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# 173. Akira Minato, Seiyu Hirose, and Shoryo Hayashi*[1] : Studies on Proteases in Submaxillary Gland. I. Fractionation and General Characteristics of Rat Submaxillary Proteases.*[2]

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By chromatography on DEAE-cellulose column, five protease fractions were separated from the rat submaxillary gland, and tentatively named rat submaxillary protease A, B, C, D and E. The casein-hydrolyzing activities of the protease A, B, C, D and E were optimal at pH 8.3, 10.2, 9.6, 8.3 and 9.6, respectively. The rat submaxillary proteases were most stable at neutral pH, and were not affected by the addition of EDTA, rat submaxillary dialyzate, monoiodoacetate or *p*-chloromercuribenzoate. All of the five proteases were inactivated by diisopropyl fluorophosphate. The activities of protease A, B and E were not inhibited by soybean-trypsin-inhibitor, whereas chymotrypsin-type inhibitions were observed in the cases of the protease C and D. The protease A had no less milk-clotting activity than that of chymotrypsin, while the protease B expressed about one-tenth activity of that of chymotrypsin, and only neglectable activities were observed in the cases of the protease C, D and E.

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The proteolytic activity of salivary glands had not been demonstrated until Junqueira, *et al.*[1] showed in 1949 that the extract of the submaxillary glands of mice could degrade casein. Subsequently, Shafer, *et al.*[2,3] reported that the proteolytic activity of rat submaxillary gland, as measured by the casein-hydrolyzing activity at pH 7.6, was suppressed by hypophysectomy, and partially restored by thyroxine-testosterone treatment. Similar findings of the hormonal control of this enzyme activity was also reported by Sreebny, *et al.*[4]. Kim[5] reported the rapid degradation of $^{131}$I-labeled casein, growth hormone and parotin by the homogenate of rat submaxillary gland at pH 7.4, as compared with the hydrolysis of these radioiodinated proteins by kidney, liver, spleen, pancreas, parotid gland, muscle and testis homogenates. In this connection, Ito, *et al.*[6] demonstrated the presence of proteolytic activity in human saliva using $^{131}$I-labeled casein as substrate. On the other hand, the presence of kallikrein activity which liberates a vasodilator polypeptide from plasma protein, was demonstrated in the submaxillary glands of mouse, cat, dog, pig, cattle and man.[7~9] Hilton and Lewis[10] have proposed that the kallikrein release is responsible for the functional vasodilation in the salivary gland during activity. However, the information concerning the detailed enzymatic properties of the rat submaxillary kallikrein has been scarcely reported.

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*2 Presented at the 85th Annual Meeting of the Pharmaceutical Society of Japan at Tokushima, Oct. 28, 1965.

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Sreebny, *et al.*[11] studied the properties of over-all proteolytic activity of the homogenate of rat submaxillary gland and reported that the activity was optimal at between pH 8.5 and 9.5, mildly sensitive to acids, markedly sensitive to alkali, activated by cysteine and ascorbic acid, inhibited by iodine and dialysis, and unaffected by limabean-trypsin-inhibitor.

This paper describes a method for the column-chromatographic separation of five protease fractions from the rat submaxillary gland and the general characteristics of these proteases.

## Materials and Methods

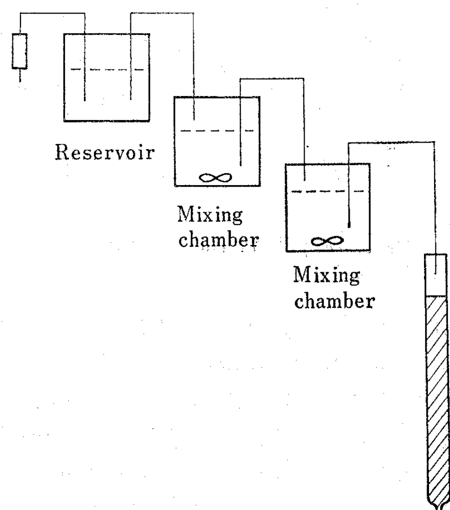
**Material**——The submaxillary glands of mature male and female rats of Wistar strain were used for this experiment. DEAE-cellulose, purchased from Brown Company, was used after the purification by successive washings with 1*M* NaCl-0.1*M* HCl, water, 0.5*M* NaOH and water. Crystalline trypsin and chymotrypsin were obtained from C.F. Boehringer, and casein (nach Hammarsten) from E. Merk. Diisopropyl fluorophosphate (DFP) was synthesized by the method of Saunders, *et al.*[12,13] Soybean-trypsin-inhibitor was isolated from soybeans according to the method of Kunitz.[14]

**Assay of Proteolytic Activity**——Under standard conditions, the reaction mixture containing, in a total volume of 2 ml., 20 mg. of casein, 100 μmoles of sodium phosphate-borate buffer, pH 8.6 (or optimal pH), and 1 ml. of enzyme solution, was incubated at 37° for 60 minutes (trypsin and chymotrypsin : 30 minutes), and the reaction was stopped by the addition of an equal amount of 10 per cent perchloric acid (PCA). The mixture was kept at room temperature for about 30 minutes, and the precipitate was removed by centrifugation. The optical density of the supernatant solution was measured at 275 mμ with 1 cm. light path against the blank without the incubation.

## Results and Discussions

### Preparation of Five Protease Fractions from Rat Submaxillary Gland

Rat submaxillary gland was homogenized in 9 volumes of isotonic KCl solution in a Waring Blendor at ice-cold temperature, and centrifuged at 100,000 ×*g* for 60 minutes. The supernatant solution was dialyzed against distilled water for 3 days in the cold, and




Scheme 1. The Two-step-gradient Elution Method

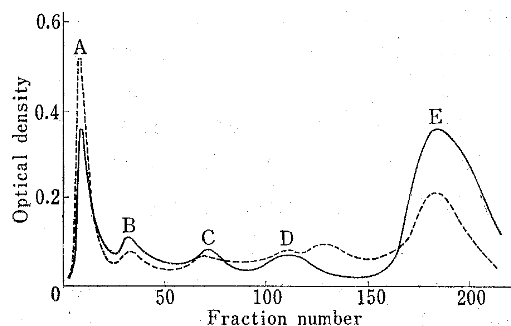



Fig. 1. A Typical Result of the Column-chromatographic Separation of Five Rat Submaxillary Protease Fractions

——— Proteolytic activity measured by the standard method (pH 8.6)
-------- Protein concentration represented by the optical density of each fraction at 280 mμ

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lyophilized. A 100 mg. portion of this preparation was dissolved in 5 ml. of 5 m$M$ sodium phosphate buffer, pH 6.0, and dialyzed against the same buffer solution in a refrigerator. The dialyzed solution was then applied on the DEAE-cellulose column (0.9×40 cm.) which had been equilibrated previously with the above buffer solution, and the two-step-gradient elution was performed using two mixing chambers (each containing 1,600 ml. of 5 m$M$ sodium phosphate buffer, pH 6.0) and a reservoir (containing 3.3 $M$ sodium chloride-5 m$M$ sodium phosphate buffer, pH 6.0) as shown in Scheme 1. The flow rate was about 12 ml. per hour, and fractions of 4 ml. each were collected. By this procedure, five proteolytic enzyme fractions were obtained and tentatively named rat submaxillary protease A, B, C, D and E (Fig. 1).

### pH Optimum

The effect of pH on the casein-hydrolyzing activity of the rat submaxillary proteases is shown in Fig. 2. Buffers used at incubation were 40 m$M$ sodium phosphate-borate or sodium borate-carbonate buffer. Although the over-all proteolytic activity of the rat submaxillary homogenate was maximal at pH 8.6, the protease A, B, C, D and E had the optimal pH of 8.3, 10.2, 9.6, 8.3 and 9.6, respectively.

### Stability

The relation between pH and stability of the rat submaxillary proteases was studied by heat-treatments at 37° for 20 hours and at 50° for 3 hours. In contrast to trypsin and chymotrypsin which were more stable as the acidity of the solution was raised, the rat submaxillary proteases were most stable at neutral pH with the gradual decrease on ether side of that pH (Fig. 3). In neutral solutions, no significant drop of the activity of the five rat submaxillary proteases was observed after the heat-treatment at 37° for 20 hours, although 15~20 per cent decrease in the activity was recognized following the treatment at 50° for 3 hours.

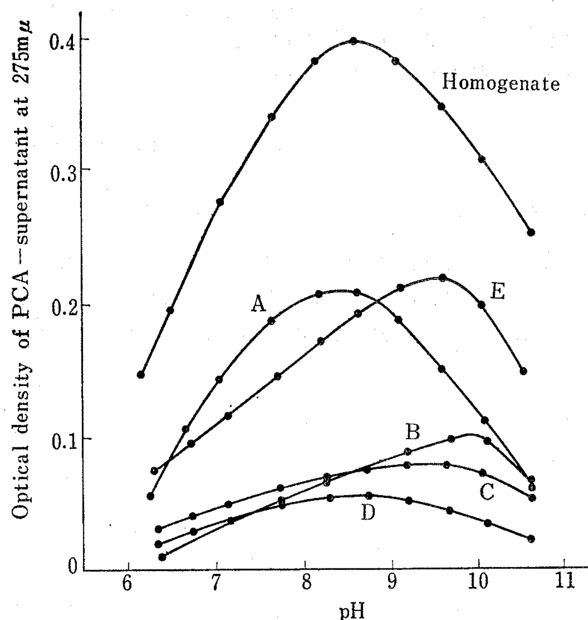


Fig. 2. Effect of pH on the Activity of Rat Submaxillary Proteases

### Effect of EDTA and Submaxillary Dialyzate

In order to determine whether the rat submaxillary proteases belong to a group of the proteases whose activity is dependent on metal ions, *e.g.*, $Mg^{++}$, $Mn^{++}$ and others, the effect of EDTA and the rat submaxillary dialyzate on the activity of the five proteases was examined. The dialyzate was prepared by dialyzing 6 per cent homogenate of the rat submaxillary gland against 2 volumes of isotonic NaCl solution. The results shown in Table I suggest that the submaxillary proteases do not belong to the group of the proteases which require special metal ions.

### Effect of Iodoacetate and *p*-Chloromercuribenzoate

For the purpose of estimating the necessity of thiol group for the activity of the rat submaxillary proteases, the influence of iodoacetate and *p*-chloromercuribenzoate on the activity of the five proteases was tested as follows: each protease solution (2 ml.)

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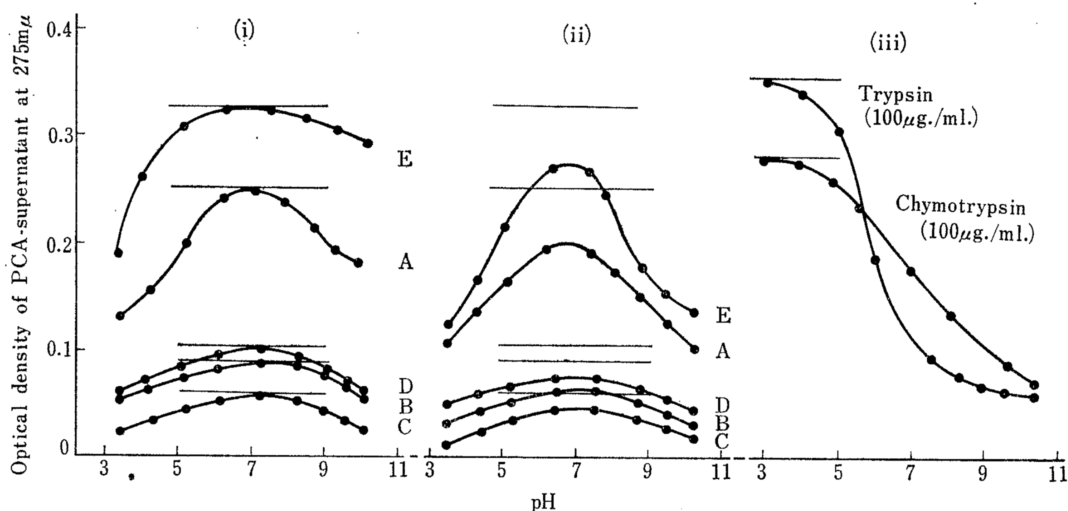


Fig. 3. Relation between pH and Stability of Rat Submaxillary Proteases

A reaction mixture containing 10μ moles of buffer of various pH and 1 ml. of each enzyme solution, in a total volume of 2 ml., was incubated at 37° for 20 hr. (i), at 50° for 3 hr. (ii), or at 37° for 2 hr. (iii). The remaining proteolytic activity was assayed by the standard method at each optimal pH where trypsin and chymotrypsin solutions were diluted 10-fold prior to the assay.

was mixed with 0.5 ml. of 10 m*M* iodoacetate or 0.6 m*M* *p*-chloromercuribenzoate, kept at room temperature and pH 8.0 for about 30 minutes, and dialyzed against distilled water for one day in the cold. The volume of the dialyzed solution was then adjusted to 5 ml., and the proteolytic activity of each solution was measured by the standard method at each optimal pH. As shown in Table II, no significant decrease in the ac-

Table I. Effect of EDTA and Submaxillary Dialyzate on the Activity of Rat Submaxillary Proteases

| Solutions added | Proteolytic activity[a) (Optical density of PCA-supernatant at 275 mμ) Rat submaxillary protease | | | | |
|---|---|---|---|---|---|
| | A | B | C | D | E |
| EDTA | 0.296 | 0.122 | 0.106 | 0.063 | 0.324 |
| Submaxillary dialyzate | 0.273 | 0.113 | 0.090 | 0.066 | 0.317 |
| NaCl | 0.267 | 0.115 | 0.088 | 0.062 | 0.315 |

a) The proteolytic activity was measured by the standard method at each optimal pH with the addition of 0.5 ml. of 10 m*M* EDTA, 2 per cent submaxillary dialyzate or 0.9 per cent NaCl (control).

Table II. Effect of Iodoacetate and *p*-Chloromercuribenzoate on the Activity of Rat Submaxillary Proteases

| Experimental number | Treatments | Proteolytic activity[a) (Optical density of PCA-supernatant at 275 mμ) Rat submaxillary protease | | | | |
|---|---|---|---|---|---|---|
| | | A | B | C | D | E |
| 1 | Iodoacetate | 0.108 | 0.027 | 0.031 | 0.031 | 0.189 |
| | Control | 0.107 | 0.028 | 0.030 | 0.028 | 0.189 |
| 2 | *p*-Chloromercuribenzoate | 0.125 | 0.031 | 0.031 | 0.026 | 0.169 |
| | Control | 0.127 | 0.032 | 0.032 | 0.022 | 0.163 |

a) Proteolytic activity was assayed by the method described in the text.

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tivity of the five proteases was found after the treatment with iodoacetate or *p*-chloromercuribenzoate within the limits of the experimental conditions.

**Effect of DFP**

The effect of DEP on the activity of rat submaxillary proteases is shown in Fig. 4. Definite inhibitions by DEP were observed in all of the five proteases. It was also known that a higher concentration of DFP was required to produce 50 per cent inhibition of the activity of each submaxillary protease than that of DFP producing the same amount of inactivation of the trypsin solution (5 μg./ml.), which expressed, without the inhibitor, no less proteolytic activity than that of any submaxillary protease solution. Therefore, the ratio of DFP concentration causing the 50 per cent inhibition of the proteolytic activity to the protein concentration of each enzyme solution expressed by the optical density at 280 mμ was determined. The values of the protease D and E were 0.027 and 0.026 which were nearly equal to that of trypsin (0.025), whereas the higher values (0.11, 0.25, 0.13) were obtained in the cases of the protease A, B and C. In this connection, it must be considered that the five protease fractions prepared by the column chromatography are not always free from inactive proteins. Therefore, the effect of a certain concentration of DFP on the proteolytic activity of the solutions containing each submaxillary protease and trypsin collectively or separately was examined by the method described in Table Ⅲ to determine whether any essential difference exists between each submaxillary protease and trypsin in the susceptibility to DFP. Distinct differences were found between the rat submaxillary proteases and trypsin in DFP-inhibition, because it was considered that the activity of each submaxillary protease was inhibited only slightly by the designated concentration of DFP which produced strong inactivation of trypsin contained in the same reaction mixture (Table Ⅲ).

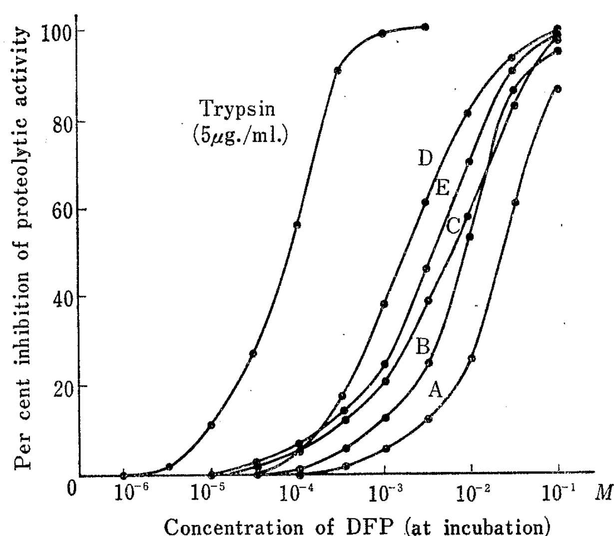


Fig. 4. Effect of DFP on the Activity of Rat Submaxillary Proteases

A reaction mixture containing 1 ml. of each enzyme solution and 0.1 ml. of DFP dissolved in isopropanol at various concentrations, was incubated at 37° for 20 minutes. The remaining proteolytic activity was assayed by the standard method at each optimal pH.

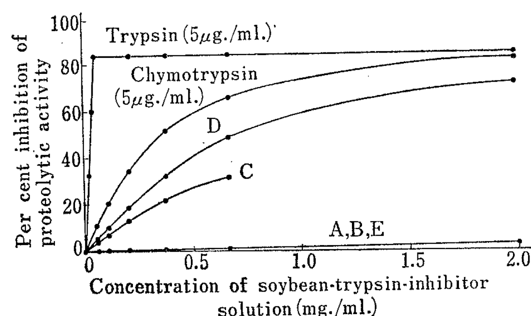


Fig. 5. Effect of Soybean-trypsin-inhibitor on the Activity of Rat Submaxillary Proteases

A mixture of 1 ml. of each enzyme solution and 0.5 ml. of soybean-trypsin-inhibitor solution of the designated concentration was kept at 0° for 20 minutes, and the remaining proteolytic activity was measured by the standard method at each optimal pH.

**Effect of Soybean-trypsin-inhibitor**

In order to obtain further distinctions between rat submaxillary proteases and trypsin, the influence of soybean-trypsin-inhibitor on the activity of the five proteases was

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Table III. Comparison between Rat Submaxillary Proteases and Trypsin in DFP-inhibition

| Composition of reaction mixture (ml.) | | | | | Proteolytic activity[a] (Optical density of PCA-supern. at 275 mμ) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Rat Submaxillary protease | | | | |
| Trypsin solution (10 μg./ml.) | Rat submax. protease solution | Water | $3.3\times10^{-3}M$ DFP in isopropanol | Isopropanol | A | B | C | D | E |
| 0.5 | — | 1.0 | — | 0.1 | 0.562 | 0.351 | 0.496 | 0.562 | 0.496 |
| 0.5 | — | 1.0 | 0.1 | — | 0.139 | 0.083 | 0.095 | 0.140 | 0.095 |
| — | 1.0 | 0.5 | — | 0.1 | 0.183 | 0.070 | 0.080 | 0.052 | 0.343 |
| — | 1.0 | 0.5 | 0.1 | — | 0.179 | 0.066 | 0.070 | 0.043 | 0.296 |
| 0.5 | 1.0 | — | — | 0.1 | 0.667 | 0.412 | 0.682 | 0.873 | 0.968 |
| 0.5 | 1.0 | — | 0.1 | — | 0.295 | 0.124 | 0.110 | 0.207 | 0.424 |

a) Proteolytic activity was assayed by the standard method where incubations were carried out for 60 minutes at 37° and pH optimal for each submaxillary protease.

examined. As shown in Fig. 5, the protease A, B and E were not inactivated by this inhibitor, while chymotrypsin-type inhibitions were observed in the cases of the protease C and D.

**Milk-clotting Activity**

The milk-clotting activity of the rat submaxillary proteases was estimated by the following method comparing with that of chymotrypsin. The reaction mixture containing, in a total volume of 2 ml., 1 ml. of fresh milk, 50 μ moles of sodium acetate buffer, pH 5.5, and 1 ml. of each submaxillary protease solution or chymotrypsin solution of varying concentration, was incubated at 37° until the precipitate appeared. The concentration of chymotrypsin equivalent to each submaxillary protease solution in milk-clotting activity was determined by finding out a pair of reaction mixtures which clotted at about the same time. On the other hand, the concentration of chymotrypsin equivalent to each submaxillary protease solution in casein-hydrolyzing activity was determined after the incubation of protease solutions with casein at 37° and optimal pH for 30 minutes. Judging from the ratio of the two chymotrypsin concentrations equivalent to each submaxillary protease solution in milk-clotting and casein-hydrolyzing activities, the protease A had no less milk-clotting activity than that of chymotrypsin, while the protease B expressed about one-tenth activity of that of chymotrypsin, and only neglectable activities were observed in the cases of the protease C, D and E (Table IV).

Table IV. Milk-clotting Activity of Rat Submaxillary Proteases

| Rat submax. protease | Concentration of chymotrypsin equivalent to each submaxillary protease solution[a] (μg./ml.) | | Ratio (M.A./C.A.) |
|---|---|---|---|
| | Milk-clotting activity | Casein-hydrolyzing activity | |
| A | 7 | 4.5 | 1.6 |
| B | 0.16 | 1.6 | 0.1 |
| C | <0.08 | 0.92 | <0.09 |
| D | <0.08 | 0.95 | <0.08 |
| E | <0.08 | 4.1 | <0.02 |

a) The details of the methods are described in the text.

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