[Chem. Pharm. Bull. 30(12)4429—4434(1982)]

Purification of a Leukocytosis Promotion-inhibiting Factor from Bovine Bile

Masato Shinoda,* Yasunori Kushi, Motoyuki Shimizu, Shizuo Nakajin, and Saburo Yamashita

Department of Biochemistry, Hoshi College of Pharmacy, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142, Japan

(Received April 23, 1982)

A leukocytosis promotion-inhibiting factor (LPIF) was isolated and purified from crude bovine bile. The purified LPIF was homogeneous on 15% polyacrylamide gel electrophoresis. It inhibits the effects of leukocytosis-promoting substance in rabbits. The molecular weight of LPIF was estimated to be 1.2×10^4, and the isoelectric point was at pH 8.2. The active component dissociated into two subcomponents with molecular weights of 9000 and 3000 on sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis.

Keywords——leukocytosis promotion-inhibiting factor; bovine bile; leukocytosis inhibition; leukocytosis; leukocytes

It is well known that the bovine parotid gland extract contains a leukocytosis-promoting activity in rabits.[1] The authors have purified the leukocytosis-promoting substance (LP-substance) from a crude bovine parotid gland extract.[2] The molecular weight of the purified LP-substance was estimated to be 4.5×10^4 (ref. 2), and the activity was also demonstrated in splenectomized, adrenalectomized, nephrectomized or CCl_4-treated rabbits.[3] The LP-substance was inactivated by incubation with rabbit serum or bile at 37°C for 30 min.[4]

In this paper an attempt was made to isolate and purify from bovine bile the leukocytosis promotion-inhibiting factor (LPIF) against bovine parotid LP-substance.

Materials and Methods

LP-substance was prepared according to the previously described method.[2] The effective dose for leukocytosis promotion of the preparation was 100 μg/kg in rabbits: that is, this dose increased the number of circulating leukocytes by 100% or more within 8 h after the *i.v.* injection of the preparation.

Bile used in the present experiment was prepared from the normal bovine gall bladder. The supernatant of the bile, after centrifugation at 3000 rpm for 15 min, was diluted ten times with 0.9% NaCl and stored at −20°C until required.

Determination of the Leukocytosis Promotion-inhibiting Activity——Three male albino rabbits, weighing about 3—3.5 kg, were used in one group. The LP-substance and the bile preparation were dissolved in 0.9% NaCl, and the mixture was incubated at 37°C for 1 h. The concentration of the mixture was adjusted to 100 μg of the LP-substance per 0.5 ml of 0.9% NaCl, and 0.5 ml of this solution per kg body weight of the rabbit was injected into the ear vein. Specimens were collected from the other ear vein before and at 2, 4, 6 and 8 h after the *i.v.* injection.

Numbers of circulating leukocytes were counted with a microcell counter (Toa Electric Co., Ltd.). The activity of LPIF was determined according to the method reported previously.[5]

That is, 0.5—1 ml of the LPIF was incubated with 12 mg/10 ml of bovine parotid LP-substance at 37°C for 60 min, then injected into rabbits, and the numbers of leukocytes were counted. The following criteria were used to judge the effect of the LPIF; that is, when the LP-activity exerted by 100 μg/kg of LP-substance was completely inhibited, the result was judged as (+), when the transient decrease disappeared within 2 h after the injection and an inhibition of the subsequent increase of leukocytes was caused by LP-substance, the result was judged to be (±), and when the LP-activity was unaffected, the result was (−) (see Table I). "Complete inhibition" means that there was no statistically significant increase in leukocyte count during 8 h after injection ($n=3$), and "no inhibition" means that the leukocyte counts up to 8 h showed no statistically significant difference from the control (given 0 μl of bile).

Estimation of the Amount of Protein——The amount of protein was estimated by the method of Lowry *et al.*[6] with BSA (bovine serum albumin) as the standard. Spetrophotometric absorbance at 280 nm was used to monitor the relative protein concentration of the effluents from column chromatography.

Disc Electrophoresis——Electrophoresis was carried out according to the method described by Ornstein and Davis.[7] Samples were applied to 15% polyacrylamide gels (0.5×7 cm) and were electrophoresed at 2 mA/tube for 1.6 h in Tris buffer, pH 8.9. After the electrophoresis, a gel was stained with Coomassie blue by the method reported by Fazekas *et al.*[8] For preparative electrophoresis, gels were sliced in 3 mm thickness without staining, and each slice was homogenized with 1 ml of 0.9% NaCl in the cold. The homogenate was centrifuged at 10000 rpm for 30 min, and the LPIF activity in the supernatant was determined.

Estimation of the Molecular Weight of dansylated LPIF by SDS-electrophoresis——Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was carried out according to the method described by Weber and Osborn[9] with a minor modification.

After dansylation, protein was denatured for electrophoresis in 10 mM Na phosphate buffer, pH 7.2, containing 0.1% NaSDS, 2-mercaptoethanol and 8 M urea. Six reference proteins were used for calibration; phosphorylase b (9.4×10^4), BSA (6.7×10^4), ovalbumin (4.3×10^4), carboxyanhydrase (3×10^4), trypsin inhibitor (2.0×10^4) and α-lactalbumin (1.4×10^4). Ten micrograms of each protein was applied to the 10% polyacrylamide gel. Electrophoresis was performed at 8 mA/tube for 4 h at room temp. in 0.1 M Na phosphate buffer, pH 7.2, containing 0.1% SDS. The molecular weight of LPIF was estimated from the calibration curve, which was prepared by plotting the mobilities of the dansylated reference proteins detected under ultraviolet (UV) light.

Isoelectric Focusing in Gel Tubes——Two polyacrylamide gel tubes, 0.5×10 cm, containing 1% ampholine, pH 3.5—10, and the samples were prepared as described in the preceding section for SDS-electrophoresis. After the electrophoresis, one of the gel tubes was stained with 0.1% Coomassie brilliant blue R-250.[10] The other was sliced as in the previous section, and each of the slices was homogenized with 1 ml of water. The homogenates were incubated for 24 h at 4°C with occasional shaking. After centrifugation, LPIF activity in the supernatant was determined.

Results and Discussion

LPIF Activity in the Bovine Bile

The effect of the bovine bile on the LP-activity of the bovine LP-substance was determined. As shown in Table I, the LP-activity of the LP-substance was completely inhibited by 10 μl of bile per 100 μg of LP-substance. The leukocyte level in rabbits was not changed significantly by *i.v.* injection of 0.9% NaCl in control experiments. The time course of the induction of complete inhibition of LP-substance was examined with the minimum amount of bile that showed LPIF activity. The inhibition of the LP-substance by bile was already apparent after 15 min, and was complete after 30 min as shown in Table II.

Stability of LPIF in Bovine Bile

The LPIF activity was stable after heat treatment at 100°C for 5 min, and 56°C for 30 min. The activity was not dialyzable, as shown in Table III.

Table I. Leukocytosis Promotion-inhibiting Activity in Bovine Bile

Dose of bile[a] (μl)	Change in leukocyte counts after *i.v.* injection of LPIF [Mean (%) ± S.E., $n=3$]				LPIF activity[b]
	2 h	4 h	6 h	8 h	
40	−24± 5	−7± 9	−18± 2	−9± 6	+
20	−9± 6	−18±26	22± 7	−13± 7	+
10	9±15	−6± 5	−15±13	3±13	+
5	43±13	45±11	39±19	30±11	±
2.5	−40±14	−24± 7	66±36	87±25	−
1.25	−41±12	13±19	49±37	186±62	−
0	−35± 3	1±24	66±48	124±38	−

a) The dose is shown as the volume per 100 μg of LP-substance.
b) Bile (800—25 μl) and LP-substance (2000 μg) were dissolved in 10 ml of 0.9% NaCl and incubated at 37°C for 1 h. The injection dose of the treated sample solution was 0.5 ml/kg of rabbit. The LPIF activity of bile on LP-substance (100 μg/kg) is expressed as follows: (+) complete inhibition, (±) partial inhibition, and (−) no inhibition. For details, see "Materials and Methods."

Table II. Influence of Incubation of LPIF in Bile on LP-substance

Incubation time (min)	LPIF activity[a]
0	−
15	±
30	+
45	+
60	+

a) Bile (200 μl) and LP-substance (2000 μg) were dissolved in 10 ml of 0.9% NaCl, and at 37°C for 0—60 min. The amount injected and aciteria of LPIF activity were the same as described in Table I.

Table III. Influence of Various Treatments on LPIF Activity of Bile

Treatment	LPIF activity[a]
100°C, 5 min	+
56°C, 30 min	+
Dialysis[b]	
Inner solution	+
Outer solution	−
Lyophilization	+

a) LPIF activity was determined as described in Table I.
b) The bile was dialyzed against 0.05 M phosphate-buffered saline (pH 7.4) at 4°C for 2 d.

Purification of LPIF from Bovine Bile

Unless otherwise stated, all operations were carried out in a cold room at 4°C, or in an ice bath.

Four liters of bovine bile were centrifuged at 3000 rpm for 15 min and the supernatant was fractionated with acetone. Table IV shows the results of the acetone fractionation.

The most active fraction was fraction A-4, and this fraction was further purified by a glacial acetic acid extraction method; lyophilized fraction A-4, 764 mg, was extracted with ten volumes of glacial acetic acid for 2 h at 50°C, and the mixture was centrifuged. Saturated NaCl solution was then added to the supernatant to 0.06% (w/w) and ether was then added to the supernatant to 0.06% (v/v). The resulting precipitate was dissolved in water and lyophilized. The lyophilized product, fraction B, had LPIF activity at a dose of 25.2 μg/100 μg of LP-substance. Fraction B, 329 mg, was dissolved in 0.2 M pyridine–acetate buffer, pH 6.0, and the suspension was applied to a Sephadex G-25 column (3.1×44 cm) and eluted with the same buffer (flow rate, 18.6×44 cm). The LPIF active fraction, C-1 (Fig. 1), 325 mg, was dissolved in 0.2 M pyridine–acetate buffer, pH 6.0, then applied to a Sephadex G-50

Table IV. Fractionation of LPIF in Bile with Acetone

Acetone (%)	Protein[a]		LPIF activity[b]
	(mg)	Yield (%)	
0—30(A-1)	421	6.4	−
30—50(A-2)	725	11.1	±
50—75(A-3)	1143	17.4	−
75 soluble (A-4)	764	11.6	+

a) Values are protein contents and percentages of total protein.
b) LPIF activity was determined as described in Table I.

column (2.9×95 cm) and eluted with the same buffer (flow rate, 18.6 ml/h). The LPIF active fraction, D-2 (Fig. 2), was rechromatographed on the same column and under the same conditions as in Fig. 2. The E-1 (Fig. 3) fraction thus obtained, 19 mg, was applied to DEAE-Sephadex A-25 previously equilibrated with 0.05 M ammonium bicarbonate buffer, pH 9.0. Elution was carried out with a gradient, from 0.05 to 0.5 M, of ammonium bicarbonate buffer, and the LPIF fraction, F-2, was finally obtained. The results are illustrated in Figs. 1—4.

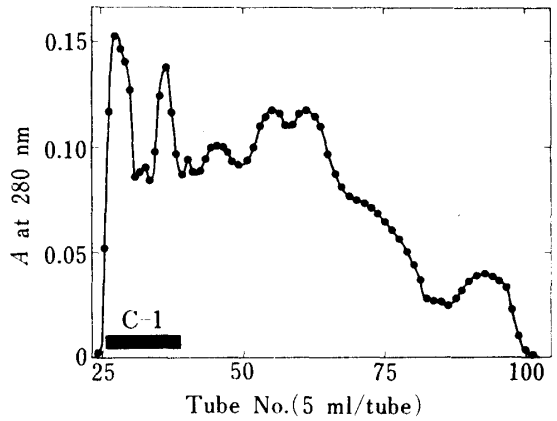

Fig. 1. Gel Filtration of Fraction B on Sephadex G-25

Fractions B (329 mg) was dissolved in 0.2 M, pH 6.0 pyridine acetate buffer and applied to a Sephadex G-25 column (3.1×44 cm), which was eluted with the same buffer (flow rate 18.6 ml/h).

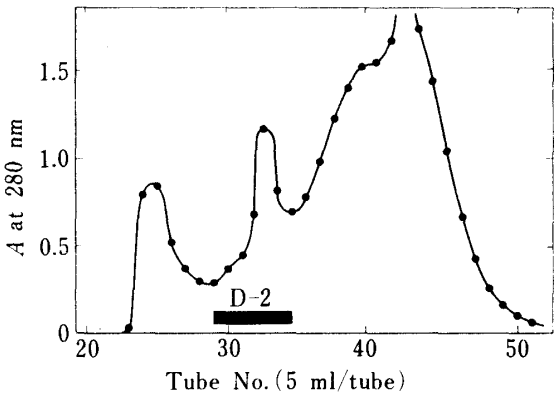

Fig. 2. Gel Filtration of Fraction C-1 on Sephadex G-50

Lyophilized fraction C-1 (325 mg) was dissolved in 0.2 M, pH 6.0 pyridine acetate buffer and applied to a Sephadex G-50 column (2.9×95 cm), which was eluted with the same buffer (flow rate 18.0 ml/h).

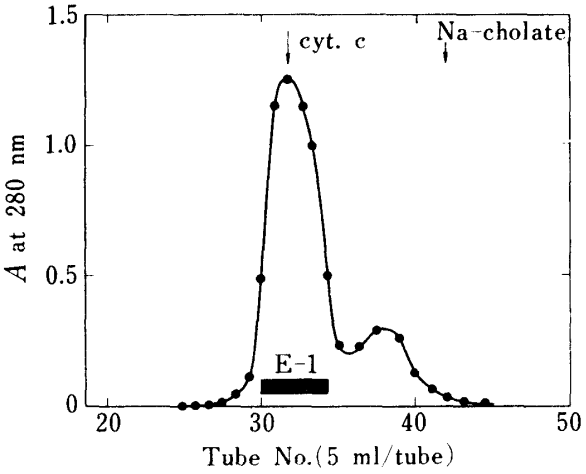

Fig. 3. Second Gel Filtration of Fraction D-2 on Sephadex G-50

The experimental conditions were the same as described in Fig. 2.

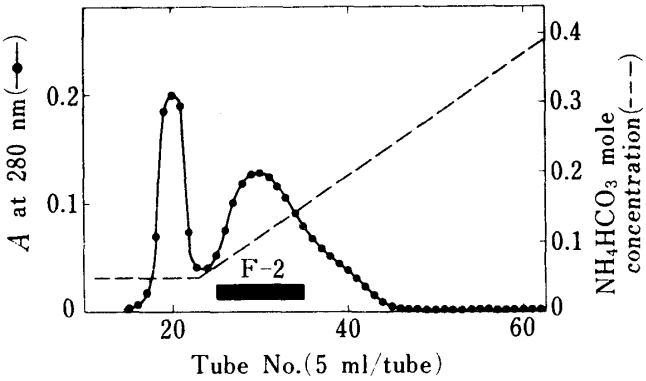

Fig. 4. DEAE-Sephadex A-25 Column Chromatography of Fraction E-1

DEAE-Sephadex A-25 was fully equilibrated with 0.05 M ammonium bicarbonate buffer (pH 9.0) and packed into a column (3.2×25 cm). Fraction E-1 (19 mg) was dissolved in the same buffer and applied to the column. Elution was carried out with a gradient of ammonium bicarbonate from 0.05 to 0.5 M.

The homogeneity of the products was examined by disc electrophoresis with 15% polyacrylamide, and fraction F-2 showed a single band. Fraction F-2 was further purified by preparative electrophoresis as described in "Materials and Methods." The degree of purification at each step is shown in Table V. In the present experiment, the final LPIF was found to have been purified approx. 200-fold from the strating material.

TABLE V. Purification of LPIF from Bovine Bile

Purification step	Protein[a] (mg)	Protein[a] Yield (%)	Effective dose[b] (μg/100 μg LP-substance)
Starting material	6552	100	187.2
Acetone fractionation (A-4)	764	11.6	68.4
Acetic acid extraction (B)	329	5.0	26.2
Gel filtration on Sephadex G-25 (C-1)	325	4.9	12.3
Gel filtration on Sephadex G-50 (D-2)	52	0.8	8.3
Second gel filtration on Sephadex G-50 (E-1)	19	0.29	4.2
DEAE-Sephadex A-25 (F-2)	2.8	0.04	1.3
Preparative disc electrophoresis (LPIF)	1.4	0.02	1.0

a) The yield (protein content) is expressed as percentage (w/w) recovery from the starting material.
b) Effective dose is expressed as the minimum bile protein dose (μg) able to completely inhibit 100 μg of LP-substance (see "Materials and Methods").

Some Properties of Bovine Bile LPIF

Reference standard proteins of known molecular weight were applied to the Sephadex G-50 column, under the conditions shown in Fig. 3. K_{av} of the LPIF was comparable to that of cytochrome c, as shown by an arrow in Fig. 3. The molecular weight of the purified LPIF was estimated to be 1.2×10^4 by polyacrylamide gel disc electrophoresis. The LPIF was separated into two components, having molecular weights of 9×10^3 and 3×10^3, on SDS-polyacrylamide gel electrophoresis.

The isoelectric point of the LPIF was found to be 8.2 by the isoelectric focusing method in gel tubes. The bile acid content in the purified LPIF was determined by an enzyme method, but no bile acids could be detected.

Since it was possible that LPIF might be a proteolytic enzyme in the bile, the decomposition of LP-substance by LPIF was examined by the method described by Ito and Shinoda,[11] 10 mg of LP-substance solution and 0.1 mg of LPIF were dissolved in 10 ml of physiological saline, and the mixture was incubated at 37°C for 1, 2 and 4 h. Then, the mixture was cooled in an ice bath to stop the reaction, if any, and 4 ml of 5% perchloric acid was added to 1 ml of the reaction mixture and to the control, which contained no LPIF. The mixtures were centrifuged, and amino acids and peptides in the supernatant were examined. No evidence of protein digestion was found, so that the possibility of LPIF being a protease or LP-substance decomposing enzyme was excluded.

LP-factor and LPIF are both found in the serum of rabbits,[5] but their origins and physiological significance are not fully understood. In the present study, the authors purified a specific LPIF, whith should prove useful in further studies of the above problems.

References and Notes

1) Y. Ito, *Ann. N.Y. Acad. Sci.*, **85**, 228 (1960).
2) M. Shinoda, S. Nakajin, T. Oikawa, M. Shimizu, and A. Kamogawa, *Biochim. Biophys. Acta*, **584**, 116 (1979).
3) M. Shinoda, M. Shimizu, S. Nakajin, Y. Kushi, and A. Kamogawa, *Yakugaku Zasshi*. **98**, 1119 (1978).
4) M. Shinoda, M. Shimizu, and Y. Kushi, *Yakugaku Zasshi*, **98**, 553 (1978).

5) M. Shinoda, M. Shimizu, Y. Kushi, and S. Nakajin, *Yakugaku Zasshi*, **98**, 997 (1978).
6) O.H. Lowry, A.L. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
7) L. Ornstein and B.J. Davis, *Ann. N.Y. Acad. Sci.*, **121**, 404 (1964).
8) S. Fazekas, S.T. DeGroth, R.G. Webster, and A. Datyner, *Biochim. Biophys. Acta*, **71**, 377 (1963).
9) K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1963).
10) R.M. Zacharius, T. Zell, J.H. Morrison, and J.J. Woodlock, *Anal. Biochem.*, **30**, 148 (1969).
11) Y. Ito and A. Shinoda, *Endocrinol. Jpn.*, **8**, 9 (1961).