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Note

Anti-inflammatory effect of the polysaccharide from the fruit bodies of *Auricularia* species*

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Previous studies in this series have dealt with the characterization[1], anti-tumor[2], and anti-inflammatory[3,4] activities of polysaccharides isolated from various fungi. In the preceding articles, we reported that a branched (1→3)-β-D-glucan[4] and partially *O*-acetylated (1→3)-α-D-mannan[3] isolated from the fruit bodies of *Dictyophora indusiata* showed a significant anti-inflammatory effect on both carrageenan-induced edema and hyperalgesia in scald-induced, edematous lesions in the hindpaws of rats. Anti-inflammatory effects of polysaccharide preparations have been observed with complex polysaccharides[5,6], polysaccharides containing protein[7], and sulfated polysaccharides[8]. However, few papers have been published on the activity of the pure polysaccharides[9,10].

The fruit bodies of *Auricularia* species (Chinese name: Yū ěr) belonging to the Auriculariaceae have been used as a food and as a drug in China. The present investigation was undertaken to isolate a pure, acidic polysaccharide from the fungus, and assay its anti-inflammatory effects.

The fruit bodies were extracted successively with hot methanol and hot, 70% aqueous ethanol, and the residue was extracted with hot water. Compounds of low molecular weight and proteins in the extract were removed by Pronase treatment, dialysis, and the Sevag procedure[11]. The solution thus obtained was mixed with ethanol, to precipitate the crude polysaccharide (U-3-EP) in ~7% yield. U-3-EP contained a mixture of acidic (40%) and neutral (60%) polysaccharides. It was

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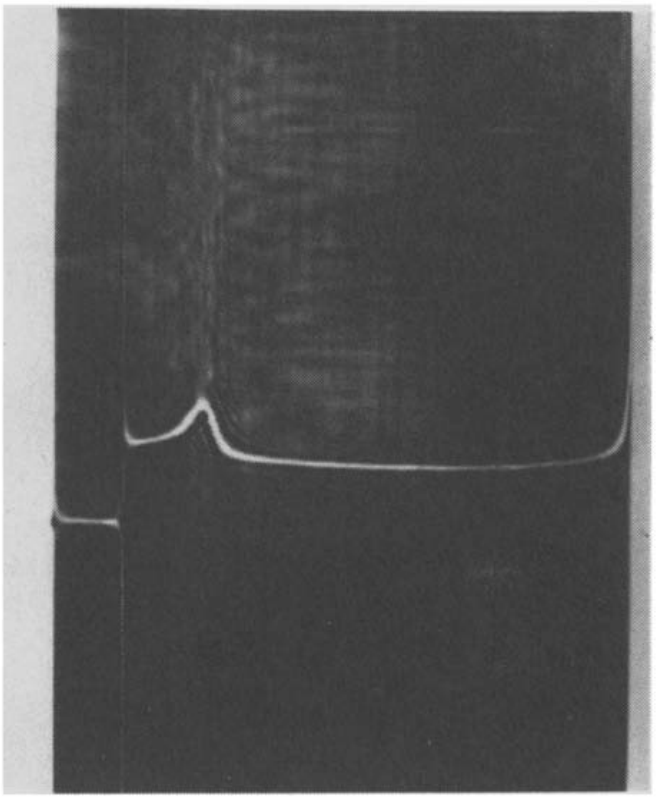

Fig. 1. Ultracentrifugal pattern of U-3-A (5 mg/mL in 0.5M sodium hydroxide) after 30 min at 60,000 r.p.m.

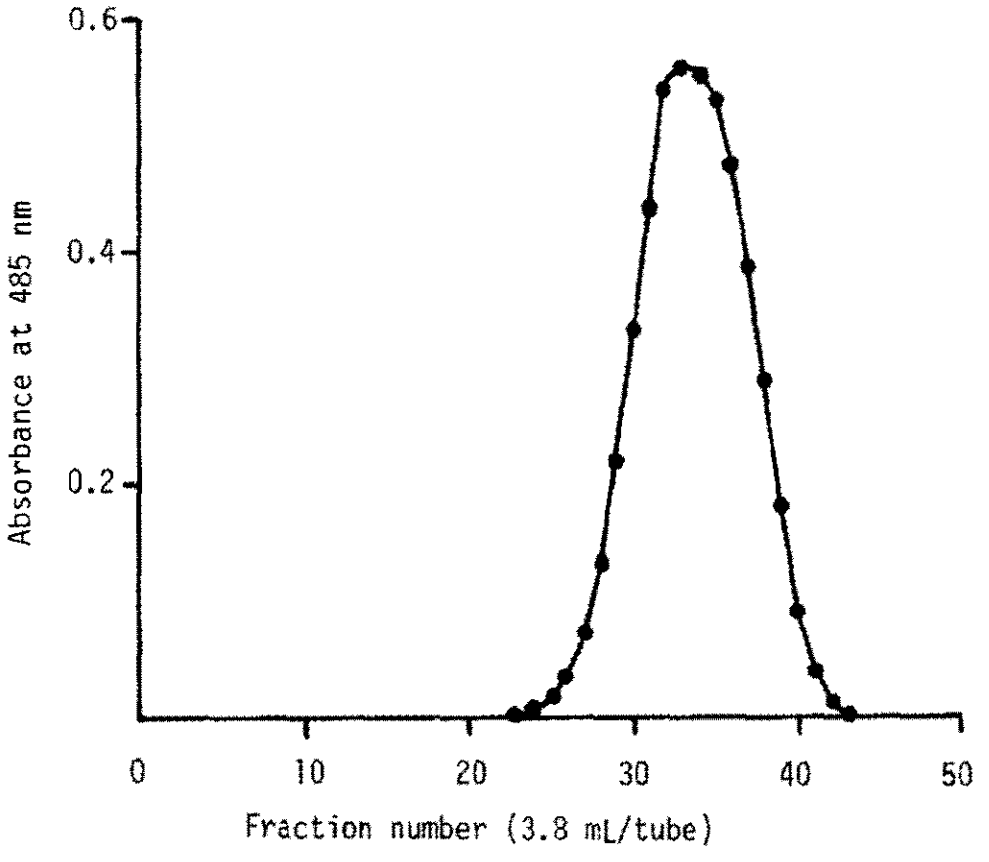

Fig. 2. Chromatogram of U-3-A on Sephacryl S-1000. The column (1.5 × 97 cm) was eluted with 0.1M sodium hydroxide.

fractionated by treatment with cetyltrimethylammonium bromide (CTAB), to provide, in ~2.6% yield, an acidic polysaccharide (U-3-A) that showed gelatinous properties and high viscosity in water.

U-3-A was found to be homogeneous, as judged by ultracentrifugal analysis (see Fig. 1) and by gel filtration on Sephacryl S-1000 in 0.1M sodium hydroxide (see

Fig. 2). U-3-A, which had $[\alpha]_D^{20}$ −8.7° (*c* 0.168, 0.05M sodium hydroxide), was composed of glucuronic acid, xylose, mannose, and glucose in the molar ratios of 1.0:1.9:2.9:1.8, as determined by paper chromatography (p.c.) of the hydrolyzate, gas–liquid chromatography (g.l.c.) of alditol acetate derivatives of the hydrolyzate, and colorimetric determination of glucuronic acid[12] in the polysaccharide solution. The infrared (i.r.) spectrum of U-3-A showed, at 1725 and 1250 cm^{-1}, characteristic absorption bands which disappeared after alkali treatment. This result indicated the presence of *O*-acyl groups. The total *O*-acetyl content was determined to be 0.3% by g.l.c. of the acetic acid in the hydrolyzate (obtained with M hydrochloric acid during 3 h at 100°), as previously described[13].

Furthermore, pyruvic acid was detected by paper chromatography (p.c.) after mild hydrolysis, and the content was determined to be 0.3% by the enzymic method using lactate dehydrogenase[14]. The molecular weight of U-3-A was estimated to be 2,400,000 by use of the calibration curve obtained by gel filtration on Sepharose CL-2B. Thus, U-3-A is an acidic heteroglycan of high molecular weight which contains neither protein nor lipid.

The anti-inflammatory activity of the polysaccharide preparations (U-3-EP and U-3-A) was tested in the carrageenan-induced, hindpaw-edema assay and scald-induced, hyperalgesia assay in rats. The results, given in Tables I and II, show that both U-3-EP and U-3-A possess significant inhibitory effects. The purified acidic polysaccharide U-3-A exerted stronger inhibitory effect than the crude polysaccharide U-3-EP at lower doses.

Intraperitoneal (i.p.) administration of both samples at these doses caused neither change in gross behavior nor death of the animals within a period of 14 days. Moreover, as no abdominal-writhing response to the i.p. injection of U-3-A

TABLE I

EFFECT OF CRUDE (U-3-EP) AND PURIFIED (U-3-A) POLYSACCHARIDES ON CARRAGEENAN-INDUCED, HINDPAW EDEMA IN RATS

Compounds	*Trial no.*	*Dose[a] (mg/kg × per injection)*	*100 × Hindpaw thickness (mm)[b]*			*Area under time-response curve (edema) (0–3 h)*	*Change in the area under the curve (%) (0–3 h)*
			Time (h) after carrageenan injection				
			0	*2*	*3*		
Vehicle	I	5 mL/kg	360 ±6	637 ±26	738 ±39	466 ±36	—
control	II	5 mL/kg	336 ±4	684 ±9	701 ±19	531 ±20	—
	III	5 mL/kg	343 ±7	708 ±34	767 ±32	577 ±59	—
U-3-EP	I	50	352 ±1	491 ±22[c]	545 ±11[c]	236 ±31[d]	−50[d]
U-3-A	II	6.25	338 ±1	622 ±21[c]	631 ±20[c]	431 ±31[c]	−19[c]
	II	12.5	334 ±4	550 ±11[e]	563 ±9[e]	331 ±10[e]	−37.6[e]
	III	25	335 ±3	525 ±14[d]	530 ±11[e]	288 ±16[d]	−50.1[d]

[a]The polysaccharides dissolved in water were given i.p immediately, and again 1 h after injection of 0.1 mL of 5% carrageenan suspension into the right hindpaw. [b]The data indicate the means ±s.e.m. of 100 × the paw thickness (mm) obtained from 5 rats per group. [c]A significant difference from the corresponding vehicle controls, $p < 0.05$ [d]$p < 0.01$. [e]$p < 0.001$.

TABLE II

EFFECT OF CRUDE (U-3-EP) AND PURIFIED (U-3-A) POLYSACCHARIDES ON SCALD-INDUCED HYPERALGESIA IN THE HINDPAW OF RATS

Compounds	*Trial no.*	*Dose[a] (mg/kg × per injection)*	*Pain threshold (in g of pressure on the inflamed paw)[b]*				*Area under time-response curve (hyperalgesia) (0–4.5 h)*	*Change in the area under the curve (%) (0–4.5 h)*
			Time (h) after immersing the hindpaw in the hot water					
			0	*3*	*3.5*	*4.5*		
Vehicle	I	5 mL/kg	369 ±5	213 ±9	223 ±14	230 ±17	267 ±15	—
control	II	5 mL/kg	365 ±10	259 ±11	240 ±17	213 ±17	223 ±17	—
	III	5 mL/kg	362 ±7	235 ±2	224 ±8	209 ±11	243 ±11	—
U-3-EP	I	50	352 ±7	245 ±16	237 ±7	229 ±20	200 ±7[c]	−25[c]
U-3-A	II	6.25	355 ±15	282 ±14	271 ±20	259 ±13	148 ±16[c]	−33.8[c]
	II	12.5	364 ±12	312 ±15[c]	269 ±18	258 ±16	122 ±31[c]	−45.4[c]
	III	25	363 ±7	337 ±10[d]	304 ±11[e]	301 ±16[e]	87 ±16[e]	−64.1[e]

[a]The polysaccharides, dissolved in water, were given i.p. twice, 1.5 and 2.5 h after immersion of the left hindpaw for 12 s in water at 54°. [b]The data shown indicate the means ±s.e.m. of pain threshold (g/pin) obtained from 5 rats per group. [c]A significant difference from the corresponding vehicle controls, $p < 0.05$. [d]$p < 0.01$. [e]$p < 0.001$.

TABLE III

ANTI-INFLAMMATORY ACTIVITIES[a] OF THE POLYSACCHARIDE IN BOTH INFLAMMATORY MODELS

Compounds	*Dose (mg/kg per injection) at 1-h intervals)*	*Scald hyperalgesia*				*Carrageenan edema*
		ED_{50} (in mg/kg; 95% confidence limits)				*ED_{30} (in mg/kg, per injection; 95% confidence limits)*
		Time (h) after immersing the hindpaw in the hot water			*Area (0–4.5 h)*	*Area (0–3 h)*
		3	*3.5*	*4.5*		
U-3-A	6.25–25	12.1 (5.73–20.2)	12.5–25	12.5–25	13.7 (6.48–46.8)	23.8 (18.3–38.3)
Phenylbutazone	12.5–50	29.8 (18.6–73.9)	>50	>50	>50	45 (30–206)

[a]Anti-inflammatory ED_{50} (scald hyperalgesia) and ED_{30} (carrageenan edema) values indicate the doses required to increase the pain threshold by 50%, or to decrease the thickness of the hindpaw by 30%, respectively. These values were determined for dose–response curves obtained by administering the compounds at 3 dose levels (5 animals per group). Each animal received two i.p. injections of drug, at 1 h intervals, as described in the Experimental section.

was observed, the anti-inflammatory activity is probably not a secondary effect caused by counter-irritation. The results in Table III show that the anti-inflammatory potency of U-3-A (given i.p.) on carrageenan edema (ED_{30}) and scald hyperalgesia (ED_{50}) is stronger than that of phenylbutazone. On oral administration (200 mg/kg × 1), however, neither U-3-EP nor U-3-A inhibited carrageenan edema or scald hyperalgesia in the hindpaws of rats.

Our previous studies on the anti-inflammatory effect of various polysaccharides[3,4,15] demonstrated that two homoglycans, *i.e.* (1→3)-β-D-glucan[16] and partially *O*-acetylated (1→3)-α-D-mannan[17], showed considerable inhibitory activity in the aforementioned two inflammatory models, but several other polysaccharides were barely effective. U-3-A is an acidic polysaccharide consisting of glucuronic acid, mannose, xylose, and glucose, and it showed even greater anti-inflammatory effect than the foregoing polysaccharides. The acidic polysaccharide[18] (a glucuronoxylomannan) isolated from the fruit bodies of *Auricularia auricula-judae,* belonging to the same family (Auriculariaceae), exhibited only a marginal inhibitory effect. Therefore, the structural features of U-3-A are interesting in comparing anti-inflammatory and antitumor[2] biological activities of polysaccharides.

EXPERIMENTAL

Materials. — The dried fruit bodies of *Auricularia* species (Chinese name: Yū ěr, Auriculariaceae) are commercially available in Hong Kong. Pronase (70,000 p.u.k./kg) was purchased from Kaken Chemical Ind., lactate dehydrogenase (from rabbit muscle, type II) from Sigma Chemical Co., and NADH from Nakarai Chemicals. Sephacryl S-1000 and Sepharose CL-2B were purchased from Pharmacia Fine Chemicals.

General methods. — Specific rotations were measured with a JASCO DIP-4 polarimeter. I.r. spectra were recorded with a JASCO IRA-1 spectrometer. P.c. was conducted by the ascending method, using Toyo No. 51 filter paper and the following solvent systems: *A,* 6:4:3 1-butanol–pyridine–water and *B,* 18:3:1:4 ethyl acetate–acetic acid–formic acid–water. Detection was achieved with an alkaline silver nitrate reagent[19] or *o*-phenylenediamine reagent[20]. G.l.c. was performed with a Shimadzu 4CM apparatus equipped with a flame-ionization detector. For sugar analyses, a glass column (0.3 × 150 cm) packed with 3% of ECNSS-M on Gaschrom Q (100–120 mesh) was used, with nitrogen as the carrier gas at a flow rate of 50 mL/min, at 175°, and, for acetic acid analyses, a column (0.3 × 200 cm) packed with 2% phosphoric acid–Porapack Q (80–100 mesh), at 205° and a flow rate of nitrogen of 50 mL/min. Analysis of acetyl groups was achieved as previously described[18]. Ultracentrifugal analysis was conducted in 0.5M sodium hydroxide with an MOM 3170/b analytical ultracentrifuge at 60,000 r.p.m.

Isolation of the polysaccharide. — The dried fruit-bodies (85 g) of *Auricularia* species were pulverized, and successively extracted with hot methanol and 70% aqueous ethanol in a boiling-water bath. The residue was extracted 4 times with

water for 5 h in a boiling-water bath. The extracts were combined, and de-proteinized by Pronase treatment, as previously[21] described, and by the Sevag procedure[11]. The aqueous phase from the Sevag procedure was dialyzed against de-ionized water for 5 d, and the non-dialyzable fraction was concentrated to a small volume. Ethanol (3 vol.) was then added to the concentrate, and the resulting precipitate was collected by centrifugation for 30 min at 5,000 r.p.m. A solution of the precipitate in water was lyophilized to give the crude polysaccharide, U-3-EP (~6 g).

The crude polysaccharide was dissolved in 15mM sodium sulfate (1% solution), and 2% CTAB and a small amount of Celite as an adjunct for precipitation were added until no further precipitation occurred. The mixture was kept for 24 h at 37°, and then the precipitate was collected by centrifugation, and dissolved in 5M sodium chloride. The solution was mixed with ethanol, and the precipitated fraction was dialyzed, and lyophilized, to give the acidic polysaccharide, U-3-A (~2.2 g).

Gel filtration and estimation of molecular weight. — A solution of U-3-A (2 mg) in 0.1M sodium hydroxide (1 mL) was applied to a column (1.5 × 97 cm) of Sephacryl S-1000 which was then eluted with 0.1M sodium hydroxide. Fractions (3.8 mL each) were collected, and an aliquot of each fraction was analyzed by the phenol–sulfuric acid method[22]. Gel filtration of U-3-A and standard dextrans on a column of Sepharose CL-2B was conducted in the same way for construction of the calibration curve[1]. The dextrans used were Dextran T-2000 (mol. wt., 2,000,000), T-500 (495,000), and T-250 (253,000).

Hydrolysis of the polysaccharide. — (*a*) After hydrolysis of U-3-A with 90% formic acid for 12 h at 100° and 0.5M sulfuric acid for 38 h at 100°, p.c. (solvent *A*) showed the presence of xylose, mannose, glucose, and glucuronic acid. For analysis of the neutral sugars, an aqueous solution of the hydrolyzate was reduced with sodium borohydride, the alditols were acetylated, and the resulting alditol acetates analyzed[23] by g.l.c.

(*b*) After hydrolysis with 0.01M hydrochloric acid for 1.5 h at 100°, p.c. (solvent *B*) of the concentrated solution showed a fast-moving spot, chromatographically identical with that for pyruvic acid and having the same characteristic fluorescence as the authentic compound when sprayed with *o*-phenylenediamine[20]. A solution of the hydrolyzate obtained with 0.02M oxalic acid for 4 h at 100° was treated with lactate dehydrogenase and NADH, and the absorbance was read[14] at 340 nm.

Assay of anti-inflammatory activities. — Scald hyperalgesia and carrageenan edema were induced on the left and right hindpaws, respectively, of each Sprague Dawley rat, weighing 190–200 g, as described previously[3,24]. Briefly, the left hindpaw was immersed for 12 s in water at 54°, and, 1.5 h later, the animals received a subcutaneous injection of 5% carrageenan suspension (0.1 mL) into the right hindpaw. The polysaccharides dissolved in water were administered i.p. twice, immediately after, and 1 h after the injection of carrageenan. The inhibitory effects on scald hyperalgesia and carrageenan edema of each hindpaw were determined by

using a modified, Randall–Sellito instrument at 0.5, 1, and 2 h, and 1 and 2 h after administration of the second sample, respectively. For oral administration, 200 mg of sample per kg was given once. Phenylbutazone was used as the reference drug.

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