ANTI-INFLAMMATORY POLYSACCHARIDE
PRODUCED BY SERRATIA PISCATORUM

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Abstract—Crude preparations of protease and culture filtrate of Serratia piscatorum IFO 12527 contained a heat-resistant, large molecular and non-proteinous anti-inflammatory ingredient(s) (PLS). The active ingredient was precipitated with Rivanol or Cetavlon, and purified by extraction with n-butanol or by adsorption with Lloyd’s reagent. From these and other chemical data, PLS is considered to be an acid polysaccharide. PLS injected i.m. inhibited the edema induced by carrageenin and anti-serum in rat feet. The effect on carrageenin edema was 37 times as potent as that of indomethacin and 200 times that of hydrocortisone acetate. These anti-edema effects were not observed by oral administration. PLS, i.m., inhibited cotton pellet granuloma more markedly than hydrocortisone acetate, without decreasing the weight of the adrenals and thymus. It had weak activating effect on kinin-forming systems in the rat plasma in vitro. Additional pharmacological properties of PLS are also described.

In the course of studies on anti-inflammatory properties of Serratia protease (TSP) (1, 2) obtained from culture filtrate of Serratia piscatorum IFO 12527 (formerly named Serratia sp. E-15) (3, 4), it was found that a considerable portion of anti-inflammatory activity of crude TSP preparations remained after complete destruction of enzyme activity by heating. Such a phenomenon was not observed with purified TSP. The active ingredients of heat-denatured crude TSP could not be rendered permeable through a cellophane membrane by digesting with Nagarse, a protease having broad substrate specificity. These results suggested that Serratia piscatorum produced, besides TSP, heat-resistant and large molecular anti-inflammatory substance which is different from protein. The present paper describes the results of experiments on the isolation of active substance and on its anti-inflammatory and some other pharmacological properties. A brief report of this work has already been published (5).

MATERIALS AND METHODS

1. TSP
   Origin and anti-inflammatory properties of TSP have been described in a previous report (2). In the present experiments, crude TSP preparations were used, mainly of lot no. 20.

2. Isolation of anti-inflammatory polysaccharide
   Isolation from crude TSP: Native or heat-denatured and Nagarse-treated TSP (2)

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was dissolved in distilled water or in 0.01 M phosphate buffer (pH 7.4) at concentrations of 1-2%. One tenth volume of 3% ethacridine lactate (Rivanol) solution was added, and the mixture was centrifuged at 10,000 g for 15 min. The precipitate was thoroughly washed with distilled water and dissolved in 2 M ammonium formate solution. After removal of Rivanol by extraction with n-butanol or by adsorption with Lloyd's reagent, the solution was dialyzed against water through a cellophane membrane until ammonium ion could not be detected with Nessler's solution. Anti-inflammatory substance was thus isolated and lyophilized.

Isolation from culture filtrate of Serratia piscatorum: This anti-inflammatory substance could also be isolated from the culture filtrate. The active substance obtained in larger quantities from this source was provided for us by Takeda Research Laboratories, Osaka. For the isolation, the following two methods were used. In the first method, the microorganisms were cultured in the medium composed of 2.5% defatted soybean flour, 2.0% NaH₂PO₄·2H₂O, 0.1% CaCl₂·2H₂O, 0.02% KCl, 0.02% MgCl₂·6H₂O and 0.5% soybean oil, at 28°C for 48 hr with shaking (Kita, Y.: personal communication). They were removed from culture broth by centrifugation, and acetone was added to the supernatant solution to a final concentration of 75%. The precipitate formed was suspended in water. The supernatant separated by centrifugation was boiled for 30 min and recentrifuged. The resultant supernatant was dialyzed against water, concentrated in vacuo, Rivanol added, then centrifuged. The precipitate formed was washed with water, extracted with 2 M acetate buffer (pH 6.0) and centrifuged. After removal of Rivanol by the addition of Japanese acid clay, the supernatant was dialyzed and lyophilized (PLS-1). In the second method, the culture medium was composed of 2% sucrose, 0.2% (NH₄)₂SO₄, 0.2% K₂HPO₄, 0.15% KH₂PO₄, 0.02% MgSO₄·7H₂O, 0.5% CaCO₃ and 0.1% yeast extract (6). Cetyltrimethylammonium bromide (Cetavlon) was used instead of Rivanol to precipitate active substance. Other procedures were similar to those in the first method. Lyophilysate obtained was called PLS-5.

3. Detection and determination of sugars and ninhydrin reaction

The active substance isolated from crude TSP by Rivanol precipitation was hydrolyzed with 4 N HCl at 100°C for 6 hr. A small volume of aqueous solution containing about 10 μg of active substance or its hydrolysate was applied on filter paper and dried. Aniline hydrogen oxalate prepared by shaking 100 ml of 0.1 N oxalic acid with 0.9 ml aniline (7) was sprayed on the paper, and reducing sugars were detected by heating. Ketohexoses and aldohexoses were distinguished by the color change of spot when applying acid potassium permanganate and heating again (8). The content of sugars was determined by the phenol-sulfuric acid method (9), using glucose as a standard.

To detect ninhydrin-positive substances, samples were applied on filter paper and dried. Ninhydrin solution was sprayed on the paper which was subsequently heated at 100°C for 3-5 min. The reagent used was 0.2% ninhydrin solution in water-saturated n-butanol or the solution composed of 90 ml n-butanol containing 0.5 g of ninhydrin, 0.6 ml formic acid, 0.6 ml pyridine and 4.0 ml water.
4. Assay of anti-inflammatory effects

**Determination of volume of foot edema:** Male Wistar rats weighing 140–160 g were used. Measurement of the volume of the feet was taken by the plethysmographic method described previously (1, 2). Five rats were used for each set of experiments and these were injected with 0.05 ml of 1% carrageenin or 16% lyophilized anti-rat serum (10) dissolved in 0.9% NaCl solution into both feet. The effects of drugs were expressed in terms of per cent inhibition of edema in the treated group as compared with the control at peak time of edema (2 hr for anti-serum and 3 hr for carrageenin edema). Drugs to be tested were usually given 1 hr prior to the injection of phlogistic agent.

**Determination of effects on granuloma:** The method of Robinson and Robson (11) was modified (2). Four male rats, 140–150 g, were used for each group. Cotton pellets for implantation were prepared by cutting dental rolls (Geigy) of diameter 7 mm into disks 0.5–0.8 mm thick. Cotton pellets were implanted s.c. into both axillae and inguinal regions. Drugs to be tested were administered i.m. once daily for 6 days. The animals were sacrificed on day 7, and the cotton pellets with adhering granulation tissue were removed, dried, and weighed. Final dry granuloma weight was calculated by subtracting the weight of the cotton pellet.

5. Formation of plasma kinins

Male rats, about 250 g, were used. Two ml of blood was collected by heart puncture into a syringe containing 0.2 ml of 2% sodium oxalate solution, and plasma was separated by centrifugation. Siliconized needles and glassware were used in these experiments. One tenth ml of 0.2 M Tris-HCl (pH 7.8), 0.3 ml of 0.7% 8-hydroxyquinoline (12) in 0.9% NaCl solution, and 0.1 ml of 0.9% NaCl solution containing the substance to be tested were added to 0.5 ml of plasma. The mixture was incubated at 37° for 30 min. Kinin-like substances formed were assayed on the isolated guinea-pig ileum suspended in a Tyrode bath containing mepyramine maleate (2 × 10−7 g/ml), methysergide bimaleate (2 × 10−7 g/ml) and atropine sulfate (10−7 g/ml), at 37°C. Synthetic bradykinin was used as a standard.

6. Assay of pyrogenic activity of PLS

Six male rats, about 150 g, with a rectal temperature of 37.9–38.5°C comprised each group. The experiments were performed at a room temperature of 25°C. Rectal temperature was taken before i.m. administration of PLS-1, and at hourly intervals thereafter for 5 hr.

7. Recording of blood pressure of rats

Male rats, 250–330 g, were anesthetized with pentobarbital Na (40 mg/kg, i.p.), and blood pressure was recorded by inserting a fine polyethylene tubing connected to a pressure transducer, into the carotid artery.

8. Acute toxicity and histological examination

For testing acute toxicity, male dd mice weighing 18–20 g were used, and LD₅₀ was calculated by the method of Litchfield and Wilcoxon (13). For the study of histological changes in the rat, tissues were fixed in 10% formalin solution and embedded in paraffin.
Sections of 5 μ thickness were stained with hematoxylin and eosine.

9. Drugs

Carrageenin (Viscarin®, Marine Colloids, Inc.), indomethacin and hydrocortisone acetate (Nippon Merck-Banyu), Nagarse (Nagase Industries), and bradykinin triacetate (Protein Research Institute, Osaka University).

RESULTS

I. Isolation of anti-inflammatory polysaccharide

1. Isolation of anti-inflammatory substance from crude TSP sample

In a previous report (2), it was found that crude TSP preparations contained a heat-stable, large molecular anti-inflammatory substance different from protein. On the assumption that this ingredient could be an acid polysaccharide, the quantitative relation existing between the amount of precipitate formed by adding Rivanol to the solution of

| Lot no. | Proteolytic activity (PU/ml)* | Degree of precipitation† | Anti-inflammatory activity (% inhibition of carrageenin edema)‡ |
|---------|-----------------------------|--------------------------|---------------------------------------------------------------|
| No. 20  | 400                         | #                        | 98.5                                                          |
| No. 21  | 3,612                       | -                        | 63.0                                                          |
| No. 23  | 4,000                       | ++                       | 85.7                                                          |
| No. 24  | 3,157                       | +                        | 49.2                                                          |
| No. 40  | 2,680                       | -                        | 87.7                                                          |
| No. 81  | 4,000                       | -                        | 14.3                                                          |

* PU is a protease unit, the definition of which is given in Ref. 1.
† 0.3% solution of TSP was centrifuged and clear supernatant solution was mixed with an equal volume of 0.1% Rivanol solution.
‡ 10 mg/kg, i.m., 1 hr prior to carrageenin injection.

FIG. 1. Anti-inflammatory effects of crude TSP (no. 20) and its Rivanol-precipitated fraction on carrageenin-induced edema in rat feet. Both crude TSP and its Rivanol-precipitated fraction were heated at 100°C for 1 hr before i.m. injection. Five rats were used at each dose level of each drug.
each TSP sample and the anti-inflammatory potency of the corresponding heat-denatured sample was studied. As shown in Table 1, there was some correlation between them, but a relationship was not observed between enzyme activity of native samples and anti-inflammatory activity of heat-denatured respective samples. Therefore, the active ingredient could be isolated by Rivanol-precipitation. Crude TSP no. 20 contained Rivanol-precipitated substance (referred to as RPS) amounting to 14-17% of original native material by weight.

Heating and Nagarse-treatment of crude TSP had no appreciable effect on the anti-inflammatory activity of RPS. As shown in Fig. 1, anti-inflammatory activity of RPS was considerably more potent than that of heat-denatured crude TSP (no. 20) from which the former had been derived.

2. Analysis of anti-inflammatory substance isolated from TSP

Fig. 2 shows ultraviolet absorption spectra of RPS, crude (no. 20) and purified TSP sample (no. 81). The crude sample showed broad absorption with a maximum between 250-260 m\(\mu\), whereas with the purified TSP an absorption peak was seen at 278 m\(\mu\). Absorption spectrum of RPS had no peak between 240-310 m\(\mu\), suggesting a limited content of nucleic acids, proteins and related substances.

Sugar content of RPS was 47%, when determined with glucose as a standard. Reducing sugars were not detected in RPS with aniline hydrogen oxalate; however, acid hydrolysate of RPS showed a positive reaction of reducing sugars (aldohexoses). RPS did not give a color spot with ninhydrin solution in water-saturated n-butanol; but, a positive color reaction was obtained when using ninhydrin solution in n-butanol acidified with formic acid. RPS, thus, seems to be an acid polysaccharide(s).
3. Isolation of anti-inflammatory polysaccharide (PLS) from culture filtrate of Serratia piscatorum

Culture filtrate of Serratia piscatorum was more suitable for obtaining the anti-inflammatory polysaccharide in larger quantities. The active substance obtained by Rivanol precipitation from the culture filtrate (PLS-1) contained 60% sugars as glucose. A similar substance was also obtained with Cetavlon as well as Rivanol (PLS-5).

II. Anti-inflammatory properties of PLS
1. Inhibitory effect on edema in the rat feet

The polysaccharide sample obtained from crude TSP no. 20 and PLS-1 and PLS-5 were compared regarding the anti-inflammatory potency. With an injection of 0.25 mg/kg i.m., carrageenin edema was inhibited by 68, 70 and 68%, respectively. Further experiments were performed employing mainly PLS-1.

![Figure 3](image1.png)

**Fig. 3.** Anti-inflammatory effects of i.m. injection of PLS-1, indomethacin and hydrocortisone acetate on carrageenin-induced edema in rat feet. Five rats were used at each dose level of each drug.

![Figure 4](image2.png)

**Fig. 4.** Inhibition of carrageenin-induced edema in rat feet by PLS-1 administered i.m. at different periods of time before and after carrageenin injection. Each curve represents the mean from 5 rats.
As shown in Fig. 3, anti-inflammatory effect of PLS-1 administered i.m. on carrageenin edema was 37 times as potent as that of indomethacin and 200 times that of hydrocortisone acetate given by the same route, in terms of ED50.

When PLS-1 was given i.m. 1–3 hr before injection of carrageenin, a marked inhibition of edema was observed 1–5 hr after carrageenin (Fig. 4). Simultaneous administration of PLS-1 with carrageenin caused an inhibition which was evident after 2 hr. Even when PLS-1 was administered after injection of carrageenin, it showed an inhibitory effect on a completely edematous state.

Anti-serum edema was also inhibited by PLS-1, but this effect was weaker than that on carrageenin edema (Fig. 5).

PLS-1 given s.c. or i.v. was nearly as effective on carrageenin edema as when given i.m., but, it was ineffective when given orally 40–100 mg/kg 0–12 hr before the injection of carrageenin.

![Inhibitory effects of PLS-1 administered i.m. on edemas induced by carrageenin and anti-serum in rat feet. Five rats were used at each dose level of each drug.](image)

2. Inhibition of granuloma

As shown in Table 2, a significant inhibition of cotton pellet granuloma was obtained

| Drug* | Granulation tissue (mg ± S.E.) | Inhibition (%) | Body weight (g ± S.E.) | Adrenals (mg ± S.E.) | Thymus (mg ± S.E.) |
|-------|-------------------------------|----------------|------------------------|----------------------|-------------------|
|       |                               |                | initial                | final                |                   |
| Control | 12.0 ± 0.4                   |                | 148 ± 2                | 164 ± 5              | 26 ± 1            | 413 ± 21         |
| PLS-1 0.25 mg/kg | 8.7 ± 0.2†                   | 27.5           | 146 ± 4                | 168 ± 11             | 24 ± 1            | 401 ± 40         |
| PLS-1 1.0 mg/kg | 8.2 ± 0.3†                   | 31.7           | 140 ± 1                | 158 ± 3              | 25 ± 1            | 406 ± 91         |
| Hydrocortisone acetate 10 mg/kg | 8.5 ± 0.2†                   | 29.2           | 139 ± 3                | 152 ± 5              | 15 ± 1†           | 209 ± 43‡        |

Four rats per group. * Drugs were administered i.m. once daily for 6 days and animals were sacrificed on day 7. † P<0.001 and ‡ P<0.01 by Student's t-test.
by injecting 0.25–1.0 mg/kg of PLS-1 i.m. once daily for 6 days. The effect of 1.0 mg/kg of PLS-1 was slightly superior to that of 10 mg/kg of hydrocortisone acetate. In contrast to a marked decrease in the weight of adrenals and thymus of animals given with hydrocortisone, PLS-1 did not significantly influence the weight of these organs.

III. Inflammation-producing action and activation of plasma kinin-forming systems

To quantitatively test the inflammation-producing action, 0.05 ml of 0.4–5.0% PLS-1 dissolved in 0.9% NaCl solution was injected into the subcutaneous tissues of the plantar side of both feet of the rat. Time course of the resultant edema was recorded (Fig. 6). Edema-producing action of PLS-1 was far weaker than that of carrageenin (1%, 0.05 ml).

Incubation of 1.0 mg/ml (final concentration) of PLS-5 with rat blood plasma caused the formation of 0.12 μg bradykinin-equivalent activity per ml of the plasma. At 0.5 mg/ml PLS-5 had no effect. When carrageenin 0.25 mg/ml was incubated with the plasma, 0.75 μg bradykinin-equivalent was released per ml. Glass fibers (10 mg/ml) had the same degree of activity as that of 0.25 mg/ml carrageenin.

![Fig. 6. Edema-producing activity of PLS-1 injected s.c. into rat feet. Each group included six rats and 0.05 ml of test solutions was injected into both feet.](image)

IV. Pyrogenic action of PLS

After i.m. injection of 0.25 and 1.0 mg/kg of PLS-1 in the rat, body temperature rose gradually, the maximums (0.9° and 1.4°C, respectively) being reached about 4–5 hr after the injection.

V. Circulatory and respiratory effects of PLS

Intravenous administration of 1 mg/kg of PLS-1 caused a fall in blood pressure of rats by 18–53% of the pre-injection level. Two rats given 10 mg/kg of PLS-1 showed a 42–52% decrease in blood pressure. Blood pressure began to fall at 1–1.5 min after the injection of PLS-1, reaching the lowest level at 3–4 min later. Sustaining this level for several min, it was restored gradually. When the injection of PLS-1 was repeated, the effect became much weaker.
A marked stimulation of respiration was observed in 2 out of 8 rats injected i.v. with 1 mg/kg of PLS-1. This effect was not decreased by repeated injections.

VI. Acute toxicity of PLS

The LD_{50} values of PLS-1 in mice by i.v., i.m. and s.c. injections were 44, 185 and 575 mg/kg, respectively. The main symptoms of intoxication were motor paralysis and prostration.

Macro- and microscopic changes in organs of the rat caused by i.m. injection of different doses of PLS-1 were examined, using 4 animals per each dose level. With a dose of less than 1.0 mg/kg, there was no macroscopic change in any organ 5 hr after injection. In doses of 10–100 mg/kg, petechial hemorrhage in the lung, hyperemia, edema, ulcer formation in the gastrointestinal tract, and hyperemia in the adrenal glands were observed at autopsy performed after 12–24 hr. Only the lung and small intestine were examined histologically after i.m. administration of PLS-1. Changes in the lung consisted of thickening and hyperemia of alveolar walls and leukocyte infiltration in these structures; and in the small intestine partial abrasion of epithelium of villi, leukocyte infiltration and hyperemia in submucosal tissues were observed. These changes were noted, though very slightly, at a dose as low as 0.25 mg/kg.

With an oral administration of PLS-1 at doses not higher than 10 mg/kg, macro- and microscopic changes were not observed in any organs. By this route, PLS-1 at doses higher than 10 mg/kg induced slight leukocyte infiltration in submucosal tissues of the small intestine.

DISCUSSION

The present experiments showed that Serratia piscatorum produces, besides a protease (TSP), anti-inflammatory substance(s) which was precipitated with Rivanol or Cetavlon. This substance is heat-resistant, has high molecular weight, and chemical analyses indicated it to be acid polysaccharide. This was confirmed, after the present experiments, by Nakanishi and Kita (6, 14) who made further detailed investigations into the composition of the active substance and found it to be complex polysaccharide consisting of sugars, lipids and a small amount of protein.

The anti-inflammatory polysaccharide (PLS) obtained from the culture filtrate of the Serratia showed a more potent inhibitory effect on carrageenin edema than on the edema induced by anti-serum. Similar differences in the effects on these edemas have been observed with indomethacin and phenylbutazone (2). This may be due to a certain reaction, which is effectively inhibited by PLS or other anti-inflammatory agents, playing a more important role in carrageenin edema than in anti-serum edema.

Of the bacterial polysaccharides, endotoxin of E. coli has been most extensively studied for the effect on plasma kinin-forming systems in vitro (15, 16). The results of present experiments showed in vitro activation of kinin-forming systems in the rat blood plasma by PLS. This effect was much weaker as compared with that of carrageenin, although further studies are necessary to determine the significance in the anti-inflammatory mecha-
nism of kininogen depletion by PLS due to direct activation of kinin-forming systems in the plasma.

The edema-producing effect of PLS injected s.c. into the rat feet was far weaker than that of carrageenin. However, histological examination of tissues from the lung and small intestine demonstrated that PLS administered i.m. caused general tissue injuries. Injections of irritants such as formalin and croton oil have been shown to inhibit carrageenin edema (2) and cotton pellet granuloma (17). Consequently, it is possible that tissue-injurious effects of PLS may partly contribute to the anti-edema and anti-granuloma effects. PLS injected i.v. caused a fall of the rat arterial blood pressure which lasted for less than 30 min. Such a hemodynamic change might not be the causal event for the anti-edema effect of PLS administered i.m. as early as 2-3 hr before the injection of phlogistic agent. The relationship of all of these known pharmacological properties to the anti-inflammatory action of PLS cannot be evaluated entirely at present; there may be unknown factors concerning the anti-inflammatory effects of this polysaccharide.

Endotoxins from some gram-negative bacilli such as E. coli were known to induce fever, hypotension and tissue injuries (18). These were observed in PLS as well. It is possible that certain other gram-negative bacilli may also produce anti-inflammatory polysaccharides similar to PLS; these polysaccharides, thus, may be mitigative to the inflammatory response of the host infected with such microorganisms.

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