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REPRESSION OF FIBRINOLYSIS IN SCALDED RATS BY ADMINISTRATION OF *SERRATIA* PROTEASE*

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Abstract—The oral administration of *Serratia* protease (SP), an antiinflammatory oral drug, to scalded rats markedly repressed the activation of fibrinolysis induced by the scalding. The repression was most effective when SP was given 3 hr prior to scalding, and a statistically significant repression was observed at a dose of 5 mg/kg. The repressive effect of SP was dependent on its proteolytic activity and was far stronger than those of other proteases tested. The repression was observed also by the intravenous injection of SP at a dose as low as 0.2 μg/kg, which corresponded to a blood concentration of about 4 ng/ml. In this case, too, the proteolytic activity was essential. In rat blood, SP existed as a complex with a plasma protease inhibitor, α_1-macroglobulin (α_1M), with a molar binding ratio of 1:1, still retaining about 20% of its original caseinolytic activity. This ratio, together with the α_1M concentration in rat plasma and the molecular weights of SP and α_1M, enabled the estimation that at the effective SP concentration (4 ng/ml) only 1 out of 20,000 parts of α_1M molecules in plasma took part in the complex formation.

Scalding induces the abnormal activation of fibrinolysis [1]. In the course of our study on the effects of proteases on the rat fibrinolytic system, we unexpectedly observed a significant repression of the activation of fibrinolysis, induced in scalded rats, by the oral administration of a bacterial protease called "*Serratia* protease" (SP). The protease is the active entity of Danzen [2, 3] which is used clinically in several countries including Japan as an effective antiinflammatory oral drug.

Several papers have appeared so far which refer to the systemic effects of orally given proteases in experimental animals, such as repression of paw edema formation induced by carrageenin, dextran or histamine, and repression of blood vessel permeability induced by histamine or bradykinin [4–6]. However, the effects on fibrinolytic activity have not yet been reported.

In this paper, we describe the repression of the activation of fibrinolysis, due to scalding, by the oral or intravenous administration of SP and some observations on the behavior of SP in rat blood.

MATERIALS AND METHODS

Proteases. SP is a metalloprotein containing a zinc atom per molecule; its molecular weight is about 50,000. The SP preparation used throughout the present investigation was obtained in a homogeneous form from the culture filtrate of a *Serratia* sp. according to the procedure described by Miyata *et al.* [2]. Bovine trypsin and bovine α-chymotrypsin, both twice crystallized, were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A., and pronase, from the Kaken Chemical Co., Tokyo, Japan. These proteases were dissolved in saline and administered to scalded rats either orally or intravenously.

Animals. Male JCL-SD rats, 9–11 weeks in age and weighing 310–400 g, were used in all the experiments. They were housed for at least a week under constant conditions (temperature, 23 ± 3°; humidity, 55 ± 5%) and were provided with a chew diet (CE-2, Clea Japan Inc., Osaka, Japan) and tap water *ad lib.*

Scalding and blood collection. Rats were anesthetized with ether, fixed to a wire-netting by binding their limbs, and soaked in hot water of 55° for 1 min to scald their back to the extent of 30% of the body surface. One hour after the scalding, blood samples were drawn from the tail vein and abdominal aorta of each rat with a plastic syringe under ether anesthesia.

Assay of fibrinolytic activity. Fibrinolytic activity was assayed by thrombelastography [7] or by measurement of S-2251 (H-D-Val-Leu-Lys-*p*-nitroanilide, Kabi AB, Uppsala, Sweden) hydrolytic activity [8]. From the thrombelastogram obtained using a blood sample from the tail vein with a thrombelastograph (Fa. Hellige, Freiburg, West Germany) the fibrinolytic rate was calculated according to the following equation [9]: the fibrinolytic rate (%) = $[(ma - ma')/ma] \times 100$ (*ma*, the maximum amplitude; *ma'*, the amplitude 60 min after the maximum amplitude was attained). The S-2251 hydrolytic activity was measured as follows. A reaction mixture consisting of 0.25 ml of the euglobulin fraction, 0.02 ml of a 3 mM S-2251 solution, and 0.23 ml of

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0.05 M Tris · HCl–0.012 M NaCl (pH 7.4) was incubated at 37° for 30 min, the reaction was stopped by the addition of 0.05 ml of 50% (v/v) acetic acid, and the absorbance at 405 nm was read. The hydrolytic activity was expressed in terms of the increase of the absorbance at 405 nm of the reaction mixture. The euglobulin fraction was prepared from a blood sample from abdominal aorta according to the method described previously [10]. Briefly, a cold 0.02% (v/v) acetic acid solution (9.5 ml) was added to a plastic tube containing 0.5 ml of citrated plasma to adjust the pH of the mixture to 5.2. The mixture was kept at 5° for 30 min and centrifuged at 500 *g* for 10 min. The precipitate was dissolved in 0.5 ml of 0.067 M phosphate buffered saline (pH 7.4). Interference with the assay of S-2251 hydrolytic activity by fibrinogen was not taken into consideration since we confirmed that the administration of SP did not affect the fibrinogen concentration in the euglobulin fraction.

Assay of proteolytic activity and SP-inhibiting activity. Proteolytic activity was assayed using casein (E. Merck, Darmstadt, West Germany) as substrate according to the method of Kunitz [11]. One unit of caseinolytic activity (CU) was defined as that which gave, in 1 min, an increase of the absorbance at 275 nm equivalent to that of 1 μg of tyrosine.

SP-inhibiting activity was estimated by measuring the residual caseinolytic activity of the reaction mixture that was obtained after SP and an inhibitor-containing solution had been preincubated in 0.05 M phosphate buffer (pH 7.0) at 5° for 1 hr. One unit of SP-inhibiting activity (IU) was defined as that which inhibited the caseinolytic activity of 4 μg of SP by 50%.

Fractionation of rat plasma and detection of SP inhibitor. A blood sample was drawn from the abdominal aorta of anesthetized rats with a plastic syringe containing one-ninth volume of 3.8% sodium citrate. The citrated blood was centrifuged at 1200 *g* for 10 min at 5° to obtain citrated plasma. The citrated plasma (44 ml) was applied to a Sepharose CL-6B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) column (4.6 × 54 cm) equilibrated with 0.85% NaCl–0.38% sodium citrate. The column was developed with the same buffer, and each fraction was monitored for the absorbance at 280 nm and SP-inhibiting activity. For estimation of the molecular weight of the SP inhibitor on the column, bovine serum albumin (Miles Laboratories Inc., Elkhart, IN, U.S.A.), human γ-globulin (Schwarz/Mann, Orangeburg, NY, U.S.A.) and ferritin (Boehringer Mannheim GmbH, Mannheim, West Germany) were used as marker proteins.

Purification of SP inhibitor from rat plasma. SP inhibitor was purified homogeneously from the normal rat plasma by monitoring its SP-inhibiting activity. All the purification procedures were carried out at 5°. To 127 ml of citrated rat plasma, 22 ml of 55% (v/v) ethanol was added with gentle stirring. The solution was stirred for an additional 30 min and centrifuged at 90 *g* for 10 min. To the supernatant fraction (140 ml), 460 ml of distilled water was added, and the resulting aqueous solution was applied to a DEAE-cellulose (DE-11, Whatman Inc., Clifton, NJ, U.S.A.) column (3.3 × 40 cm) equilibrated with 0.01 M phosphate buffer (pH 7.0). After the column was washed with the same buffer, a linear gradient from 0.05 to 0.2 M NaCl in 0.01 M phosphate buffer (pH 7.0) was applied. Fractions containing SP-inhibiting activity were pooled (510 ml) and concentrated to 20 ml with a Diaflo ultrafiltration membrane PM 10 (Amicon Co., Lexington, MA, U.S.A.). The concentrated solution was applied to a Sepharose CL-6B column (4 × 68 cm) equilibrated with 0.01 M phosphate buffer (pH 7.0). Active fractions were pooled (93 ml) and concentrated to 16 ml by ultrafiltration as described above. The concentrated solution was treated again on the same Sepharose CL-6B column and the active fractions were pooled (58.5 ml).

Electrophoresis. Analytical polyacrylamide gel electrophoresis was carried out in 4.0% acrylamide gel (pH 9.4) as described by Gabriel [12]. Immunoelectrophoresis was carried out for 2 hr at 4 mA/cm using 1% agar (Difco Laboratories, Detroit, MI, U.S.A.) gel according to the method of Grabar and Williams [13].

RESULTS

Repression of the activation of fibrinolysis in scalded rats by oral administration of SP. Three rats were anesthetized with ether and two of them were scalded. SP dissolved in saline (3 ml) was administered orally to one of the scalded rats 3 min after the scalding at a dose of 150 mg/kg. The other one of the scalded rats and the unscalded rat received saline (3 ml) only. The thrombelastograms of blood samples from the normal control rat, the scalded rat, and the scalded and SP-administered rat are depicted in Fig. 1, which shows a drastic change of the thrombelastogram due to the scalding and its dramatic restoration due to the oral administration of SP at a dose of 150 mg/kg body weight. To confirm that these changes in the thrombelastogram reflected changes in fibrinolytic activity, both the fibrinolytic rate obtained from thrombelastograms and the S-2251 hydrolytic activity were compared on blood samples from normal rats, scalded rats, and scalded and SP-administered rats. Scalding and oral administration of SP to these groups, each consisting of six rats, were carried out as described above. When the fibrinolytic rate obtained from the thrombelastogram of a blood sample and the S-2251 hydrolytic activity of its euglobulin fraction are presented on the abscissa and ordinate, respectively, the plots for normal rats came together in the low fibrinolytic region, and those for scalded rats moved to the higher fibrinolytic region. But when SP was given orally to the scalded rats, the plots returned to almost the same region as that for normal rats (Fig. 2). A high correlation ($r = 0.82$, $P < 0.001$) was obtained between the fibrinolytic rate and the S-2251 hydrolytic activity. These results clearly demonstrated that the oral administration of SP to scalded rats repressed the activation of fibrinolysis induced by scalding. The same dose and route of administration of SP did not affect the fibrinolytic activity of normal, unscalded rats (data not shown).

The extent of the repressive effect of SP was compared with those of several other antiinflam-

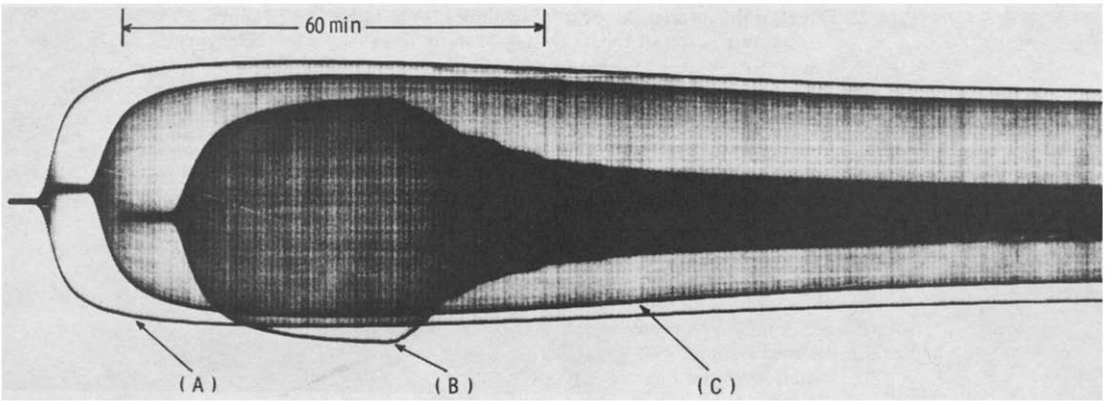

Fig. 1. Change of thrombelastogram by scalding and its restoration by oral administration of SP. Key: (A) an unscalded and saline-administered rat, (B) a scalded and saline-administered rat, and (C) a scalded and SP-administered (150 mg/kg) rat.

matory proteases. SP, bovine trypsin, bovine α-chymotrypsin and pronase were all given orally to scalded rats 3 min after scalding at doses that were equivalent in *in vitro* caseinolytic activity. As shown in Table 1, SP was far more effective than the other proteases tested. Trypsin was slightly effective, while α-chymotrypsin and pronase were ineffective. Next, the most effective time of administration for the repression was investigated. For this purpose, SP was given orally to scalded rats at a dose of 5 mg/kg at various times before or after scalding. As shown in Table 2, administration at 3 hr before scalding was most effective, and a remarkable repression was observed. At this dose, administration after scalding was ineffective. In order to know the dose dependence of the repressive effect of SP, SP was administered orally at various doses (0–50 mg/kg) 3 hr before scalding. The repressive effect was dose dependent, and a statistically significant repression was observed at doses of, and above, 5 mg/kg (Table 3). Proteolytic activity was found to be essential for the repression, since the heat-inactivated preparation of SP was ineffective (Table 3).

Repression of the activation of fibrinolysis in scalded rats by intravenous administration of SP. The effect of intravenous administration of SP on the activation of fibrinolysis in scalded rats was examined. SP was administered intravenously at various doses (0–200 μg/kg) 3 min after scalding. Blood samples were collected 1 hr after scalding, and the fibrinolytic rate was measured.

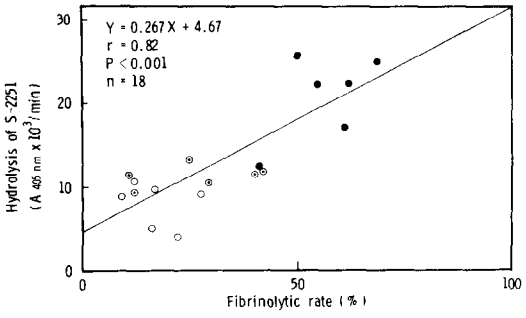

Fig. 2. Repression of the activation of fibrinolysis in scalded rats by oral administration of SP, as measured by both fibrinolytic rate of thrombelastogram and S-2251 hydrolytic activity. Key: (○) unscalded and saline-administered rats, (●) scalded and saline-administered rats, and (⊙) scalded and SP-administered (150 mg/kg) rats.

As shown in Table 4, the activation of fibrinolysis in scalded rats was repressed by the intravenous administration of SP at a dose as low as 0.2 μg/kg.

Table 1. Comparison among several proteases of their repressive effects on the activation of fibrinolysis in scalded rats*

Rat†	Protease	Dose (mg/kg)	Fibrinolytic rate (%)
Scalded			64.0 ± 10.4‡
Scalded	SP	150	13.3 ± 7.7§
Scalded	Trypsin (bovine)	260	30.7 ± 17.6‖
Scalded	α-Chymotrypsin (bovine)	260	62.7 ± 14.7
Scalded	Pronase	600	61.0 ± 12.3
Normal			12.0 ± 4.1

* Proteases were administered orally 3 min after scalding at doses equivalent in *in vitro* caseinolytic activity.

† Six animals were used in each group.

‡ Mean ± standard deviation.

§ Significantly different from the scalded control group at $P < 0.001$.

‖ Significantly different from the scalded control group at $P < 0.01$.

Table 2. Effect of the interval between oral administration of SP and scalding on the repression of the activation of fibrinolysis in rats*

Rat†	Hours between SP administration and scalding	Fibrinolytic rate (%)
Scalded		65.5 ± 15.1‡
Scalded	5	25.7 ± 9.6§
Scalded	3	17.6 ± 10.1§
Scalded	1	74.9 ± 16.5
Scalded	0	59.0 ± 6.2
Normal		12.4 ± 4.1

* SP was administered orally at a dose of 5.0 mg/kg at the same time as, or 1, 3 or 5 hr before scalding.
† Six animals were used in each group.
‡ Mean ± standard deviation.
§ Significantly different from the scalded control group at $P < 0.001$.

Table 3. Dose dependence of the repression by oral administration of SP of the activation of fibrinolysis in scalded rats*

Rat (number)	Dose (mg/kg)	Hydrolysis of S-2251 ($A_{405nm} \times 10^3$/min)
Scalded (10)	0	12.7 ± 2.4†
Scalded (5)	1	14.4 ± 3.5
Scalded (4)	5	6.0 ± 3.4‡
Scalded (5)	10	3.4 ± 1.2§
Scalded (5)	50	2.8 ± 1.7§
Scalded (5)	50 (heated‖)	13.6 ± 2.8
Normal (5)	0	2.3 ± 0.4

* SP was administered orally at various doses 3 hr before scalding.
† Mean ± standard deviation.
‡ Significantly different from the scalded control group at $P < 0.01$.
§ Significantly different from the scalded control group at $P < 0.001$.
‖ SP was completely inactivated by treatment at 65° for 1 hr.

Table 4. Repression by intravenous administration of SP of the activation of fibrinolysis in scalded rats*

Rat†	Dose (μg/kg)	Fibrinolytic rate (%)
Scalded	0	57.7 ± 8.5‡
Scalded	0.02	58.7 ± 14.7
Scalded	0.2	14.0 ± 5.6§
Scalded	2	27.0 ± 26.3‖
Scalded	20	46.8 ± 31.1
Scalded	200	49.8 ± 22.8
Scalded	0.2 (heated¶)	58.3 ± 13.7
Normal	0	12.0 ± 4.1

* SP was administered intravenously at various doses 3 min after scalding.
† Six animals were used in each group.
‡ Mean ± standard deviation.
§ Significantly different from the scalded control group at $P < 0.001$.
‖ Significantly different from the scalded control group at $P < 0.05$.
¶ SP was completely inactivated by treatment at 65° for 1 hr.

Calculated in terms of the concentration in blood, this dose corresponded to about 4 ng/ml, an extremely low concentration. At doses above 0.2 μg/kg, a gradual increase in fibrinolytic activity was observed due to the degradation of fibrin by SP itself. In this case, too, proteolytic activity was essential.

Complex formation of SP with rat α_1-macroglobulin (α_1M). In order to know how such a trace amount of SP in rat blood can repress the activation of fibrinolysis, we studied the fate and function of intravenously injected SP. The first observation, that its caseinolytic activity was partially depressed in the presence of rat plasma (data not shown), indicated the existence of an SP inhibitor in the plasma and prompted us to isolate the inhibitor. Rat plasma was fractionated by gel filtration on a Sepharose CL-6B column, and the SP-inhibiting activity of each fraction was assayed. It was detected in the macromolecular region and, from its eluting position, this inhibitor protein was expected to be α_1M (Fig. 3). So, the purification of the SP inhibitor from rat plasma was carried out as described in Materials and Methods. A summary of the purification from 127 ml of rat plasma is given in Table 5. The final preparation represented a 28.7-fold purification from the original plasma with a 56% recovery. It gave a single band on polyacrylamide gel electrophoresis (Fig. 4A) and a single precipitin line with a rabbit anti-rat

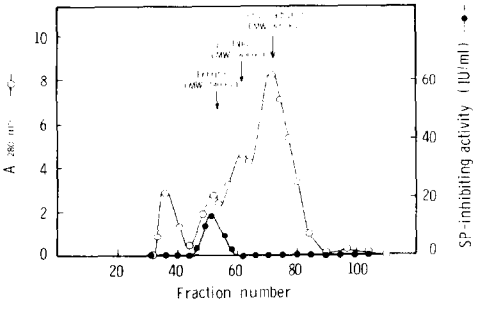

Fig. 3. Inhibition of SP proteolytic activity by macromolecular protein(s) in rat plasma. Citrated rat plasma (44 ml) was fractionated by gel filtration on a Sepharose CL-6B column (4.6 × 54 cm) equilibrated with 0.85% NaCl–0.38% sodium citrate, and the fractions (10 ml/tube) were monitored for the absorbance at 280 nm (—○—) and SP-inhibiting activity (—●—).

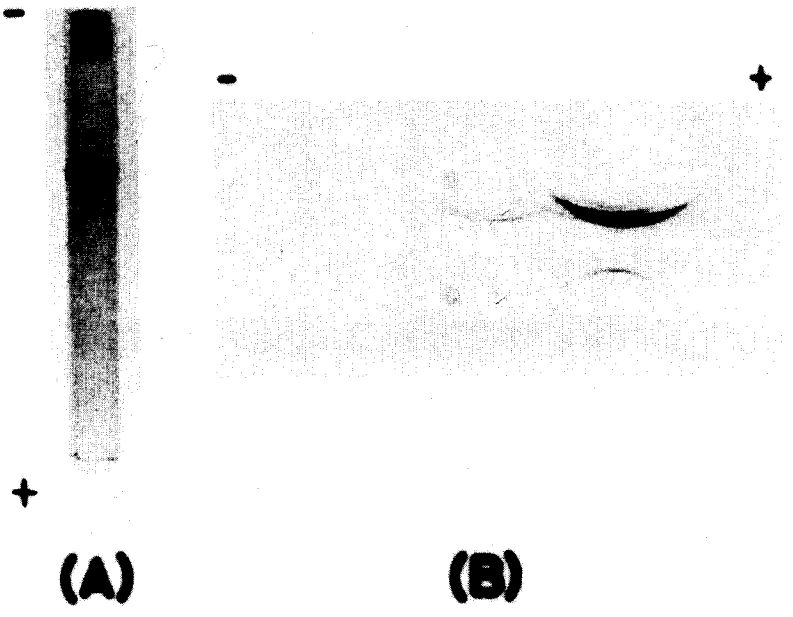

Fig. 4. Polyacrylamide gel electrophoresis (A) and immunoelectrophoresis (B) of the purified SP inhibitor. (A) The inhibitor was electrophoresed in 4% acrylamide gel (pH 9.4) at 2 mA/tube for 2.5 hr. (B) Normal rat plasma (upper well) and the inhibitor (lower well) were electrophoresed in 1% agar gel at 4 mA/cm for 2 hr and developed with rabbit anti-rat serum antiserum.

serum antiserum (Miles Laboratories Inc.) on immunoelectrophoresis (Fig. 4B). The molecular weight was calculated to be 770,000 by gel filtration on Sepharose CL-6B. Based on the molecular weight, electromobility and protease inhibition spectrum (data not shown), this purified inhibitor was identified as α_1M [14].

Using this purified rat α_1M preparation, complex formation between SP and α_1M was studied by gel filtration. When 0.06 mg of SP and 3.77 mg of α_1M were applied separately to a Sephacryl S-200 column,

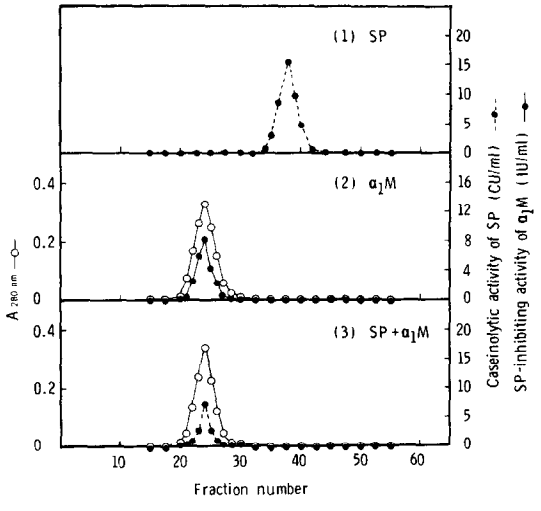

Fig. 5. Complex formation between SP and rat α_1M. SP, 0.06 mg (1), α_1M, 3.77 mg (2), and SP, 0.06 mg, plus α_1M, 3.77 mg (molar ratio 1:4) (3) were applied to a Sephacryl S-200 (Pharmacia Fine Chemicals AB) column (2.2 × 47 cm) equilibrated with 0.01 M phosphate buffer (pH 7.0), and the fractions (3 ml/tube) were monitored for the absorbance at 280 nm (—○—), caseinolytic activity of SP (- -●- -) or SP-inhibiting activity (—●—).

they were eluted as a single peak [Fig. 5 (1) and (2) respectively]. But when they were mixed (molar ratio of SP/α_1M 1:4) and incubated prior to application, they were eluted as a complex, and the complex still retained about 20% of the original caseinolytic activity of SP [Fig. 5 (3)]. A titration curve of SP with α_1M is given in Fig. 6. The caseinolytic activity of 4 μg of SP decreased linearly with the increase of the amount of α_1M until it reached 63 μg. Thereafter, no more inhibition was observed, and about 20% of the original activity remained. At this saturation point, their molar ratio was nearly 1. These data indicated that SP in blood existed as an equimolar complex with α_1M, still retaining weak proteolytic activity.

DISCUSSION

Oral administration of SP to scalded rats repressed the activation of fibrinolysis induced by the scalding. The activation was repressed also by the intravenous injection of SP at a dose giving a blood concentration

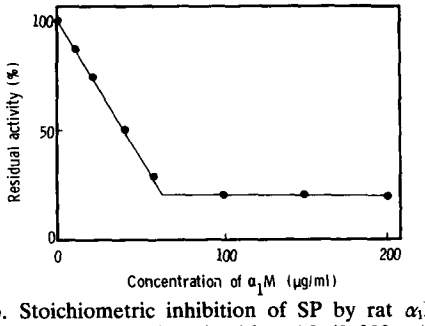

Fig. 6. Stoichiometric inhibition of SP by rat α_1M. SP (4 μg/ml) was preincubated with α_1M (0–200 μg/ml) in 0.05 M phosphate buffer (pH 7.0) at 5° for 1 hr, and the residual caseinolytic activity was measured.

Table 5. Summary of purification of SP inhibitor from rat plasma*

Step	Volume (ml)	SP-inhibiting activity (IU/ml)	(IU)	Specific activity (IU/A_{280nm})	Recovery (%)
Rat plasma	127	57.0	7240	1.32	100
EtOH supernatant fraction	140	50.2	7030	1.45	97
DEAE-cellulose eluate	510	10.0	5100	4.46	70
1st Sepharose CL-6B eluate	93.0	51.6	4800	23.1	66
2nd Sepharose CL-6B eluate	58.5	68.8	4030	28.7	56

* The purification procedure is described in Materials and Methods.

as low as 4 ng/ml. In both cases, the proteolytic activity of SP was essential.

The abnormal activation of fibrinolysis affects the clotting system, the kallikrein-kinin system, and the complement system [15] and brings about complicated abnormal symptoms, such as bleeding tendency, shock, inflammation and decline of immunological responses [16]. Therefore, the above described repression of the abnormal activation of fibrinolysis in scalded rats by orally administered SP may explain, at least partly, the clinical effectiveness of SP as an antiinflammatory oral drug.

Several papers demonstrated that horse radish peroxidase [17], elastase [18], and pancreatic lipase [19] were absorbed through the intestinal tract into blood or lymph in rats and dogs. As to the intestinal absorption of SP, Miyata *et al.* [20] have reported recently that, in rats and dogs, intraduodenally administered SP was detected in blood by radioimmunoassay (maximum level in blood 4–5 ng/ml). From both our present data, that the intravenous administration of SP to scalded rats repressed the activation of fibrinolysis at an extremely low dose, and the above observation by Miyata *et al.*, it is possible to say that a trace amount of the orally administered SP may be transferred to the blood stream in a catalytically active form that exerts the repressive effect.

SP injected into rat blood has been shown to exist as an equimolar complex with α_1M, still retaining weak caseinolytic activity corresponding to about 20% of that of the native SP. This can be explained by the characteristic manner in which human α_2-macroglobulin inhibits endopeptidases [21, 22]: it does not form a covalent acyl bond with the active site serine of the enzymes as other plasma inhibitors do, but entraps the enzyme molecules to form a kind of inclusion compound, leaving their active sites alive. Taking into account the calculated α_1M concentration in rat plasma (about 2.8 mg/ml), the binding ratio, and the molecular weights of α_1M and SP, it is estimated that, at the effective concentration of SP in blood (4 ng/ml), only 1 out of 20,000 parts of α_1M molecules in plasma takes part in complex formation with the enzyme. We found that the complexes disappeared rapidly from the blood stream, with a half-life of around 10 min. The intravenous injection of SP–α_1M complexes prepared *in vitro* exerted the same repressive effect (data not shown).

It is of particular interest how such a tiny amount of SP–α_1M complex brings about such a dramatic repression of the activation of fibrinolysis induced by scalding. The speculative explanations for the roles of the complex in the repression include (1) repression of the release of plasminogen activator(s) into the blood stream, (2) inhibition of the plasminogen activation by the activator(s), (3) inhibition of the activity of the generated plasmin, and (4) an increase in the level of plasmin inhibitor(s).

The elucidation of the mechanism of the repression is now under study.

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