A NEW SERUM COMPONENT WHICH SPECIFICALLY INHIBITS THIOL PROTEINASES

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SUMMARY

A serum proteinase inhibitor specific for thiol proteinases was prepared in a functionally pure state by Sephadex G-200 gel filtration, starch block electrophoresis and immunoaffinity chromatography. This component was distinct from the known serum proteinase inhibitors. It was demonstrated by immunoelectrophoresis that the incubated mixture of thiol proteinase and this inhibitor produced a soluble complex possessing both antigenicities. The molecular weight of the inhibitor was found to be 90,000 by gel filtration on Sephadex G-150 column, and the electrophoretic mobility was in the α_2-region. A tentative term, α_2-thiol proteinase inhibitor, was given because of its mobility and inhibition spectrum.

Most serum proteinase inhibitors that have been characterized interact with serine proteinases (1) with the exception of α_2-macroglobulin, which binds not only serine proteinases but also other proteinases including thiol proteinases. In a previous investigation (2) we noticed that an inhibitory activity specific to thiol proteinases appeared between the second and third peaks of Sephadex G-200 fractionation. The purpose of this study was to elucidate the characteristics of this component as a proteinase inhibitor.

MATERIALS AND METHODS

Materials. Bovine trypsin[EC 3.4.21.4] and α-chymotrypsin [EC 3.4.21.1] were purchased from Worthington Biochemical Corporation and nagarse (subtilisin BPN') [EC 3.4.21.14] from Nagase Company Limited. Papain [EC 3.4.22.2] and ficin [EC 3.4.22.3] were purified in our laboratory by the methods of Kimmel and Smith (3) and of Englund et al (4), respectively, from crude enzyme preparations from Worthington Biochemical Corporation. Cathepsin B1 [EC 3.4.22.1] was purified from fresh porcine liver according to the procedure of Greenbaum and Fruton (5). Stem [EC 3.4.22.4] and fruit bromelains were prepared as described previously (6). Casein for enzyme assay was a product of Wako Company limited. Hemoglobin for the assay of cathepsin B1, was purchased from Miles Laboratories Incorporation. Pure agar, Agarose, monospecific antisera and immunoplates (Partigen) were preparations of Behringwerke Höchst. The γ-

Abbreviations used: α_2TPI, α_2-thiol proteinase inhibitor; α_2M, α_2-macroglobulin; α_1AT, α_1-antitrypsin; α_1X, α_1-antichymotrypsin; Ath III, Antithrombin III; IαI, inter α-trypsin inhibitor; C_1sI, C_1 esterase inhibitor; Cp, ceruloplasmin; and α_2HS, α_2-heat stable glycoprotein.

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globulin fractions of these specific antisera were fractionated by ammonium sulfate precipitation (7) (40% saturation once and 33% saturation twice) in order to exclude the coexistent proteinase inhibitors in the α_1- and α_2-regions of these antisera. Antiwhole human serum and anti-α_2TPI were prepared in our laboratory by immunizing rabbits (8). Human serum was collected from more than ten healthy persons.

Enzyme assay. Caseinolytic activity was assayed by Kunitz's method (9) with an appropriate modification. To 1.0 ml of enzyme solution (enzyme, 10-20 μg) was added 3.0 ml of 1.2% casein in 0.05 M potassium phosphate buffer, pH 7.5 (In the case of thiol proteinases, substrate solution contained 6 mM cysteine for maximum activation of enzyme.). After 30 min-incubation at 30°C, 4.0 ml of 6% trichloroacetic acid in 0.05 M acetate buffer, pH 4.5 was poured into the reaction mixture. Coagulated precipitate was removed by filtration after standing for an additional 30 min. The absorption of the filtrate was then determined at 275 nm using a Hitachi Perkin-Elmer 139 spectrophotometer. Proteolytic activity of cathepsin B1 (10) was assayed by using hemoglobin as substrate in 0.05 M acetate buffer, pH 4.5, instead of the casein solution.
Inhibition assay. Enzyme was first incubated at room temperature for 30 min with an appropriate amount of the serum fraction containing proteinase inhibitor. The subsequent procedures were the same as for the enzyme assay described above.
Suppression of inhibition activities by specific antibodies. The inhibitor was incubated for 30 min with monospecific antibody (γ-globulin fraction), followed by adding 15 μg of enzyme and adjusting the volume to 1.0 ml. After 30 min-incubation, casein and then trichloroacetic acid were added, followed by the procedures described above.
Inhibition effect of α_2TPI on factor XIII. The effect of α_2TPI on factor XIII activity was measured by means of an assay system from Behringwerke Höchst (a kit for determining coagulation factor XIII).
Immobilization of protein. Antibody was attached to BrCN-activated Chromagel A-2 (Dojin Yakkagaku Institute) for immunoaffinity chromatography (11).
Starch block electrophoresis. It was performed in 0.05 M veronal-acetate buffer, pH 8.6, with a voltage gradient of 5 V/cm for 60 hr at 4°C.
Immunoelectrophoresis. It was carried out in 1.2% agar (1:1 mixture of Pure agar and Agarose) dissolved in 0.025 M veronal-acetate buffer, pH 8.6, with a voltage gradient of 8 V/cm for 90 min at 4°C.

RESULTS

Partial Purification. Fresh human serum was fractionated by Sephadex G-200 gel filtration. The inhibitory activity in every fraction toward thiol proteinases was estimated using casein as substrate. Dominant inhibitory activities were found both in the first peak and between the second and third peaks (figure 1). The former inhibitory activity coincided with α_2-macroglobulin activity and the latter depended on a new proteinase inhibitor. The elution positions of other proteinase inhibitors and of two additional serum components, ceruloplasmin and α_2-heat stable glycoprotein (which show very similar behaviors to those of α_2TPI in chromatographic and electrophoretic analyses), were determined by measuring their antigenicities on immunoplates containing specific antisera

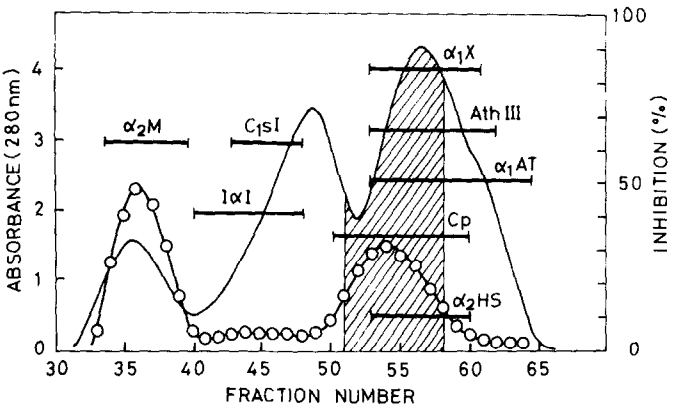

Figure 1. Distribution of the inhibitory activity against thiol proteinase and of several plasma proteins on Sephadex G-200 gel filtration. 25 ml of human serum was applied on Sephadex G-200 gel column (3 x 140 cm) equilibrated with 0.02 M borate buffered saline, pH 8.0. Gel filtration was performed with the same buffer at a flow rate of 10 ml/hr. Each fraction volume was 15 ml. Inhibitory activity was estimated as described in the Materials and Methods section. Open circles denote inhibitory activity against thiol proteinase and solid line UV absorption at 280 nm. The locations of several plasma proteins estimated by radial immunodiffusion plates are shown with bar symbols.

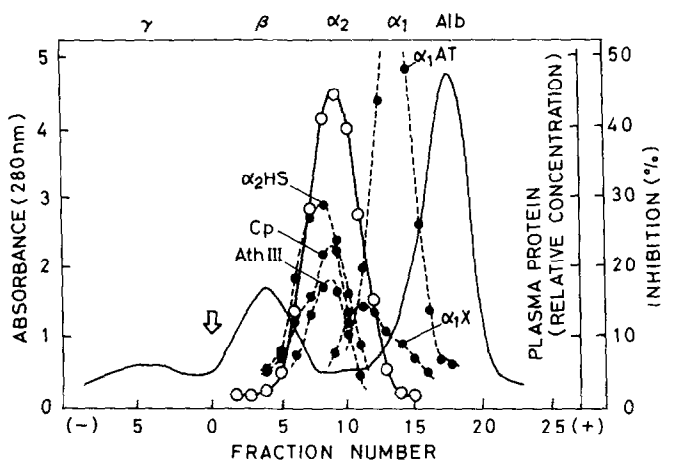

Figure 2. Distribution of the inhibitory activity against thiol proteinase and of several plasma proteins on starch block electrophoresis. The shaded portion of figure 1 was subjected to starch block electrophoresis. The size of the block was 4 x 1.5 x 45 cm. An arrow indicates the position the sample was applied. Electrophoresis was carried out as described in the Materials and Methods section. After electrophoresis the starch was cut into 1 cm-long small blocks, and the protein was extracted with chilled borate buffered saline, pH 8.0. Open circles denote inhibitory activity against thiol proteinase, solid circles, the amount of plasma proteins estimated by immunoplates, and solid line, UV absorption.

Table 1

Suppression of the inhibitory activity of α_2TPI by corresponding antibody

Experimental systems	Absorbance (275nm)	Inhibition (%)	Suppression (%)	Suppression corrected(%)
Fic	0.542	0	–	–
Fic + (α_2TPI)	0.116	78.6(100.0)	0	0
Fic + (α_2TPI + anti-α_2TPI)	0.354	34.7(44.1)	55.9	58.4
Fic + (α_2TPI + anti-Cp)	0.138	74.5(94.8)	5.2	7.8
Fic + (α_2TPI + anti-Ath III)	0.100	81.5(103.7)	−3.7	−1.1
Fic + (α_2TPI + anti-α_1X)	0.104	80.8(102.8)	−0.3	−0.3
Fic + (α_2TPI + anti-α_2HS)	0.118	78.2(99.5)	0.5	3.0
Fic + (α_2TPI + normal IgG)	0.105	80.6(102.5)	(−2.5)	–

The extent of inhibition and suppression was estimated as described in the Materials and Methods section.
Assay system: Enzyme; ficin, 15 μg/15 μl. Buffer; 0.02 M borate buffered saline, pH 8.0, 0.2 ml (In the case without added antibody or without antibody and inhibitor, 0.7 or 1.0 ml of buffer solution was used in place of 0.2 ml of buffer solution). Inhibitor; α_2TPI fraction purified by immunoadsorbent column, 0.3 ml. Antibody; γ-globulin fraction and rabbit normal IgG, 0.5 mg/0.5 ml. Substrate; 1.2% casein in 0.05 M phosphate buffer, pH 7.0, 3.0 ml. Reagent for stopping the reaction; 6% trichloroacetic acid in 0.05 M acetate buffer, pH 4.5, 4.0 ml. Fic; ficin.

(shown as bar symbols). The gel filtration procedure made it possible to eliminate three of six known proteinase inhibitors from the α_2TPI fraction (shaded area), which was then subjected to starch block electrophoresis after concentration. As shown in figure 2 the inhibitory activity was found in the α_2-region being distinguished from the electrophoretic positions of α_1-antitrypsin and α_1-antichymotrypsin. One of the proteinase inhibitors, antithrombin III, and other serum components such as α_2-heat stable glycoprotein and ceruloplasmin, were still inseparable by these two successive steps of fractionations from α_2TPI activity.

Identification of inhibitor by suppression effect. The remaining components not separated by the above procedure were tested by the effects of suppression using specific antibodies corresponding to each component. The result is listed in table 1. In this assay system 15 μg of ficin digested casein to an

absorption value of 0.542 at 275 nm, but in the presence of α_2TPI (mixture of fractions No.8, 9 and 10 in starch block electrophoresis in figure 2) the digestion was reduced to as low as 21% of the original activity (absorption value, 0.116). Preincubation of the α_2TPI fraction with anti-antithrombin III, anti-α_1-antichymotrypsin or anti-α_2-heat stable glycoprotein antibody did not affect the inhibitory activity of α_2TPI. Only antibody preparation against the α_2TPI fraction displayed a significant suppression of the inhibitory activity. Anti-ceruloplasmin showed a slight but insignificant suppression of proteinase inhibitor activity. Accordingly, these data demonstrated that α_2TPI differed from the known six serum proteinase inhibitors, either in their molecular weight, electrophoretic mobility or antigenicity.

Inhibition spectrum. It is necessary to obtain a functionally pure sample for studying the inhibition spectrum. For this purpose γ-globulin fractions were isolated from commercially available monospecific antisera (anti-α_1-antitrypsin, anti-α_1-antichymotrypsin and anti-antithrombin III) by ammonium sulfate precipitation (see Materials and Methods). A mixture of these γ-globulins was attached to BrCN-activated Chromagel A-2 providing an immunoadsorbent column. The sample prepared by gel filtration followed by electrophoresis was repeatedly passed through this immunoadsorbent column (1.2 x 4 cm) in order to thoroughly eliminate the above three proteinase inhibitors. The nonadsorbed fraction was concentrated and used for the inhibition assay. Although papain, ficin and cathepsin B1 were inhibited almost completely when they were preincubated with sufficient amounts of this fraction (not shown), the extent of inhibition of fruit and stem bromelains was considerably lower than that of papain and ficin (about one-third). On the other hand, the inhibition toward serine proteinases such as trypsin, chymotrypsin and nagarse was approximately 0 but up to 10% at the highest. Unlike thiol proteinases, a thiol enzyme (factor XIII in the blood coagulation system) was totally unaffected when estimated by the use of coagulation assay system (see Materials and Methods).

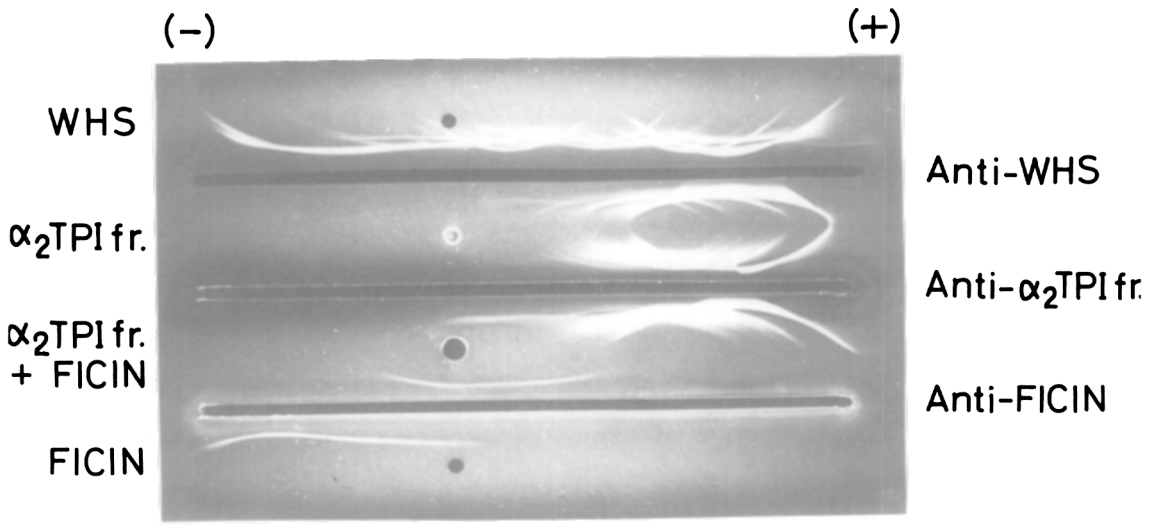

Figure 3. Immunoelectrophoretic demonstration of ficin-α_2TPI complex. 3 µl of each antigen was applied in the wells as indicated. Only for the mixed solution a total of 6 µl was used after 15 min-incubation at room temperature. Ficin was activated with 5 mM cysteine before incubation. α_2TPI fr.; mixture of fractions No.8, 9 and 10 in starch block electrophoresis. WHS; whole human serum. Electrophoretic condition, see Materials and Methods section.

Molecular weight. The molecular weight of this inhibitor was measured by gel filtration through a Sephadex G-150 column (0.9 x 100 cm) (12) in comparison with some reference proteins (α-chymotrypsin, egg albumin, bovine serum albumin and human IgG). The inhibition activity emerged in the position corresponding to a molecular weight of 90,000.

Proteinase-inhibitor complex. For the elucidation of a proteinase-inhibitor complex, an incubated mixture of ficin and α_2TPI fraction was applied on a agar plate for immunoelectrophoresis. Figure 3 shows the immunoelectrophoretic patterns when ficin, α_2TPI fraction and the above mixture were run simultaneouly. Ficin migrated toward the anode, while all the serum components in the α_2TPI fraction migrated toward the cathode. However, in the mixture of ficin and the α_2TPI fraction a new component appeared which migrated in an intermediate position between ficin and α_2TPI and had antigenic properties of both. This indicates that α_2TPI formed a soluble complex with ficin.

DISCUSSION

α_2TPI was separated from α_2-macroglobulin, inter α-trypsin inhibitor and C_1 esterase inhibitor by the difference of their molecular weights, and from α_1-antitrypsin and α_1-antichymotrypsin by their electrophoretic mobilities. The examination of antigenicities further distinguished α_2TPI from another inhibitor, antithrombin III, and from two other serum components (ceruloplasmin and α_2-heat stable glycoprotein) that had very similar physicochemical properties to α_2TPI. Thus, it appears that this inhibitor is a new serum component. α_2TPI inhibited very selectively thiol proteinases of animal and plant origins. The extent of the inhibitory effect was, however, different among several thiol proteinases investigated. Ficin and papain were most sensitive, cathepsin B1 was less sensitive, and fruit and stem bromelains were considerably less sensitive. Such a difference of susceptibility among thiol proteinases may suggest that the inhibition is not due to a simple stoichiometric combination. It is well known that α_2-macroglobulin inhibits not only serine proteinases but also other proteinases like thiol (2, 13) and acidic proteinases (14). In contrast, α_2TPI inhibits selectively thiol proteinases indicating that the mechanism of inhibition by α_2TPI is substantially different from that of α_2-macroglobulin. The biological function of α_2TPI is not yet known. However, one of the probable role of this inhibitor may be in the control of the thiol proteinases released at inflammatory sites. In fact, Beloff (15) and Tokaji (16) have indicated that the prolonged phase of inflammation, which depends on thiol proteinases, is influenced by blood and tissue inhibitors. It is of further interest to determine whether α_2TPI interacts with the proteinases released in the inflammatory loci and by what mechanism this occurs.

REFERENCES

1. Heimburger, N., Haupt, H. and Schwick, H.G. (1971) Proceedings of the International Research Conference on Proteinase Inhibitors, pp.1-22, Walter de Gruyter, Berlin-New York.
2. Sasaki, M., Yamamoto, H., Yamamoto, H. and Iida S. (1974) J. Biochem. 75, 171-177.
3. Kimmel, J.R. and Smith, E.L. (1954) J. Biol. Chem. 207, 515-531.
4. Englund, T.R., King, T.P. and Craig, L.C. (1968) Biochemistry 7, 163-175.

5. Greenbaum, L.M. and Fruton, J.S. (1957) J. Biol. Chem. 226, 173-180.
6. Iida, S., Sasaki, M. and Ota, S. (1973) J. Biochem. 73, 377-386.
7. Arnon, R. and Shapira, E. (1967) Biochemistry 6, 3942-3950.
8. Sasaki, M., Iida, S. and Murachi, T. (1973) J. Biochem. 73, 367-375.
9. Kunitz, M. (1947) J. Gen. Physiol. 30, 291-310.
10. Mycek, M.J. (1970) Cathepsins in "Methods in Enzymology" 19, 285-315.
11. Cuatrecasas, P., Wilchek, M. and Anfinsen, C.B. (1968) Proc. Nat. Acad. Sci. U.S. 61, 636-643.
12. Andrews, P. (1964) Biochem. J. 91, 222-233.
13. Starkey, P.M. and Barrett, A.J. (1973) Biochem. J. 131, 823-831.
14. Landurean, J.C. and Steinbuck, M. (1970) Z. Naturforsch. 25B, 231-232.
15. Beloff, A. (1946) Biochem. J. 40, 108-115.
16. Tokaji, G. (1971) Kumamoto Med. J. 24, 68-86.