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# The Optimal Conditions for Activations of Trypsinogen and Chymotrypsinogen of Pancreas in Coturnix Quail

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## Introduction

To study the genetic properties of activity of pancreatic proteinase in coturnix quail, it is necessary to activate the zymogens extracted from pancreatic tissue. There have been many reports on the activations of trypsinogen and chymotrypsinogen from pancreatic juice, homogenized pancreas, and intestinal contents of different species including guinea pig[18], rat[3,4,5,8,16,19], mice[21], rabbit[6,17,22], dog[13], cow[7,20], pig[15], chicken[10], man[2], and catfish[23]. However, with a few exceptions[6,7,18,23], the optimal conditions of activations of these zymogens have not been studied in those reports, and the conditions of activations of these zymogens by enteropeptidase or trypsin have shown wide differences, among animal species, in the solvents for zymogens extraction, the concentration of activator and $Ca^{2+}$, time, temperature and pH in activation. These indicate that the determination of optimal conditions of pancreatic zymogens activation for each species is necessary to assay their activities precisely.

In this paper, the activation conditions of trypsinogen and chymotrypsinogen from the homogenized pancreas in coturnix quail were studied, using the following views for optimal activation: maximal activation attainable *in vitro* and stability of activity at the level of maximal activation.

## Materials and Methods

### Coturnix quail

The coturnix quail used in this study were obtained from a random-bred control line (RR) maintained at the laboratory of animal breeding in Kagoshima University. All birds were reared in wire-floored individual cages with 14h of artificial light per day, and maintained at 22°C. Feed (Toyohashi New Quail Diet) and water were available *ad libitum*. All birds used were adult, aged from 10 weeks to 13 weeks.

### Chemicals

Enteropeptidase from porcine intestine and trypsin from bovine pancreas were purchased from Sigma Chemical Co.. Tosyl-L-arginine methyl ester hydrochloride (TAME) , benzoyl-L-tyrosine ethyl ester (BTEE) and bovine serum albumin (BSA) were obtained from Nakarai Chemical Ltd.. Other reagents were of the highest purity, commercially available.

**Homogenization of pancreas**

The bird was killed by decapitation and the pancreas was removed quickly and weighed accurately. The pancreas was homogenized in ULTRA-TURRAX TP 10 N (Janke & kunkel Gmbh & Co. KG) tissue grinder with 40 (v/w) times its weight of ice-cold 100 mM Tris-HCl buffer (pH 8.0) containing 100 $\mu$g/ml of BSA, or 0.15M NaCl containing 0.1% Triton X-100 or distilled water. The homogenate was centrifuged in 12,000 g for 30 min at 0°C. A part of the supernatant was used as trypsinogen solution, the other part of supernatant was 4 times diluted with above three solvents and used as chymotrypsinogen solution. Protein content of the pancreatic supernatant was determined by the method of Lowry *et al.*[14].

**Activations of trypsinogen and chymotrypsinogen**

Trypsinogen and chymotrypsinogen were activated with enteropeptidase and trypsin, respectively. Equal volumes of zymogen solution and activator solution were mixed. A variety of experiments were performed to define the optimal conditions for activation by independently varying the concentration of activator and $Ca^{2+}$, time, temperature and pH, during the activation.

**Enzyme assays**

The activities of trypsin and chymotrypsin were spectrophotometrically assayed by the method of Hummel[11] at 247 nm and 256 nm, respectively. For trypsin assay, 0.1 ml of activated zymogen solution was added to 3 ml of 0.5 mM TAME substrate (pH 8.0) cotaining 50 mM Tris-HCl and 10 mM $CaCl_2$. For chymotrypsin assay, 0.1 ml of activated zymogen solution was added to 3 ml of 0.5 mM BTEE substrate (pH 8.0) containing 50 mM Tris-HCl, 10 mM $CaCl_2$ and 15% methanol. Then their activities were measured at 19-21°C in a Hitachi spectrophotometer 100-20. Activities of trypsin and chymotrypsin were defined as the initial rate of hydrolysis of their respective substrates, and expressed as the change in absorbance ($\Delta$A) per minute[6]. All the experiments were performed within 24h of pancreas collection, being repeated three times at least.

## Results and Discussion

**Solvents for zymogen extraction**

The supernatant of pancreas homogenized with 100 mM Tris-HCl buffer (pH 8.0) containing 100 $\mu$g/ml of BSA resulted in the maximal activities of trypsin and chymotrypsin, and that in homogenate with 0.15M NaCl containing 0.1% Triton X-100 gave higher activity. However, the supernatant of pancreas homogenized with distilled water showed only 46-50% and 30-35% activities of trypsin and chymotrypsin, respectively, compared with the homogenate in 100 mM Tris-HCl buffer (pH 8.0) containing 100 $\mu$g/ml of BSA or in 0.15M NaCl containing 0.1% Triton X-100 (Fig. 1 and 2).

Proper enzyme assays of pancreatic tissues require preventing of the loss of zymogens and activated enzymes through adsorption to the container walls, and complete rupture of the zymogen granules. This loss was minimized by the addition of BSA (100 $\mu$g/mg) in this study. The protective effect of BSA had previously been noted in guinea pig[18] and rabbit[6]. On the other hand, the hypotonic effect of distilled water produced no maximal activities both in trypsin and chymotrypsin. The addition of 0.1% Triton X-100 increased the avtivity of each enzyme by 50 to

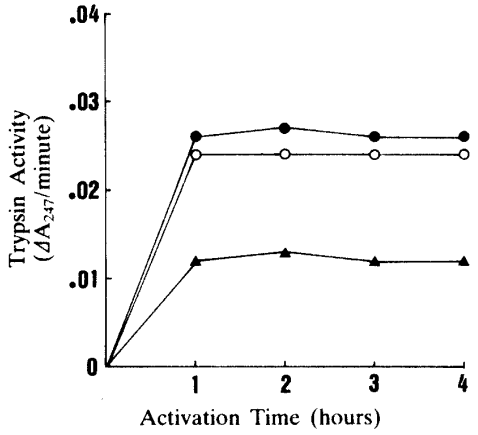


Fig. 1. Effects of solvents on trypsinogen extraction. Pancreatic tissue was homogenized and diluted to 800 $\mu$g/ml of protein in (1) 100 mM Tris-HCl buffer (pH 8.0) containing 100 $\mu$g/ml of BSA (-●—●-); (2) 0.15 M NaCl containing 0.1% Triton X-100 (-○—○-); (3) distilled water (-▲—▲-). Trypsinogen was activated at 30°C in a mixture containing 75 $\mu$g/ml of enteropeptidase, 50 mM $CaCl_2$ and 50–100 mM Tris-HCl (pH 8.0).

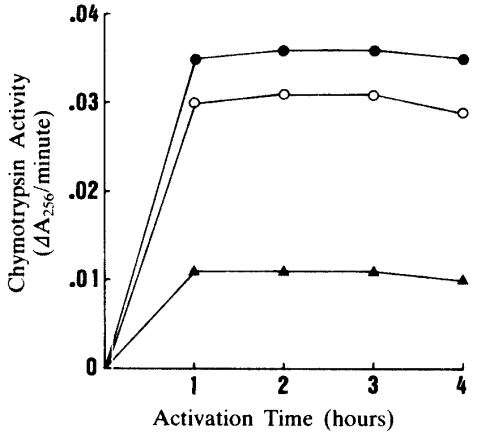


Fig. 2. Effects of solvents on chymotrypsinogen extraction. Pancreatic tissue was homogenized and diluted to 200 $\mu$g/ml of protein in (1) 100 mM Tris-HCl buffer (pH 8.0) containing 100 $\mu$g/ml of BSA (-●—●-); (2) 0.15 M NaCl containing 0.1% Triton X-100 (-○—○-); (3) distilled water (-▲--▲-). Chymotrypsinogen was activated at 4°C in a mixture containing 25 $\mu$g /ml of trypsin, 50 mM $CaCl_2$ and 50–100 mM Tris-HCl (pH 8.0).

65%, compared with that in homogenate of the distilled water. Similar effects of Triton X-100 on release and lysis of zymogen granules were reported in mouse[9], guinea pig[18] and bovine[7].

**Time and temperature**

For trypsinogen activation, a maximal activity was attained in 1h at 30°C,being maintained for 4h at least. At 38°C, a higher activity was observed after 1h of activation, getting declined with time. At 4°C, the activity increased with time of activation, becoming very low during 1–6h of activation, compared with that at 30°C (Fig. 3).

For chymotrypsinogen activation, a maximal activity was achieved after 1h of activation over the range of 4–38°C. However, a stability of enzymatic activity remained at such low temperature of 4°C for 4h, while the activity gradually decreased with time at 30°C or 38°C (Fig. 4).

The activation of zymogens required a definite time, and was sensitive to temperature

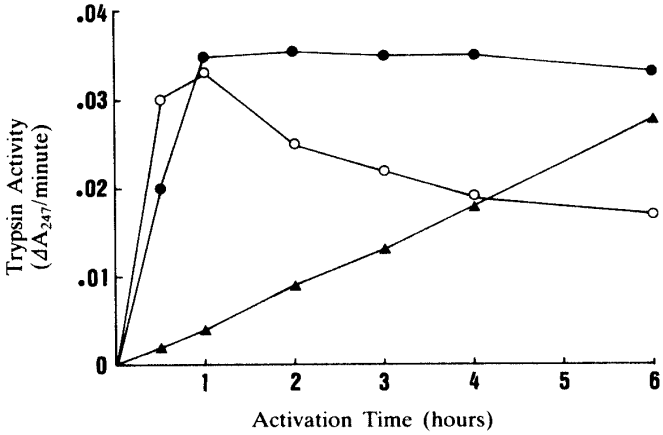


Fig. 3. Effects of time and temperature on trypsinogen activation. Incubation mixture contained 75 $\mu$g/ml of enteropeptidase, 800 $\mu$g/ml of pancreatic protein, 50 $\mu$g/ml of BSA, 50 mM $CaCl_2$ and 100 mM Tris-HCl (pH 8.0). Samples were incubated at 4°C (-▲—▲-), 30°C (-●—●-) and 38°C (-○—○-) for varying periods.

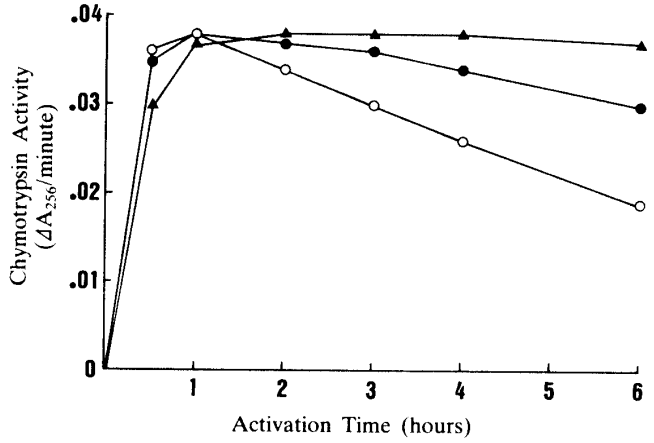


Fig. 4. Effects of time and temperature on chymotrypsinogen activation. Incubation mixture contained 25 $\mu$g/ml of trypsin, 200 $\mu$g/ml of pancreatic protein, 50 $\mu$g/ml of BSA, 50 mM $CaCl_2$ and 100 mM Tris-HCl (pH 8.0). Samples were incubated at 4°C (-▲—▲-), 30°C (-●—●-) and 38°C (-○—○-) for varying periods.

changes. In this experiment, the optimal time and temperature for activations of trypsinogen and chymotrypsinogen were 1-4h, 30°C and 4°C, respectively. These results support the study on rabbit[6], disagreeing with the results of Scheele and Palade on guinea pig[18], and Yoshinaka *et al.* on catfish[23].

**Concentration of enteropeptidase and trypsin**

75 $\mu$g/ml of enteropeptidase gave maximal activation of trypsinogen and the activity was stable through 4h; 150 $\mu$g/ml of enteropeptidase resulted also in maximal activation but the activity was unstable; 25 $\mu$g/ml of enteropeptidase caused lower activation; No activation occurred without the addition of enteropeptidase (Fig. 5). For chymotrypsinogen, 25-75 $\mu$g/ml of trypsin resulted in maximal activation of chymotrypsinogen and the activity remained constant for 4h at least. 5 $\mu$g/ml of trypsin resulted in 92% of maximal activity, and a lower activity occurred

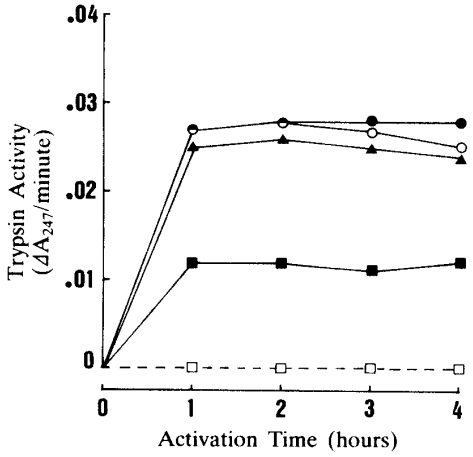


Fig. 5. Effects of enteropeptidase concentration on trypsinogen activation. Incubation mixtures contained 800 $\mu$g/ml of pancreatic protein, 50 $\mu$g/ml of BSA, 50 mM $CaCl_2$ and 100 mM Tris-HCl (pH 8.0). The concentration of enteropeptidase was 0 (-□—□-), 25 $\mu$g/ml (-■—■-), 50 $\mu$g/ml (-▲—▲-), 75 $\mu$g/ml (-●—●-), and 150 $\mu$g/ml (-○—○-). Samples were incubated at 30°C for specified periods.

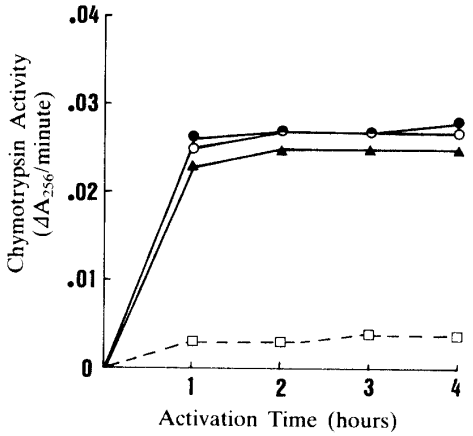


Fig. 6. Effects of trysin on chymotrypsinogen activation. Incubation mixture contained 200 $\mu$g/ml of pancreatic protein, 50 $\mu$g/ml of BSA, 50 mM $CaCl_2$ and 100 mM Tris-HCl (pH 8.0). The concentration of trypsin was 0 (-□—□-), 5 $\mu$g/ml (-▲—▲-), 25 $\mu$g/ml (-○—○-) and 75 $\mu$g/ml (-●—●-). Samples were incubated at 4°C for specified periods.

without the addition of trypsin (Fig. 6).

Activities of trypsinogen and chymotrypsinogen are not detectable in pancreatic tissue, and need to be activated by enteropeptidase and trypsin. Slower spontaneous activation of chymotrypsinogen, previously noted by Akabori[1], occurred also in this experiment. The reason is not completely ascertained, yet, but may be related with the mixed presence of chymotrypsinogen and a little amount of chymotrypsin in pancreas. Laskowski[12] separated chymotrypsinogen and chymotrpsin from pancreas, simultaneously.

**$Ca^{2+}$ concentration**

For trypsinogen activation, 25–50 mM $Ca^{2+}$ resulted in the maximal activity after 1h, but a slight decreasing of activity in the presence of 25 mM $Ca^{2+}$ was observed after 2h, whereas the activity remained stable when 50 mM $Ca^{2+}$ was used. Without addition of $Ca^{2+}$, only 31% of maximal activity was achieved (Fig. 7). For chymotrypsinogen activation, a maximal activity of chymotrypsinogen was noted in the presence of 50 mM $Ca^{2+}$. However, $Ca^{2+}$ hardly affected chymotrypsinogen activation, because 91% of maximal activity was achieved without addition of $Ca^{2+}$ (Fig. 8). This agreed with the result reported by Glazer and Steer[6].

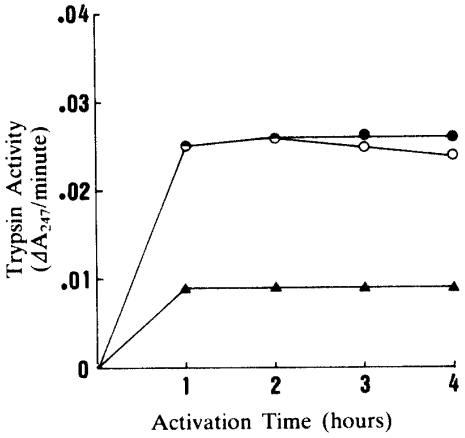


Fig. 7. Effects of $Ca^{2+}$ on trypsinogen activation. Incubation mixture contained 75 $\mu$g/ml of enteropeptidase, 800 $\mu$g/ml of pancreatic protein, 50 $\mu$g/ml of BSA and 100 mM Tris-HCl (pH 8.0). $Ca^{2+}$ concentration was 0 (-▲—▲-), 25 mM (-○—○-) and 50 mM (-●—●-). Samples were incubated at 30°C for specified periods.

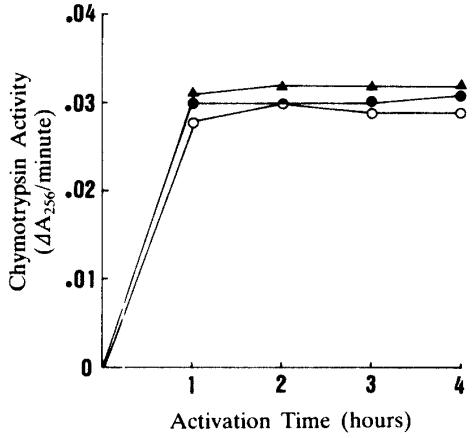


Fig. 8. Effects of $Ca^{2+}$ on chymotrypsinogen activation. Incubation mixture contained 25 $\mu$g/ml of trypsin, 200 $\mu$g/ml of pancreatic protein, 50 $\mu$g/ml of BSA and 100 mM Tris-HCl (pH 8.0). $Ca^{2+}$ concentration was 0 (-○—○-), 25 mM (-●—●-) and 50 mM (-▲—▲-). Samples were incubated at 4°C for specified periods.

**Pancreatic protein concentration**

Trypsin activity increased in proportion to the pancreatic protein concentration over the range of 200–1600 $\mu$g/ml so long as the described conditions for trypsinogen activation were maintained (Fig. 9). Chymotrypsin activity increased also with the pancreatic protein concentration over the range of 50–400 $\mu$g/ml when chymotrypsinogen activation was performed under the described conditions (Fig. 10).

Appropriate protein concentration must be determined so that the eventual proteolytic activity may give appropriate optical density changes, using the assay method of Hummel[11].

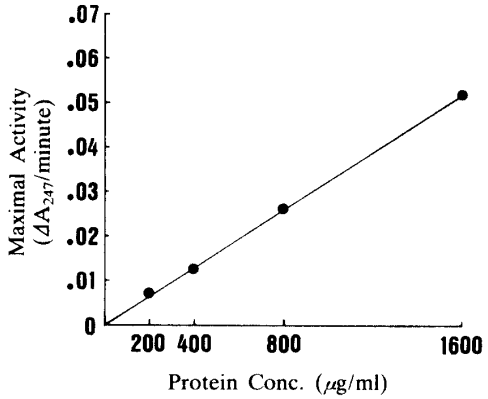


Fig. 9. Relationship between amount of pancreatic protein and maximal activity of trypsin. Trypsin was activated at 30°C for 2h in a mixture containing 75 $\mu$g/ml of enteropetidase, 50 $\mu$g/ml of BSA, 50 mM $CaCl_2$ and 100 mM Tris-HCl (pH 8.0). The concentration of pancreatic protein ranged from 200 to 1600 $\mu$g/ml.

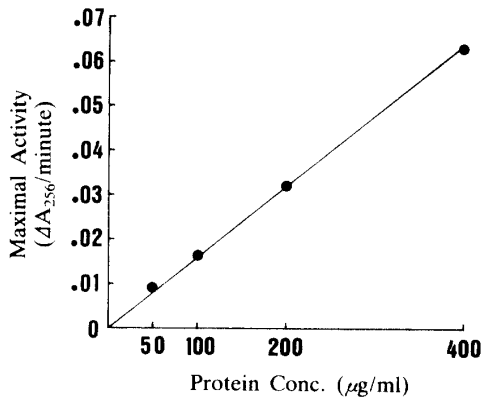


Fig. 10. Relationship between amount of pancreatic protein and maximal activity of chymotrypsin. Chymotrypsinogen was activated at 4°C for 2h in a mixture containing 25 $\mu$g/ml of trypsin, 50 $\mu$g/ml of BSA, 50 mM $CaCl_2$ and 100 mM Tris-HCl (pH 8.0). The concentration of pancreatic protein ranged from 50 to 400 $\mu$g/ml.

Higher protein concentrations were studied, but the potential activity of enzyme was beyond the sensitivity of the technique used to measure proteolytic activity. On the other hand, the enzymatic activity might be lost, when the pancreatic protein was diluted into too lower density (data not shown). This may be modified simply by the dilution with 40 times (v/w, for trypsinogen) and 160 times (v/w, for chymotrypsinogen) pancreas weight of 100 mM Tris-HCl buffer (pH 8.0) containing 100 $\mu$g/ml of BSA.

**pH**

Effect of pH on the activation of trypsinogen and chymotrypsinogen was investigated by activating these zymogens with enteropeptidase (75 $\mu$g/ml) or trypsin (25 $\mu$g/ml) at 30°C or 4°C for 2h, respectively. The results showed a wider range of pH 7.0–9.0 for the activations of trypsinogen and chymotrypsinogen because activation kinetics of these zymogens were kept unchanged over this pH range (data not shown).

In this study, the optimal conditions for trypsinogen and chymotrypsinogen activation were established as follows: For trypsinogen, pancreatic tissue is to be homogenized with 40 times (v/w) its weight of Tris-HCl buffer (pH 8.0) containing 100 $\mu$g/ml of BSA. An equal volume of 150 $\mu$g of enteropeptidase /ml in activator solution (pH 8.0) containing 100 mM Tris-HCl and 100 mM $CaCl_2$ is to be added, and the mixture is to be incubated at 30°C for 1–4h. For chymotrypsinogen, pancreatic tissue is to be homogenized with 160 times (v/w) its weight of Tris-HCl buffer (pH 8.0) containing 100 $\mu$g/ml of BSA. An equal volume of 50 $\mu$g of trypsin /ml in activator solution (pH 8.0) containing 100 mM Tris-HCl and 100 mM $CaCl_2$ is to be added, and the mixture is to be incubated at 4°C for 1–4h. After their incubations, 0.1 ml aliguot is to be taken for trypsin and chymotrypsin assays according to the method of Hummel[11], respectively.

## Summary

To assay the activities of trypsin and chymotrypsin in the pancreas of coturnix quail, the activation process of each zymogen was studied in detail in the solvents for zymogens extraction, concentration of activator and $Ca^{2+}$, temperature, time and pH, during the activation. Trypsinogen was optimally activated by enteropeptidase (75 $\mu$g/ml) at 30°C for 1–4h in 100 mM Tris-HCl buffer (pH 8.0) containing 50 $\mu$g/ml of bovine serum albumin (BSA) and 50 mM $CaCl_2$. Chymotrypsinogen was optimally activated by trypsin (25 $\mu$g/ml) at 4°C for 1–4h in 100 mM Tris-HCl buffer (pH 8.0) containing 50 $\mu$g/ml of BSA and 50 mM $CaCl_2$.The activities of trypsin and chymotrypsin through activation under the optimal conditions were noted to be directly related to the amount of pancreatic protein ranging from 200 to 1,600 $\mu$g/ml and from 50 to 400 $\mu$g/ml, respectively. The results indicate that the potential activities of trypsinogen and chymotrypsinogen in the pancreas of coturnix quail may be determined *in vitro* by these methods.



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