above classification, chymotrypsin isozyme of Japanese quail belongs to type II-B since the presence and absence of band 5 of chymotrypsin are controlled by dominant allelic genes, and gene $Prt\text{-}5^{A}$ causing the presence of band 5 is dominant to gene $Prt\text{-}5^{a}$ causing absence of band 5. On the other hand, mouse chymotrypsin isozyme showed a difference in electrophoretic mobility of band, and this

variation is controlled by codominant allelic genes, therefore, mouse chymotrypsin isozyme belongs to the type I-B. These facts suggest that the mode of inheritance of an enzyme may be different between species.

2. Structural difference of chymotrypsinogens forming chymotrypsin variants in Japanese quail

The presence of chymotrypsin variants suggests a possibility of presence of chymotrypsinogen variants, since chymotrypsin is formed from chymotrypsinogen by activation. In this study, the two chymotrypsinogens corresponding to chymotrypsin variants were purified, and it is clarified that chymotrypsin variants are derived from the activated products of different molecular structures of chymotrypsinogens.

Kaplan(1968) summarized the nature of multiple molecular forms of enzyme, and pointed out that molecular forms of enzyme controlled by allelic genes have usually small sequence differences between them, but may have striking difference in their physical and catalytic characteristics. In this study, the molecular forms of quail chymotrypsinogens also have small differences in the primary structure, since the peptide map of chymotrypsinogen from the *aa* phenotype had more two bands of peptide than that from the *AA* phenotype. However, the two chymotrypsinogens from the *AA*

and $\alpha\alpha$ phenotypes had no striking difference in their physical and enzymatic characteristics since they had the same molecular weight, the same chromatographic behaviour, and similar enzymatic activity. Therefore, it can be concluded that the difference of molecular structure of quail chymotrypsinogens would determine the formation of chymotrypsin variants, but this difference does not affect on their physical and enzymatic characteristics, and it would be belonged to neutral mutation of amino acid.

3. Development and expression of chymotrypsin variants

Electrophoretic analysis of isozyme can be used not only for determination of inheritance mode but also for development genetic study of isozyme. In the present study, the expression of pancreatic chymotrypsin locus in embryonic development of Japanese quail was determined by using agarose gel electrophoresis. The chymotrypsinogen and chymotrypsin variants were detected from the 16th day of incubation. This fact indicates that the allelic genes controlling the chymotrypsin variants express in latter period of embryonic development in Japanese quail. It is thought that the ontogeny of chymotrypsinogen closely relates to early development of digestive function, therefore, the expression of quail chymotrypsinogen in latter period of embryo would be a ready for digestive

function after hatching.

Enzyme is a product of gene, therefore, changes in isozyme patterns can be used as indicators of changes in gene expression (Whitt, 1975). The developmental studies of isozymes have been extensively carried out in lactate dehydrogenase (LDH) (Whitt, 1970), acid phosphatase (*Acp*-2) (Maeda *et al.*, 1976), and glucose 6-phosphate dehydrogenase (6PGD) (Leung and Haley, 1973), and the results indicate that patterns of some isozymes may be different in various development period. For example, mouse LDH showed only LDH-1 band in whole preimplantation development of embryo while shortly after implantation only LDH-5 band is present, then bands of LDH-4, LDH-5 and LDH-2 appeared subsequently (Auerbach and Brinster, 1967). The patterns of chymotrypsin isozyme in Japanese quail, on the contrary, had no change from first detection at the 16th day of incubation to adult quail. It is suggested that the allelic genes controlling the chymotrypsin variants are expressed in the embryo of quail with the same manner as in adult quail.

From the ontogenic point of view, the structural gene loci contained in the genome can be divided into two separate groups: 1) those which engage in transcriptional activity in most or all tissues of the body (Ohno *et al.*, 1968; Engel *et al.*, 1975), and 2) those which become active only in specific tissues (Ohno *et al.*, 1969). Therefore, the

chymotrypsin locus is belonged to the second group since the chymotrypsin precursor, chymotrypsinogen, is synthesized only in pancreas.

4. Properties of quail chymotrypsinogen

The properties of chymotrypsinogen have been extensively studied in preparations from bovines. The bovine chymotrypsinogen has served as model to examine similarities in structure and properties of chymotrypsinogens from other species.

In the present study, the properties of quail chymotrypsinogen were clarified and compared with those of bovine chymotrypsinogen A. Quail chymotrypsinogen is similar to bovine chymotrypsinogen A in active structure of enzyme, specific activity, pH and thermal stability, however, quail chymotrypsinogen is different in molecular weight, amino acid composition, peptide map and isoelectric point with bovine chymotrypsinogen A. These results indicate that the activity of this enzyme from quail and bovine is more constant than their physical characteristics.

The properties of chymotrypsins and chymotrypsinogens from various species have been compared. Zendzian and Barnard (1967b) have investigated the structure of active structure of chymotrypsins from mammals and various lower vertebrates with specific inhibitors, DFP (diisopropyl-

fluorophosphate) and TPCK (L-1-tosylamino-2-phenylethyl chloromethyl ketone). It is known that DFP reacts with one active center serine-195 of bovine chymotrypsin (Hartley, 1964), and TPCK reacts only with another active center histidine-57 of bovine chymotrypsin (Stevenson and Smillie, 1965). They have reported that both DFP and TPCK removed virtually all of chymotrypsin activity on benzoy-L-tyrosine ethyl ester (BTEE) of substrate. In the present study, the activity of quail chymotrypsin was also irreversibly inhibited by the DFP and TPCK. Therefore, it could be concluded that quail chymotrypsin is present with active structure of the same histidine-serine type as bovine chymotrypsin.

Bhargava and Barnard (1973) have compared the N-terminal sequence of bovine chymotrypsinogens with that of porcine and three species turtles, and have found that the basic point of activating cleavage in these species are the same as in the case of bovine chymotrypsinogen. Tomita *et al.*(1989) have compared the complete amino acid sequences of chymotrypsinogens from bovine, canine and human, and have found that point of activating cleavage, active center residues and ten cystine residues to establishing the tertiary structure are fully conserved among the three mammalian chymotrypsinogens.

On the other hand, comparison of N-terminal amino acid

sequence of chymotrypsinogens also indicate that a hyper-variable segment exists in N-terminal amino acid sequence of above species. It is thought that this segment is not essential for active structure, and may be for requirements for protein folding in various species (Bhargava and Barnard, 1973). Moreover, some differences of chymotrypsinogens from various species have been found in molecular weight and amino acid composition (Wilcox, 1970; Westhuizen *et al*; 1989), isoelectric point and charge (Prahl and Neurath, 1966; Bhargava and Barnard, 1973).

These studies indicate that the evolution of function and structure of chymotrypsin and chymotrypsinogen is conservative. The important segments to the maintenance of active structure of enzyme are conserved whereas some segments, having little relationship to maintenance of active structure of enzyme, tend to accumulate evolutionary changes at a rate faster than that of the rest of segments. Therefore, the function of chymotrypsin to hydrolyze protein is kept to be constant in various species although there may be some differences in physical features.

Chymotrypsin plays an important role in the digestion of protein in animal feeds. However, little information on genetics and biochemistry is known for chymotrypsin and chymotrypsinogen of avians. The genetic and biochemical

characteristics of chymotrypsin and chymotrypsinogen of quail clarified in this study filled this blank. Therefore, it will be indebtedly beneficial for future research on chymotrypsin and chymotrypsinogen from other avians and animals.

Summary and Conclusion

Chymotrypsin is one of pancreatic proteinase. Its inactive precursor, chymotrypsinogen, is synthesized and stored in pancreas. When needed for digestive purpose, chymotrypsinogen is secreted into duodenum and converted into active chymotrypsin by action of trypsin. The chymotrypsin catalyzes the protein hydrolysis into smaller peptides. Therefore, chymotrypsin plays an important role in the digestion of protein in animal feeds. Extensive studies on the properties of chymotrypsin and chymotrypsinogen have been done in bovines, and some studies on genetics of chymotrypsin have been carried out in mice. However, little information is known for chymotrypsin and chymotrypsinogen of avians.

The present study was conducted to study the genetic and biochemical aspects of chymotrypsin and chymotrypsinogen from Japanese quail (*Coturnix coturnix japonica*), a pilot animal for avian study. A genetic variation of chymotrypsin from quail pancreas was found and the mode of inheritance of chymotrypsin variants was determined. The relationship between chymotrypsin variants and chymotrypsinogens were clarified. Furthermore, the expression of chymotrypsin locus in embryonic development and localization of chymotrypsin

variants in pancreas were investigated. Finally, the biochemical properties of quail chymotrypsinogen were clarified. The contents of this thesis can be summarized as follows:

1. The mode of inheritance of chymotrypsin variation

1). By means of agarose gel electrophoresis, four bands in the anode and three or four bands in the cathode were detected in the zymogram of pancreatic proteinase, and they were designated bands 1~8 from the most rapid anodal band. An individual variation was found in the presence or absence of band 5. Furthermore, bands 5~8 were identified as chymotrypsin by the methods of chromatographic separation, substrate specificity analysis and electrophoretic detection.

2). The results of various matings indicate that the presence or absence of band 5 is controlled by a pair of allelic genes on an autosomal locus, designated the *Prt-5* locus. The gene $Prt\text{-}5^{A}$, causing expression of band 5, is dominant to gene $Prt\text{-}5^{a}$, causing absence of band 5.

3). The gene frequencies of $Prt\text{-}5^{A}$ and $Prt\text{-}5^{a}$ were estimated in a random-bred population (RR) and two lines selected for either large (LL) or small (SS) body weight of quail. The results indicate that the gene frequency of $Prt\text{-}5^{A}$ or $Prt\text{-}5^{a}$ was no significant difference among these three lines.

2. The relationship between chymotrypsin variants and chymotrypsinogens

Zymogram comparison of chymotrypsin and chymotrypsinogen indicates that bands 5~8 of chymotrypsin were formed from chymotrypsinogen by activation. However, one chymotrypsinogen showed band 5 of chymotrypsin while another chymotrypsinogen did not show band 5 after activation. It is suggested that chymotrypsin variants would be derived from different structural chymotrypsinogens. To clarify the relationship between chymotrypsin variants and chymotrypsinogen structure, a pancreatic biopsy and backcross matings were conducted to obtain the quails showing the phenotypes *AA* or *aa*. Then, the two chymotrypsinogens from the phenotypes *AA* and *aa* were purified from quail pancreas by acid extraction, salt fractionation and chromatographic separation on a CM-cellulose column and a Sephadex G-100 column. The two chymotrypsinogens had the same chromatographic behaviour, the same molecular weight and the same electrophoretic mobility. But, the result of peptide mapping indicate that the two chymotrypsinogens had different molecular structures. Therefore, this structural difference would determine the formation of chymotrypsin variants.

3. The expression of chymotrypsin locus in embryonic development and localization of chymotrypsin variants in pancreas.

1). The chymotrypsinogen and chymotrypsin variants were detected from the 16th day of incubation. The allelic genes,

controlling chymotrypsin variants, express in latter period of embryonic development with the same manner as in adult quail.

2). Chymotrypsin locus expressed in all of three lobes of pancreas, since both the presence and absence of band 5 of chymotrypsin showed the same patterns in their respective zymograms among three lobes of pancreas.

4. A quail chymotrypsinogen from the *αα* phenotype was characterized, and its properties were compared with bovine chymotrypsinogen A.

1). Quail chymotrypsinogen had amino acid residues of 242 and a molecular weight of 26100 calculated from amino acid composition data. The quail chymotrypsinogen contained larger amounts of histidine, arginine, glutamic acid and tyrosine, but smaller amounts of threonine and serine than bovine chymotrypsinogen A. The isoelectric point was 7.68.

2). The Michaelis constant (K_m) and molecular activity (k_o) of quail chymotrypsin were 3.10 mM and 40.7 sec^{-1} for tyrosine ester substrate, respectively.

3). The quail chymotrypsin had maximum activity at pH 7.0~8.0 and at 45° C, and was stable at pH 4.0~6.0 below 55° C. However, the activity was lost completely below pH 2.5 and above 70° C.

4). The effects of inhibition of specific inhibitors, DFP (diisopropylfluorophosphate) and TPCK (L-1-tosylamino-2-

phenylethyl chloromethyl ketone), on chymotrypsins of quail and bovine indicate that quail chymotrypsin had the same active structure of histidine-serine type as bovine chymotrypsin. However, quail chymotrypsin was hardly inhibited than bovine chymotrypsin by protein or specific inhibitors.

5). The property comparisons of quail and bovine chymotrypsinogens indicate that they showed similarity in specific activity, optimal pH and temperature, but differences in molecular weight, amino acid composition, peptide map and isoelectric point. It is recognized that the activity of this enzyme from quail and bovine is more constant than their physical characteristics.

Chymotrypsin plays an important role in the digestion of protein in animal feeds. However, little information on genetics and biochemistry is known for chymotrypsin and chymotrypsinogen of avians. Therefore, the genetic knowledge and biochemical properties of chymotrypsin and chymotrypsinogen of quail clarified in the present study will be indebtedly beneficial for future research on chymotrypsin and chymotrypsinogen from other avians and animals.

日本ウズラのキモトリプシンに関する遺伝生化学的研究

要　約

キモトリプシンは、動物における重要なタンパク質分解酵素のひとつである。その前駆体であるキモトリプシノーゲンは膵臓で生合成・貯蔵されており、必要に応じて十二指腸に分泌され、トリプシンによって活性化され、キモトリプシンに変換される。キモトリプシンは食餌中のタンパク質を他のタンパク質分解酵素と共にペプチドまで加水分解する。このように、キモトリプシンは食餌のタンパク質の消化過程に重要な役割を演じている。動物におけるキモトリプシンの生化学的特性に関する研究は、主としてウシを中心として行われており、また、その遺伝学的な研究は、マウスについて報告がなされているのみであり、家禽に関しての研究は殆ど見られない。

本研究は、家禽のパイロットアニマルとして種々の優れた特質を有する日本ウズラ（Coturnix coturnix japonica）を用いて、その膵臓キモトリプシンおよびキモトリプシノーゲンについて遺伝生化学的な特徴を明らかにしたものである。ウズラの膵臓キモトリプシンには遺伝的変異が存在することを見い出し、その遺伝的支配様式を明らかにした。さらに、このキモトリプシンの遺伝的変異とキモトリプシノーゲンとの関係について検討した。また、ウズラのキモトリプシンの遺伝的変異の発現時期およびキモトリプシンの組織局在性についても検討を加えた。最後にウズラのキモトリプシノーゲンの生化学的性質を明らかにした。本論文の大要を示すと次のと

おりである。

1．ウズラの膵臓キモトリプシンの遺伝的変異について

1)．ウズラの膵臓プロティナーゼのアガロース電気泳動像は陽極側に4本、陰極側に4本計8本の活性帯が検出され、これらの活性帯を陽極側より1から8とした。これらのうち、活性帯5の有無については、個体変異が認められた。分画同定した結果、活性帯5～8はキモトリプシンに属するものと判定した。

2)．交配実験の結果から、活性帯5は常染色体上の優劣関係にある1対の対立遺伝子によって支配されており、そこで、この遺伝子座をPrt-5と名付けた。遺伝子 $Prt\text{-}5^{A}$ は活性帯5を発現し、$Prt\text{-}5^{a}$ は活性帯5を発現しないことが明らかとなった。

3)．体重選抜されたウズラの3系統間（体重大、小および対照集団）について $Prt\text{-}5^{A}$ および $Prt\text{-}5^{a}$ の遺伝子頻度を推定した結果、3系統間に有意差は認められなかった。

2．キモトリプシンの変異とキモトリプシノーゲンとの関係

キモトリプシンの活性帯5～8はキモトリプシノーゲンが活性化されて発現したものであるが、あるキモトリプシノーゲンは活性化した後活性帯5を示さず、あるキモトリプシノーゲンは活性帯5を現わすことが見い出した。キモトリプシンの活性帯5の変異をキモトリプシノーゲンの分子構造レベルで解明するために、まず、膵臓バイオプシーにより活性帯5を持つ個体と持たない個体を検出し、かけ合わせにより表現型AAおよびaaのウズラを得た。次に、これらのウズラの膵臓より酸抽出、硫安分画および CM セルロースクロマトグラフィーおよびゲル濾過によって、2種類のキモトリプシノーゲンを精製した。これらのキモトリプシノーゲンは同じクロマト挙

動、電気泳動移動度および分子量を示すが、ペプチドマッピングの結果、この２種類のキモトリプシノーゲンは異なる分子構造を持つことが明らかとなり、この分子構造の違いがキモトリプシン変異を生ずるものと考えられた。

３．キモトリプシンの遺伝的変異の発現時期および組織局在性について

1)．キモトリプシノーゲンおよびキモトリプシンの変異は孵卵後の16日目に検出された。キモトリプシンの変異を支配する対立遺伝子（Prt-5^Aおよび Prt-5^a）はウズラの胚の発育の後期において発現し、その発現様式は胚から成鶉まで変わらないことを明らかにした。

2)．キモトリプシン変異体は膵臓の背葉、腹葉および第三葉の３部位ともにそれぞれ同一の電気泳動像を示し、その組織局在性は認められなかった。

４．表現型aaから精製されたキモトリプシノーゲンについて、その生化学的性質を明らかにし、さらにウシのキモトリプシノーゲンＡとの比較も行った。

1)．ウズラのキモトリプシノーゲンは、アミノ酸組成によるアミノ酸残基数が242で、分子量は26100であった。そのアミノ酸組成は、ウシのものと比べて、ヒスチジン、アルギニン、グルタミン酸およびチロシン含量が多く、スレオニンおよびセリン含量が少なかった。また、等電点は7.68であった

2)．ウズラキモトリプシンの特異的基質であるBTEE（benzoyl-L-tyrosine ethyl ester）に対する酵素活性は、Michaelis定数（Km）が3.10 mMで、分子活性（ko）は40.7 sec^{-1}であった。

3)．ウズラのキモトリプシンは、反応至適pHが7.0～8.0で、安定

pHは4.0～6.0であった。また、至適温度は45°Cであり、55°C以下で安定であった。しかしながら、pH 2.5以下および温度70°C以上で失活することが明らかとなった。

4). 活性中心阻害剤 DFP (diisopropylfluorophosphate) および TPCK (L-1-tosylamino-2-phenylethyl chloromethyl ketone) を用いて活性中心構造を検討した結果は、ウズラのキモトリプシンはウシのキモトリプシンと同じヒスチジン－セリン活性構造を持っていることが推測された。しかしながら、前者は後者よりもタンパク質阻害剤による阻害を受け難いことが判明した。

5). ウズラとウシのキモトリプシノーゲンの生化学的性質を比較検討した結果、基質的特異性および反応至適pH、反応至適温度など酵素反応の至適条件は両者間に差異は認められなかった。しかしながら、分子量、アミノ酸組成、ペプチドマップおよび等電点などに違いが認められた。

キモトリプシンは食餌タンパク質の消化に重要な役割を持つ酵素であるにもかかわらず、とくに鳥類においてはその遺伝的支配様式および生化学的性質が殆ど解明されていない。したがって、本研究で解明されたウズラのキモトリプシンおよびキモトリプシノーゲンの遺伝的知見および生化学的性質は、今後家禽や他の動物におけるタンパク質消化酵素の研究の一助となるものと考えられる。

鹌鹑胰凝乳蛋白酶的生化遗传学研究

摘　要

胰凝乳蛋白酶(chymotrypsin)是动物体内一种重要的蛋白质消化酶。其前体物胰凝乳蛋白酶原(chymotrypsinogen)合成于胰脏的腺泡细胞并储存于脂蛋白膜。动物消化饲料时，此酶原和其它胰脏酶原一起分泌到十二指肠，被胰蛋白酶(trypsin)激活成具有活性的胰凝乳蛋白酶，从而催化饲料中的蛋白质分解成多肽，这些多肽再经多肽酶催化分解成可吸收的氨基酸。因此，胰凝乳蛋白酶在动物的蛋白质消化过程中起着十分重要的作用。关于这种酶的生物化学性质的研究主要报道于牛，遗传学特征的研究仅见报道于小白鼠。但在鸟类，这种酶的生物化学特性以及遗传学特征尚未解明。

本研究以家禽研究的理想实验动物日本鹌鹑(Coturnix coturnix japonica)为材料，对鹌鹑胰凝乳蛋白酶进行了遗传学、发育学和生物化学的研究。发现了鹌鹑的胰凝乳蛋白酶存在着遗传变异，解明了它的遗传控制方式；研讨了胰凝乳蛋白酶的遗传变异与胰凝乳蛋白酶原分子结构的关系；解析了鹌鹑胰凝乳蛋白酶遗传变异体的表达时期和表达特征；最后解明了鹌鹑胰凝乳蛋白酶原的酶学及生物化学性质。本论文的主要内容可概括如下：

1．鹌鹑胰凝乳蛋白酶的遗传变异

(1) 对鹌鹑的胰脏消化蛋白酶进行琼脂凝胶电泳时可检出8条电泳带(4条位于阳极，4条位于阴极)。为方便起见，按阳极到阴极的顺序，将电泳带编号为1至8。结果发现有的个体含有电泳带5，有

的个体则不含有电泳带5。对胰脏的酶提取物进行阴离子交换树脂柱状层析(DEAE-cellulose column chromatography)、基质特异性分析和琼脂凝胶电泳分离，证实了电泳带5～8属于胰凝乳蛋白酶。

(2) 交配实验的结果判定，电泳带5的出现受体染色体上的1对显隐性关系的等位基因所控制，在此命名这个基因位点为 Prt-5。基因 $Prt\text{-}5^{A}$(表达电泳带5)对基因 $Prt\text{-}5^{a}$(不表达电泳带5)显性。

(3) 对经过50世代体重进行大、小选择的两个品系及一个随机育种的鹌鹑群体进行了基因频率估测，发现群体间 $Prt\text{-}5^{A}$ 及 $Prt\text{-}5^{a}$ 的基因频率无显著差异。

2．胰凝乳蛋白酶的变异体和胰凝乳蛋白酶原的关系

胰凝乳蛋白酶和胰凝乳蛋白酶原的电泳比较表明电泳带5～8是胰凝乳蛋白酶原激活后形成的。但是，某种胰凝乳蛋白酶原在激活后表现出电泳带5，而另一种胰凝乳蛋白酶原在激活后不表现出电泳带5。为了解明胰凝乳蛋白酶原与胰凝乳蛋白酶的遗传变异体的关系，作者首先成功地开发了鹌鹑胰脏生体检查法 (pancreatic biopsy)：即摘出小部分胰脏组织，进行胰凝乳蛋白酶的电泳检查，判定出 A-(包括AA和Aa) 表型和aa表型，接着将A-表型与aa表型回交，从A-表型中选出AA型。最后采用AA×AA 和 aa×aa表型同型交配，从而获得持有AA表型和aa表型的鹌鹑个体。第二，采用硫酸抽提，硫氨分段沉淀，阳离子交换树脂柱状层析 (CM-cellulose column chromatography)和凝胶过滤 (gel filtration on Sephadex G-100) 等手段，成功地从鹌鹑胰脏中提纯了AA和aa两种表型的胰凝乳蛋白酶原。这两种表型的胰凝乳蛋白酶原表现出相同的离子交换特性和电泳迁移率、分子量。但是它们的多肽图 (peptide map)显示出这两种酶原具有不同的分子结构，这种分子结构上的差异决定了胰凝乳蛋白酶的变异体的

形成。

3．胰凝乳蛋白酶的遗传变异的表达时期及组织分布特性

（1）鹌鹑胰凝乳蛋白酶原在孵卵后的第16天（出雏的前一天）被检出。控制胰凝乳蛋白酶的遗传变异的等位基因（Prt-5^{A}及Prt-5^{a}）表达于鹌鹑胚胎发育的后期，并且，其表达方式从胚胎到成年鹌鹑都保持不变。

（2）胰凝乳蛋白酶的遗传变异体，在胰脏的背叶、腹叶及第三叶等3个组织部位分别表现出相同的电泳带。因此，他们同时地表达于胰脏的3个组织部位。

4．对从表型aa提纯的鹌鹑胰凝乳蛋白酶原，进行了酶学及生物化学特性的研究，并将其结果与牛的胰凝乳蛋白酶原A进行了比较。

（1）根据鹌鹑胰凝乳蛋白酶原的氨基酸组成计算出它的氨基酸残基数为242个，分子量为26100；其氨基酸组成与牛相比，含有较多的组氨酸、精氨酸，谷氨酸和酪氨酸，但含有较少的苏氨酸和丝氨酸。鹌鹑胰凝乳蛋白酶原的等电点为 7.68。

（2）鹌鹑胰凝乳蛋白酶对特异性基质（benzol-L-tyrosine ethyl ester，BTEE）的酶学特性：米氏常数（Michaelis constant，Km）为3.10 mM，分子活性（molecular activity，ko）为40.7 sec^{-1}。

（3）鹌鹑胰凝乳蛋白酶的反应最适 pH为7.0～8.0，pH 4.0～6.0时安定；反应最适温度45℃，温度55℃以下时安定。但是，当pH 2.5以下以及温度70℃以上时，酶则失活。

（4）采用酶的活性中心抑制剂（diisopropyl fluorophosphate，DFP 和L-1-tosylamino-2-phenylethyl chloromethyl ketone，TPCK）对该酶活性中心结构进行了分析。结果认为：鹌鹑凝乳蛋白酶与牛的凝乳蛋白酶具有同样的组氨酸－丝氨酸活性中心结构。但是，前者比

后者难于被蛋白质抑制剂所阻碍。

（5）鹌鹑和牛的凝乳蛋白酶的酶学、生物化学性质比较结果表明，胰凝乳蛋白酶的活性，反应最适pH以及最适温度等方面在这两种动物间相似。但是它们的酶原的分子量、氨基酸组成，多肽图以及等电点等物理性质方面存在着差异。

胰凝乳蛋白酶在饲料蛋白质的消化过程中起着重要的作用。尽管如此，在鸟类，这种酶的遗传学特征以及生物化学特性几乎未得到解明。因此，本研究所解明的鹌鹑的胰凝乳蛋白酶的遗传学特征和生物化学性质，对今后鸟类及其它动物的蛋白质消化酶的研究无疑将起到推进作用。

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Publications in Relation to This Thesis

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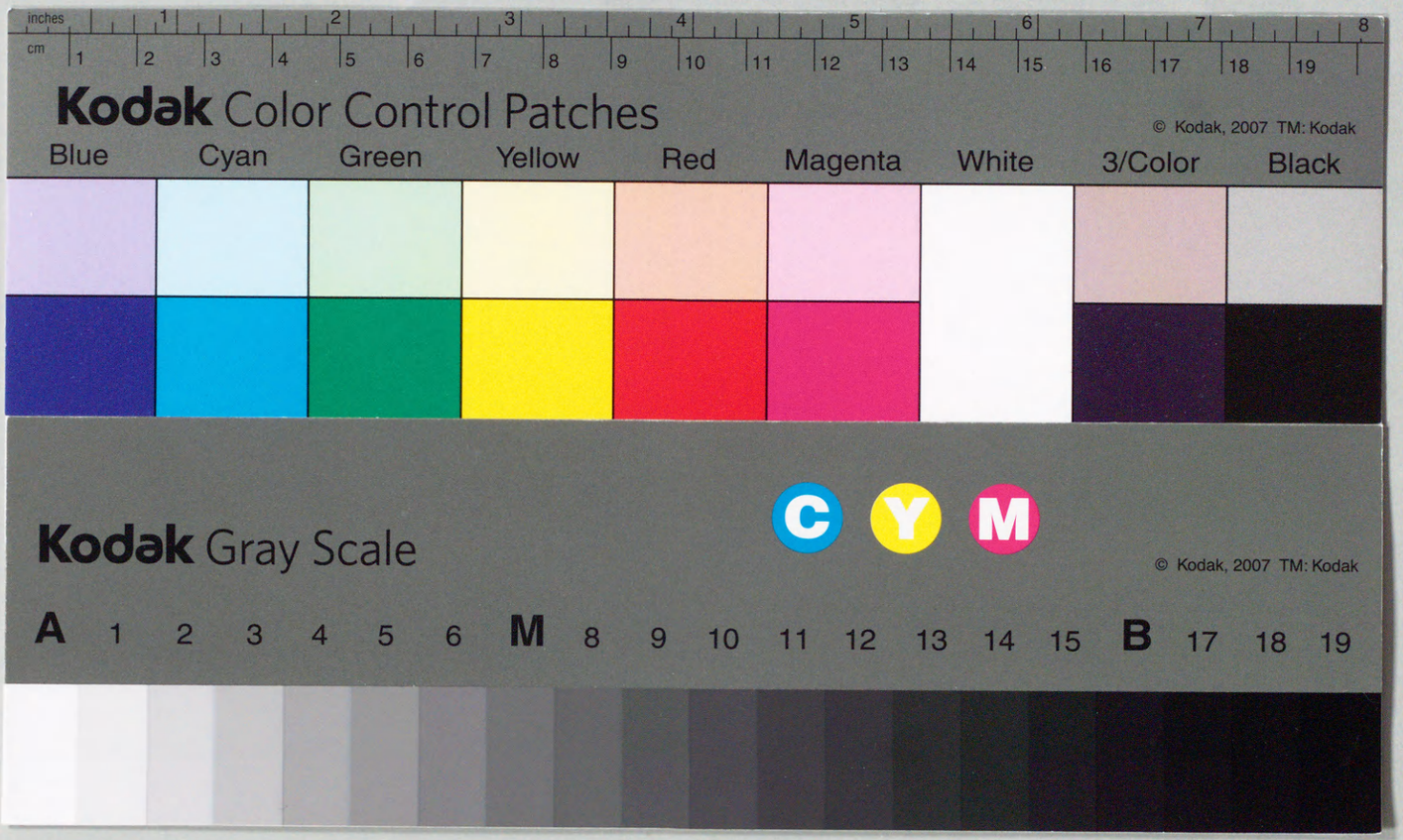
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