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Comparison of Biological and Immunological Methods for Determination of Serum α_1-Antitrypsin[1]

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Comparison of biological and immunological methods for determination of α_1-antitrypsin showed that the amount of α_1-antitrypsin determined in the presence of trypsin were not the same. From the result of the comparison of effect of heat on the amount of α_1-antitrypsin found by biological and immunological methods, it is suggested that α_1-antitrypsin and the α_1-antitrypsin–trypsin complex are indistinguishable immunologically. The investigation of the trypsin inhibitory activity of α_1-antitrypsin and molecular weight determination showed that α_1-antitrypsin and trypsin formed a 1 : 1 stoichiometric complex.

The results of immunoelectrophoresis and sodium dodecyl sulfate electrophoresis indicated that the α_1-antitrypsin–trypsin complex was broken down with incubation and the breakdown products also had immunogenicity.

Keywords——α_1-antitrypsin; α_1-antitrypsin–trypsin complex; trypsin inhibitory activity; immunoelectrophoresis; SDS electrophoresis

In 1962, Laurell and Eriksson reported that α_1-antitrypsin deficiency in human serum was associated with pulmonary emphysema.[3] This report stimulated us to examine the relationship between human serum α_1-antitrypsin and diseases. Up to now, many reports have been published on the relationship between α_1-antitrypsin and pulmonary emphysema and infantile cirrhosis of the liver.[4]

Human serum α_1-antitrypsin is now determined by a biological method of measuring the capacity of serum to inhibit trypsin activity or by the immunological method of measuring the amount of α_1-antitrypsin with a specific antiserum. By Morishita and Abels,[5] another method was developed to determine the human serum α_1-antitrypsin by the reduction exerted by trypsin in the uptake of vitamine B_{12} into a mucosal homogenate from guinea pig ileum. The human serum α_1-antitrypsin is, however, determined almost exclusively by either biological or immunological method.

Recently, Saito and Sawaki[6] reported that there was no correlation in human serum α_1-antitrypsin values determined by biological and immunological methods in various diseases, such as inflammation and cancer, and that it must rely on the biological and immunological methods to determine human serum α_1-antitrypsin. For this reason, the reliability of α_1-antitrypsin values determined by the biological and immunological methods is doubtful when studying the relationship between human serum α_1-antitrypsin values and disease. The present paper discussed the difference between the biological and immunological active site of purified human α_1-antitrypsin.

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Materials and Methods

Purified Human α_1-Antitrypsin——The purified human α_1-antitrypsin was prepared according to the method of Pannell, *et al.*[7] with a minor modification. Albumin-free human serum was prepared by chromatography over Sepharose-Blue dextran using 50 mM Tris–HCl buffer (pH 8.0) containing 50 mM NaCl, and fractionated by $(NH_4)_2SO_4$ between 40% and 90% saturation. This fraction was chromatographed over Sephadex G-150 and then chromatographed over DEAE-cellulose at both 10 mM Tris–HCl buffer (pH 8.8) and 10 mM phosphate buffer (pH 6.5). The human α_1-antitrypsin thus purified was used in the following experiments.

Trypsin——Bovine pancreatic trypsin was supplied by Eisai Co., Tokyo.

Rabbit Anti-human α_1-Antitrypsin Serum——Rabbit anti-human α_1-antitrypsin serum was purchased from Behringwerk AG.

Assay of Trypsin Activity——Trypsin activity was measured according to the method of Folin and Ciocalteau.[8] A mixture of 3 ml of 1% casein solution and the enzyme solution was incubated at 37° for 20 min. The reaction was stopped by adding 3 ml of TCA–B solution composed of 0.11 M trichloroacetic acid, 0.22 M acetic, and 0.33 M sodium acetate. The mixture was allowed to stand for 30 min at 37° and then was filtered. Five ml of 0.55 M Na_2CO_3 solution and 1 ml of Folin reagent were added to 1 ml of the filtrate, and the mixture was allowed to stand at 37° for 30 min. Absorbance of this solution at 660 nm was measured.

Assay of Trypsin Inhibitory Activity of α_1-Antitrypsin——A mixture of 0.1 ml of trypsin solution (10 μg), 0.1 ml of α_1-antitrypsin solution, and 1 ml of 0.1 M Tris–HCl buffer (pH 8.2) was preincubated at 37° for 5 min and the residual trypsin activity was determined.

Biological Method for Determination of α_1-Antitrypsin——α_1-Antftrypsin was determined by trypsin inhibitory activity of α_1-antitrypsin.

Immunological Method for Determination of α_1-Antitrypsin——α_1-Antitrypsin was determined by the single radial immunodiffusion method. In this method, 5 μl of the sample was introduced into wells in Partigen-α_1-antitrypsin Immunoplate and allowed to stand at room temperature for 48 hr. The diameter of the ring of immunoprecipitate was measured. The concentration of α_1-antitrypsin in the samples was estimated from a standard curve.

Analytical Electrophoresis on Cellulose Acetate Membrane——Cellulose acetate membrane electrophoresis was carried out using Veronal buffer of pH 8.6 and ionic strength of 0.05 at 0.8 mA/cm of membrane width and at room temperature.

Immunoelectrophoresis——Immunoelectrophoresis was carried out in 1% agar and Veronal buffer of pH 8.6 and ionic strength of 0.05, at 7.5 mA/reaction plate and at room temperature. The reaction plates were allowed to stand for 24 hr and to form an immunoprecipitate with antiserum.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS Electrophoresis)——SDS electrophoresis was performed as described by Weber and Osborn.[9] A mixture of 1 ml of the sample and 0.1 ml of a solution containing 5% SDS, 8 M urea, and 2% 2-mercaptoethanol was incubated at 37° for 60 min. Five μl of the mixture was placed on 7% polyacrylamide gel containing 0.1% SDS. The electrophoresis was carried out in 0.1 M sodium phosphate buffer (pH 7.1) containing 0.1% SDS at 6 mA/tube and at room temperature for 4 hr. Protein was stained with Amido Black 10B. As marker proteins for molecular weight estimation, β-galactosidase (130000), phosphorylase a (94000), bovine serum albumin (67000), ovalbumin (43000), and cytochrome c (13500) were used.

Results

Amount of α_1-Antitrypsin by Biological Method

Amount of α_1-antitrypsin was determined by the above described method. As shown in Fig. 1, the trypsin inhibitory activity of α_1-antitrypsin increased with increasing concentration of α_1-antitrypsin. When the concentration of α_1-antitrypsin is expressed as a molar ratio to trypsin, as shown in Fig. 2, the trypsin activity was inhibited to 90% at the same molar ratio of α_1-antitrypsin to trypsin, and α_1-antitrypsin and trypsin formed a 1:1 stoichiometric complex.

Effect of Trypsin on Amount of α_1-Antitrypsin by Immunological Method

Trypsin of various concentrations and 1.28 mg/ml of α_1-antitrypsin were mixed in the same quantity and preincubated at 37° for 5 min. The amount of α_1-antitrypsin was deter-

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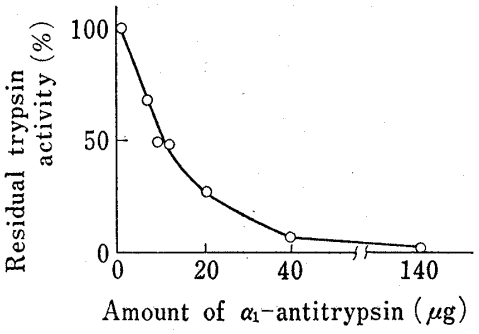

Fig. 1. Trypsin Inhibitory Activity of α_1-Antitrypsin

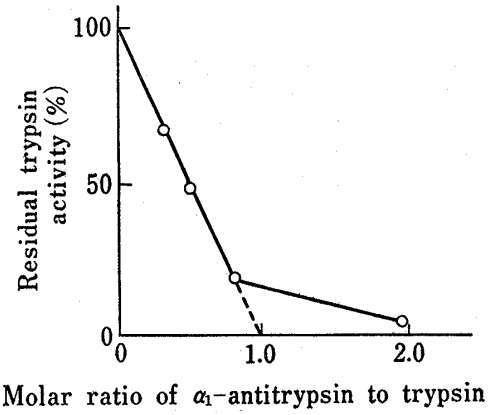

Fig. 2. Percentage of Trypsin Activity at Various Molar Ratios of α_1-Antitrypsin to Trypsin

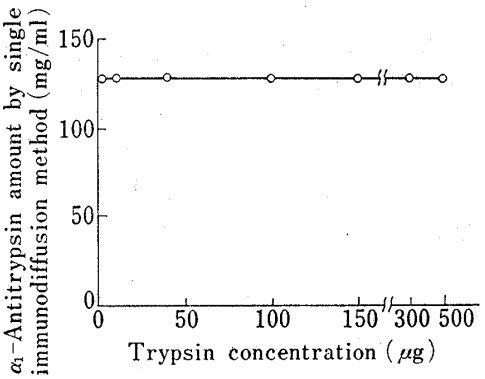

Fig. 3. Effect of Trypsin on Single Radial Immunodiffusion

The samples were introduced into wells in Partigen-α_1-antitrypsin Immunoplate. The diameter of the ring of immunoplate was measured after 48 hr. The concentration of α_1-antitrypsin in the samples was estimated from the standard curve.

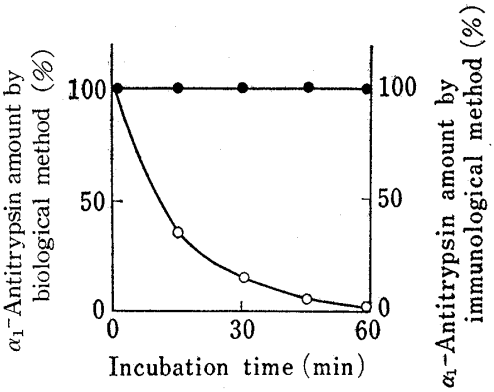

Fig. 4. Effect of Heat on the Amount of α_1-Antitrypsin Determined by Biological and Immunological Methods

○, by biological method.
●, by immunological method.

mined by the immunological method. As shown in Fig. 3, addition of increasing amounts of trypsin did not change the amount of α_1-antitrypsin.

Effect of Heat on Amount of α_1-Antitrypsin by Biological and Immunological Methods

After α_1-antitrypsin was incubated at 60°, the amount of α_1-antitrypsin determined by biological and immunological methods was compared. As shown in Fig. 4, the amount of α_1-antitrypsin by the biological method gradually decreased with increasing incubation time. On the other hand, the amount of α_1-antitrypsin by the immunological method was not affected by incubation at 60°.

Effect of Antiserum on Amount of α_1-Antitrypsin by Biological Method

A mixture of 1 ml of α_1-antitrypsin solution and 0.1 ml of antiserum of a given concentration was incubated at 37° for 2 hr, 0.1 ml of trypsin solution (100 μg/ml) was added to the reaction mixture, which was further incubated at 37° for 5 min, and the trypsin activity was determined. As shown in Fig. 5, the amount of α_1-antitrypsin by the biological method decreased with increasing concentration of antiserum.

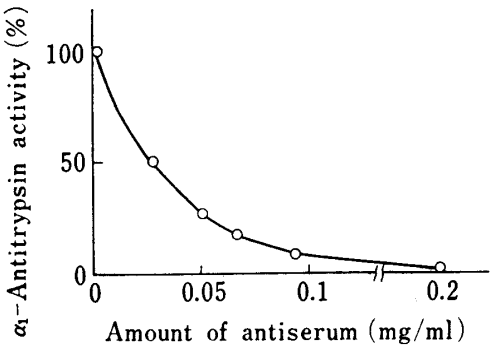

Fig. 5. Effect of Antiserum on Trypsin Inhibitory Activity of α_1-Antitrypsin

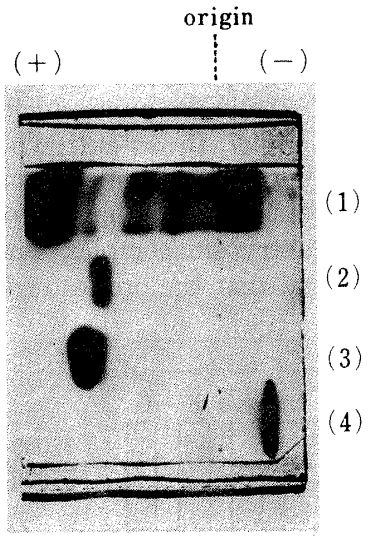

Fig. 6. Electrophoresis of Purified α_1-Antitrypsin, and Their Complex on Cellulose Acetate Membrane

Protein was stained with Amido Black 10B. (1) human serum, (2) α_1-antitrypsin-trypsin complex, (3) α_1-antitrypsin, (4) trypsin.

Electrophoresis of α_1-Antitrypsin, Trypsin, and Their Complex on Cellulose Acetate Membrane

Electrophoresis of α_1-antitrypsin, trypsin, and their complex on a cellulose acetate membrane was performed. Figure 6 showed that α_1-antitrypsin and α_1-antitrypsin–trypsin complex migrated to the α_1 and α_2 region, respectively.

Molecular Stoichiometry of Trypsin Inhibition by α_1-Antitrypsin

The molecular weight of the complex was estimated by SDS-electrophoresis. The electrophoresis showed that α_1-antitrypsin and trypsin had 55000 and 23000 molecular weight, respectively, that a new band was observed at 75000 for the complex and that the complex was in a 1: 1 molecular ratio of trypsin to α_1-antitrypsin.

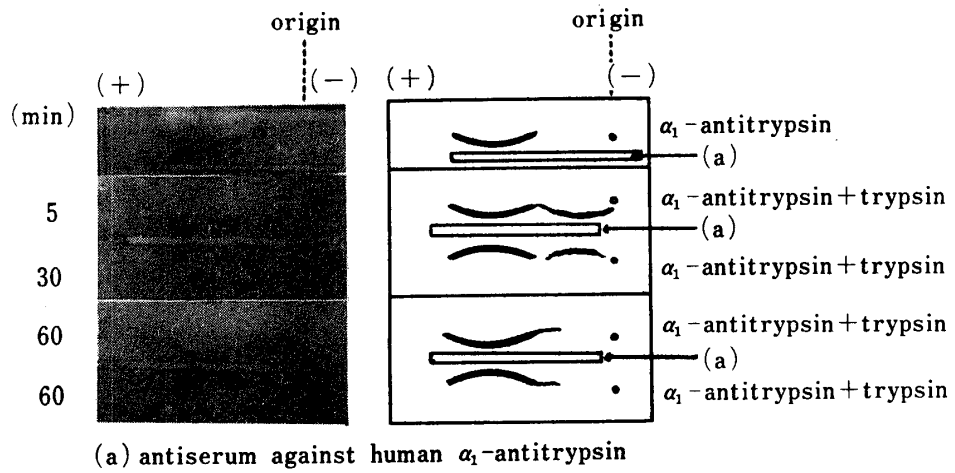

Fig. 7. Immunoelectrophoresis of α_1Antitrypsin–Trypsin Complex at Various Incubation Times

A mixture of 10 mg of trypsin and 1 mg of α_1-antitrypsin in 0.1 M phosphate buffer (pH 7.0) was incubated at 37° for indicated period of time and immunoelectrophoresis was performed.

Immunogenicity of α_1-Antitrypsin–Trypsin Complex with Excess of Trypsin

After the α_1-antitrypsin–trypsin complex with an excess of trypsin was incubated at 37° for 5, 30, and 60 min, immunogenicity of the complex was examined by immunoelectrophoresis. As shown in Fig. 7, the α_1-antitrypsin–trypsin complex migrated to the α_2 region when incubated for a short time. Longer incubation made the precipitin line of the α_2 region relatively faint and the precipitin line migrated toward the anode. This result suggests that the α_1-antitrypsin–trypsin complex with an excess amount of trypsin will be broken down by incubation and that the breakdown products also have immunogenicity.

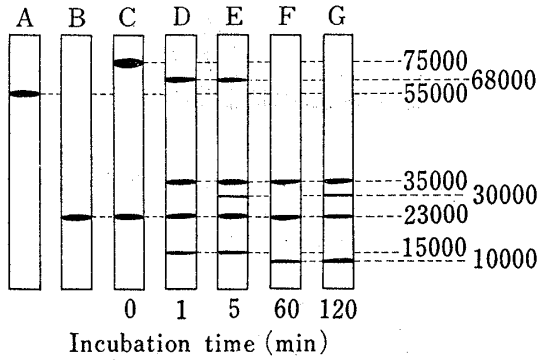

Fig. 8. SDS Electrophoresis of α_1Antitrypsin–Trypsin Complex at Various Incubation Times

A mixture of 10 mg of trypsin and 1 mg of α_1-antitrypsin in 0.1 M phosphate buffer (pH 7.0) was incubated at 37° for indicated period of time. Five ul of the mixture was applied to SDS electrophoresis.

Molecular Weight Determination of Breakdown Products of α_1-Antitrypsin–Trypsin Complex by SDS Electrophoresis

After the α_1-antitrypsin–trypsin complex with an excess of trypsin was incubated at 37° for various periods of time, the breakdowm products were determined by SDS electrophoresis for molecular weight. Figure 8 shows that the α_1-antitrypsin–trypsin complex of molecuar weight 75000 was broken down by incubation into proteins with molecular weight of 68000, 35000, 30000, 15000, and 10000 each. While the pathway of the breakdown is not clear, the determined molecular weights of the breakdown products suggests not only that α_1-antitrypsin and trypsin will dissociate, but also that the complex itself will be broken down.

Discussion

In the presence of trypsin, the amount of α_1-antitrypsin determined by the biological and immunological methods was not the same, and α_1-antitrypsin and α_1-antitrypsin–trypsin complex were indistinguishable immunologically. When incubated at 60°, the amount of α_1-antitrypsin found by the biological method decreased but that by the immunological method remained the same. Trypsin inhibitory activity of α_1-antitrypsin decreased with increasing concentration of antiserum for α_1-antitrypsin. This result shows that the biological and immunological active site of α_1-antitrypsin differs from each other. Namely, the biological active site is affected by the combination of its immunological active site with an antibody, and the immunological active site is not masked by the combination of the biological active site and trypsin. Johnson, *et al.*[10] reported that α_1-antitrypsin and trypsin formed a 1 : 2 stoichiometric complex but Moroi and Yamasaki[11] concluded from the molecular weight that α_1-antitrypsin and trypsin formed a 1 : 1 stoichiometric complex. Our investigation of the trypsin inhibitory activity of α_1-antitrypsin and molecular weight determination by SDS electrophoresis also showed that α_1-antitrypsin and trypsin formed a 1 : 1 stoichiometric complex. The differrence in experimental results may be due to the differences in the type and purity of α_1-antitrypsin and trypsin, and in the experimental conditions.

The results of immunoelectrophoresis and SDS electrophoresis indicate that the immunogenicity and molecular weight of the complex with an excess amount of trypsin decrease with incubation. This result promoted our interest to study how the amount of α_1-antitrypsin determined by biological and immunological methods would vary in various diseases.

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It was reported that fibrinogen was broken down by plasmin and that the breakdown products had antigenic determinants similar to those of fibrinogen[12] and were associated with abnormal fibrinolysis[13] and various kidney diseases.[14] It seemed important to see whether the breakdown products of the α_1-antitrypsin–trypsin complex are associated with diseases or not.

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