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DEGRADATION WITH FORMIC ACID OF CONFORMATIONALLY DIFFERENT (1→3)-β-D-GLUCANS ISOLATED FROM LIQUID-CULTURED MYCELIUM OF *Grifola frondosa*

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ABSTRACT

On formolysis of the title antitumour (1→3)-β-D-glucans (LELFD), which have different conformations (native and helix) in the solid state, the former gave a polymer fraction and oligosaccharides. The conformation of the polymer fraction was not changed as assessed by c.p.–m.a.s. ^{13}C-n.m.r. spectroscopy. In contrast, LELFD-helix was degraded readily under mild conditions (aqueous 90% HCOOH, 20 min, 95°), to give products having a molecular weight of $\sim 10^4$, suggesting an endo-mode of degradation. From the results of the formation of glucan–dye complexes, it is suggested that the formolysis products having a mol. wt. of >34,000 possess a significant content of helices. These products and those with a mol. wt. of >21,000 showed antitumour activity. These results suggested that the conformation of the glucan affected the outcome of formolysis and that the native conformer was more rigid than the helix form.

INTRODUCTION

LELFD, an extracellular polysaccharide produced from liquid-cultured mycelium of *Grifola frondosa*[1], is a (1→3)-β-D-glucan possessing a β-D-glucosyl branch at position 6 of every third unit. It is the same as schizophyllan from *Schizophyllium commune*[2], which has been clinically used for cancer immunotherapy in Japan. Lentinan also is a (1→3)-β-D-glucan with two 6-linked β-D-glucosyl side-chains for every five main-chain residues[3]. LELFD has a molecular weight of $>5 \times 10^6$ and is hardly soluble in water because of gel formation, so that, for clinical use, a low-viscosity glucan is required. Hence, the use of formolysis to decrease the gel-forming ability was studied.

Application of solid-state cross-polarisation–magic angle spinning (c.p.–m.a.s.) ^{13}C-n.m.r. spectroscopy showed that LELFD had native and helix confor-

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mations and that the transition from native to helix is effected by dissolution in 8M urea followed by dialysis and lyophilisation[4]. The chemical shift of the C-3 resonance of LELFD-native is ~86 p.p.m., which corresponds to that of the glucan prepared under milder conditions, *i.e.*, hot-water extraction of the fruit body or liquid-culture filtrate. In contrast, the C-3 signal of LELFD-helix appeared at 89 p.p.m., similar to those of curdlan powder or cold alkaline extracts of *G. frondosa*. The native conformation requires a higher molarity of urea or sodium hydroxide to denature the gel structure than does the helix form, suggesting the former to be more rigid[5]. A similar result was observed with SSG which was obtained from the culture broth of *Sclerotinia sclerotiorum* IFO 9395, and which is a (1→3)-β-D-glucan having a β-D-glucosyl branch at O-6 of every other main-chain unit[6]. However, the detailed ultrastructure of both conformations is unclear, because there are few technologies to elucidate conformation of these gels.

It has been suggested that, in addition to the primary structure, a certain molecular weight and ordered structure, such as a triple helix, are required for antitumour activity. The relationship between the molecular weight and gel formation was found in lentinan (from *Lentinus edodes*)[7], curdlan [linear (1→3)-β-D-glucan from *Alcaligenes faecalis* IFO 13140[8]][9], and schizophyllan *(Schyzophyllum commune)*[10]. The physicochemical properties of LELFD are similar to those of these glucans, but comparison of the detailed structure, such as the proportion of the triple-helix moiety, has not been investigated.

We now compare the reactivity of the native and helix conformations on formolysis, and the critical molecular weight of LELFD necessary for antitumour activity.

EXPERIMENTAL

Materials. — Toyopearl TSK-GEL HW-65F and HW-55F were obtained from Toyo Soda Mfg. Co. (Tokyo, Japan), Bio-Gel P-2 from Bio-Rad Laboratories, Aniline Blue from Chroma Gesellschaft Schmid (Stuttgart F.R.G.), and dextran T10–T2000 from Pharmacia Fine Chemicals.

Preparation of LELFD. — Liquid-cultured mycelium of *Grifola frondosa* (from 5 L) was incubated in 0.5% citrate buffer (pH 4.0) containing 5% of D-glucose (5 L) at 25°. After incubation for 6 days, the mixture was centrifuged and the supernatant solution was diluted with 1 vol. of ethanol. A solution of the resulting precipitate in water was diluted with 1 vol. of ethanol, and the precipitate was collected, dried, and used as LELFD-native (11.6 g). In order to prepare the LELFD-helix, a solution of LELFD-native in 8M urea was dialysed against water and then lyophilised.

Formolysis of LELFD. — A suspension of LELFD (1 or 2 g) in aqueous 90% formic acid (200 or 400 mL) was heated at 90 or 95° for various periods. The resulting solution was concentrated to dryness *in vacuo*. Distilled water was added to the products, and the mixtures were boiled for 3 h to remove formyl groups in

the glucan formate, then concentrated, and dried *in vacuo*. The resulting mixture was dissolved in water (200 or 400 mL) and fractionated by adding several vol. of ethanol.

Determination of molecular weight. — The molecular weight of each fraction was determined by elution from a column of TSK-GEL HW-65F (1.5 × 28 cm) or HW-55F (1.8 × 83 cm) with 0.3M sodium hydroxide. Dextran T10–T2000 were used as standards. The carbohydrate contents of fractions were monitored by phenol–sulfuric acid method[11].

N.m.r. studies. — ^{13}C-N.m.r. spectra (50.1 MHz) were recorded at room temperature for solutions in H_2O, with a JEOL FX-200 spectrometer. The spectra were obtained in the pulsed F.t. mode with complete proton decoupling. C.p.–m.a.s. ^{13}C-n.m.r. spectra were recorded with a c.p.–m.a.s. unit together with the appropriate software and with the use of a Dyflon rotor. Contact time, pulse interval, and number of pulses were 1 ms, 1–2 s, and 500–2,000 scans, respectively. Chemical shifts relative to that of Me_4Si were determined by using the signal of adamantane (29.5 p.p.m.).

Formation of complexes with Aniline Blue[12]. — The glucans were dissolved in Aniline Blue solution (10 μg/mL; 0.1M NaOH), and the intensity of fluorescence was measured at excitation 400 nm and emission 500 nm, using a Hitachi 650-40 fluorescence spectrophotometer.

Assay of antitumour activity. — Male ICR mice (6 weeks old and weighing 27–30 g) were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals, and were bred under specific pathogen-free (SPF) conditions. Sarcoma 180 tumour cells were maintained serially in the ascites form by weekly passage in ICR mice. Sarcoma 180 tumour cells (5×10^6) were inoculated subcutaneously into the right groin of ICR mice. Each glucan fraction was dissolved in saline and administered for 5 consecutive days from day 10 after the inoculation of the tumour. After 5 weeks, the mice were sacrificed and the tumours were extirpated. The inhibition ratio was calculated as follows:

$$\left(1 - \frac{\text{average tumour weight of the treated group}}{\text{average tumour weight of the control group}}\right) \times 100\,(\%)$$

RESULTS AND DISCUSSION

Formolysis. — For LELFD-native, the reaction conditions used were 90°/40 min, 95°/3 h, and 95°/12 h. LELFD-helix was formolysed with aqueous 90% formic acid at 95° for 20 min. After removal of the formic ester, the products were fractionally precipitated with ethanol. Table I shows the yields and molecular weights of the products, which were determined by gel filtration. With LELFD-native, material insoluble in formic acid was observed even after formolysis. Each product, except the supernatant fraction of the ethanol precipitation, possessed a molecular weight in the range 10^5–10^6. On treatment of LELFD-native with aqueous 90%

formic acid at 90° for 40 min, 57.8% remained insoluble. The products obtained after formolysis for 3 h had increased solubility in water, but their molecular weights were still high (750,000–2,250,000). Although formolysis of LELFD-native for 12 h provided fragments of smaller molecular weight, the difference of molecular weight between precipitates and supernatant fractions was remarkable. The fraction 12 h-3.0 sup (Table I) was fractionated on Bio-Gel P-2 (Fig. 1). The major component was glucose and there were small quantities of oligosaccharides of low mol. wt. The other ethanol supernatant fractions (40 min-1.0 sup, 3 h-0.8 sup) had compositions similar to that of 12 h-3.0 sup as shown by h.p.l.c. on silica NH_2 (data not shown). For LELFD-helix, the products were eluted from HW-55F with 0.3M sodium hydroxide (Fig. 2). The mol. wt. of each fraction was <5,000–34,000, and estimated to be ~100 times smaller than the product from LELFD-native. These results showed that the reactivities of LELFD-native and LELFD-helix on formolysis were different, and that the molecule of the former was more rigid. The products of formolysis of LELFD-helix showed uniformly distributed molecular weights, suggesting that degradation had occurred by exo-attack and that of LELFD-helix by endo-attack. The different modes of formolysis could reflect differences in hydrophilicity. LELFD-native may be more closely cross-linked to the neighboring glucan chains than LELFD-helix, and formic acid molecules cannot enter the interstices.

TABLE I

PRODUCTS OF FORMOLYSIS OF LELFD WITH AQUEOUS 90% HCOOH

Sample	*Reaction conditions*	*Fraction (EtOH vol.)*	*Yield (%)*	*Mol. wt.*[a] *(× 10^3)*
LELFD-native	90° 40 min	0 ppt	57.8	n.d.[b]
		0.5 ppt	24.1	2,250
		1.0 ppt	5.9	1,100
		1.0 sup	2.4	n.d.
	95° 3 h	0 ppt	6.8	n.d.
		0.1 ppt	1.5	2,250
		0.4 ppt	45.6	1,560
		0.5 ppt	21.7	1,100
		0.8 ppt	3.1	750
		0.8 sup	14.3	n.d.
	95° 12 h	0 ppt	7.6	750
		1.0 ppt	10.7	750
		3.0 ppt	2.0	520
		3.0 sup	65.0	Glucose
LELFD-helix	95° 20 min	0 ppt	13.1	n.d.
		1.5 ppt	23.9	34
		2.5 ppt	31.7	21
		8.0 ppt	18.3	6.2
		14 ppt	0.9	<5
		14 sup	6.7	<5

[a]Determined by elution from a column of TSK-GEL HW-65F or HW-55F with 0.3M sodium hydroxide or from Bio-Gel P-2 with distilled water. [b]Not determined.

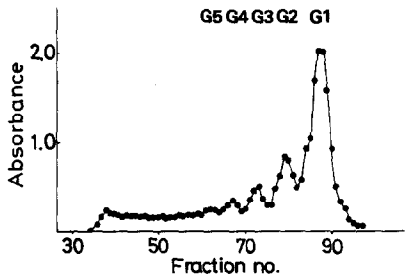

Fig. 1. Elution of the product (12 h-3.0 sup, 40 mg) of formolysis of LELFD-native from a column (1.8 × 136 cm) of Bio-Gel P-2 equilibrated with H_2O. Fractions (2.87 mL) were assayed by the phenol–sulfuric acid method: G1, glucose; G2, biose; G3, triose, G4, tetraose; G5, pentaose; elution volumes of glucose and laminari-oligosaccharides.

Conformation of the products of formolysis of the native and helix forms of LELFD in aqueous solution. — Aqueous solutions of the products of formolysis, isolated by precipitation with ethanol, were studied by ^{13}C-n.m.r. spectroscopy. Saito *et al.*[13] suggested that the ^{13}C signals of linear (1→3)-β-D-glucans in dilute alkaline solutions reflect the segments of single helical conformation and that, in >0.25M sodium hydroxide, the chemical shifts of the C-1 and C-3 resonances change to 103 and 86 p.p.m., respectively, due to the formation of a random-coil structure[13]. They showed also that the ^{13}C signals of the (1→3)-β-linked D-glucosyl residue of lentinan, a 6-branched (1→3)-β-D-glucan, in the neutral gel state were completely suppressed, due to the immobilisation of the molecular chain, mainly as a result of the presence of physical cross-links[14].

Each fraction from LELFD-helix gave a highly resolved spectrum in H_2O

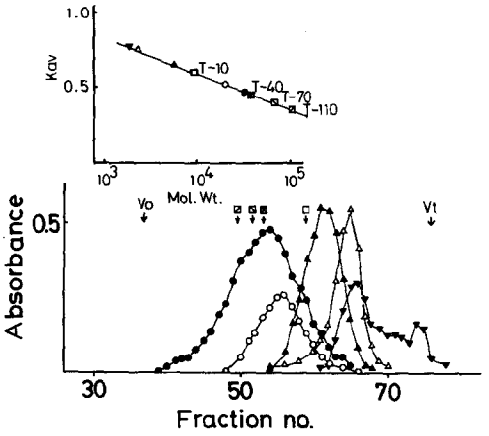

Fig. 2. Elution of the products (5 or 7 mg) of formolysis of LELFD-helix from a column (1.8 × 83 cm) of TSK-GEL HW-55F equilibrated with 0.3M sodium hydroxide. Fractions (2.8 mL) were assayed by the phenol–sulfuric acid method: ●, 1.5 ppt; ○, 2.5 ppt; ▲, 8.0 ppt; △, 14 ppt; ▼, 14 sup; V_o, void volume; V_t, total volume; □, dextran T10; ⊠, T40; ⧅, T70; ⧈, T110.

(Fig. 3). The chemical shifts were similar to that of the spectrum for a solution in 0.25M sodium hydroxide. The chemical shift of the C-3 resonance was 85 p.p.m., suggesting that the lower-molecular-weight LELFD had an almost random-coil structure and that the proportion of the sol was increased by formolysis. In contrast, the ethanol-precipitated fragment of LELFD-native, 12 h-3.0 ppt (Fig. 3e), showed broad signals suggesting the presence of physical cross-links. The supernatant fraction (12 h-3.0 sup) from LELFD-native gave signals attributed to glucose (Fig. 3f). This result coincided with that of the elution profile from Bio-Gel P-2. In order to investigate the ultrastructure of the fragments of formolysis of LELFD, the intensities of fluorescence of the glucan–Aniline Blue complexes formed in alkaline solution were measured. Aniline Blue fluoresces on interacting with (1→3)-β-D-glucans having an ordered structure (*e.g.*, curdlan) in alkaline solution. The intensity of fluorescence declined on increasing the concentration of alkali and a random-coil glucan, such as laminaran, does not cause fluorescence. Each sample was dissolved in Aniline Blue solution (10 μg/mL, 0.1M NaOH), and the intensity of fluorescence was measured at excitation (400 nm) and emission (500 nm). Grifolan LE purified from LELFD-helix [a 6-branched (1→3)-β-D-glucan[1]] was used as the positive standard. The relative intensity of fluorescence was calculated from the value at a concentration of 100 μg/mL of glucan and the relative intensity was calculated from the value of grifolan LE as 100%. The precipitates of formolysed LELFD-native, except the 12 h-3.0 ppt, exhibited an intensity similar to that of grifolan LE (Table II). In the case of LELFD-helix, even the 1.5 ppt fraction (mol. wt. 34,000) fluoresced but to a lesser extent (40.7%) (Table II). The decline of the fluorescence

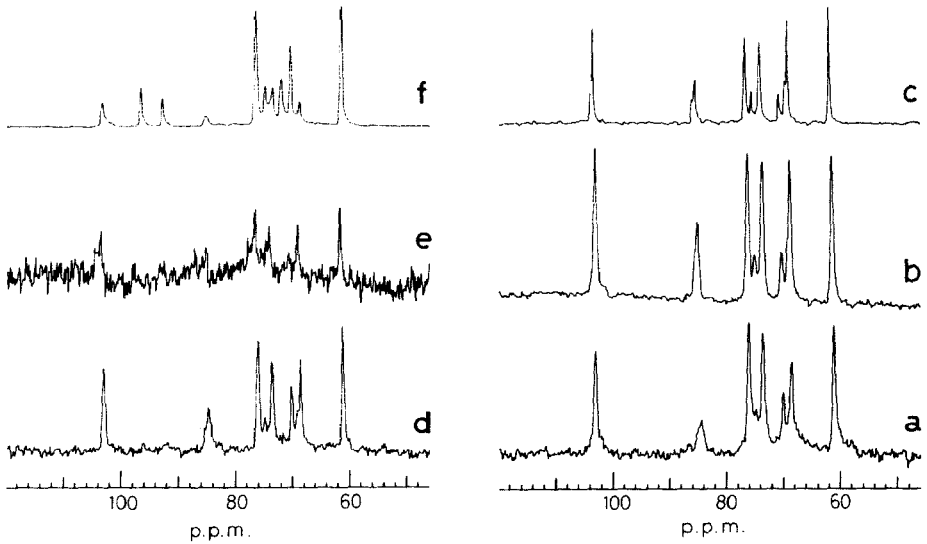

Fig. 3. ^{13}C-N.m.r. spectra (H_2O) of the products of smaller molecular weight of LELFD-helix: a, 20 min 1.5 ppt; b, 20 min 2.5 ppt; c, 20 min 8.0 ppt; d, 20 min 14 sup; LELFD-native: e, 12 h 3.0 ppt; f, 12 h 3.0 sup.

TABLE II

INTENSITY OF FLUORESCENCE OF ANILINE BLUE ADMIXED WITH THE PRODUCTS OF FORMOLYSIS OF LELFD WITH AQUEOUS 90% HCOOH[a]

Sample	*Reaction conditions*	*Fraction (EtOH vol.)*	*Relative intensity*
LELFD-native	95°, 3 h	0.4 ppt	75.9
		0.8 ppt	81.5
	95°, 12 h	1.0 ppt	88.9
		3.0 ppt	42.6
LELFD-helix	95°, 20 min	1.5 ppt	40.7
		2.5 ppt	18.5
		8.0 ppt	5.6
Grifolan LE			100

[a]Aniline Blue solution (3 mL, 10 μg/mL) was mixed with several LELFD fragments (100 μg/mL), and the intensity of fluorescence was recorded. Each value was calculated from the intensity of grifolan LE as 100%.

correlated with the reduction of molecular weight, indicating that the lower-molecular-weight fraction had lost the moiety which could form an ordered conformation. This conclusion was supported by the ^{13}C-n.m.r. spectra which contained highly resolved signals for the neutral solution (Fig. 3).

Fig. 4 shows the c.p.–m.a.s. ^{13}C-n.m.r. spectra of LELFD-native 3 h-0.4 ppt

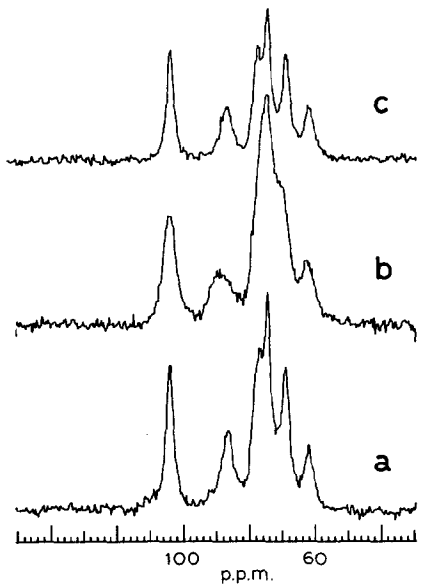

Fig. 4. C.p.–m.a.s. ^{13}C-n.m.r. spectra of a, LELFD-native; b, LELFD-helix; c, formolysed LELFD-native 3 h 0.4 ppt.

TABLE III

ANTITUMOUR ACTIVITY OF THE PRODUCTS OF FORMOLYSIS OF LELFD-NATIVE AGAINST SARCOMA 180[a]

Sample	*Dose × 5 (μg/mouse)*	*Tumour weight[b] (g, mean ±s.d.)*	*Inhibition ratio (%)[c]*	*Complete regression[c]*
40 min 0.5 ppt	100	0.18 ±0.38[d]	97.0	6/10
	500	0.57 ±0.53[d]	90.5	2/10
3 h 0.4 ppt	100	0.09 ±0.21[d]	98.5	7/10
	500	1.31 ±1.50[d]	78.2	1/10
12 h 1.0 ppt	100	0.56 ±0.93[d]	90.7	4/10
	500	1.09 ±0.93[d]	81.8	1/10
Grifolan LE	100	0.26 ±0.62[d]	95.7	7/10
Nil		6.00 ±4.00		0/26

[a]Sarcoma 180 tumour cells (5×10^6) were inoculated s.c. (day 0). Each sample was administered i.p. every other day from day 10 to 18 as a saline solution. [b]The significance was evaluated by means of Student's *t*-test against the untreated group. [c]Determined at day 35 after tumour inoculation. [d]$p < 0.001$.

TABLE IV

ANTITUMOUR ACTIVITY AGAINST SARCOMA 180 OF SMALLER MOLECULAR-WEIGHT-FRAGMENTS OBTAINED BY FORMOLYSIS OF LELFD-HELIX[a]

Sample	*Dose × 5 (μg/mouse)*	*Injection route*	*Tumour weight (g, mean ±s.d.)*	*Inhibition ratio (%)[c]*	*Complete regression[c]*
1.5 ppt	100	i.p	1.97 ±3.15[d]	71.6	0/10
	500		1.18 ±2.26[d]	83.0	3/10
2.5 ppt	100		4.76 ±3.15	31.3	1/10
	500		4.76 ±4.05	31.3	2/10
8.0 ppt	100		7.15 ±3.74	3.2	0/1
	500		6.38 ±2.51	7.9	1/10
Grifolan LE	100		0.65 ±1.32[d]	90.6	4/10
	500		0.06 ±0.05[e]	99.1	3/10
1.5 ppt	100	i.v.	3.20 ±2.66[b]	53.8	2/10
	500		1.23 ±1.78[d]	82.3	3/10
2.5 ppt	100		3.22 ±3.37[b]	53.5	1/10
	500		4.77 ±3.48	31.2	0/10
8.0 ppt	100		5.09 ±2.07	26.6	0/10
	500		5.61 ±3.28	19.1	0/10
Grifolan LE	100		1.23 ±2.60[d]	82.3	2/9
	500		1.24 ±1.73[d]	82.1	0/9
Nil			6.93 ±5.03		0/13

[a]See Table III, footnote a. [b]The significance was evaluated by means of Student's *t*-test against the untreated group: $p < 0.05$. [c]Determined at day 35 after tumour inoculation. [d]$p < 0.01$. [e]$p < 0.001$.

in the solid state. The chemical shift of the C-3 resonance was identical to those of LELFD-native, indicating no alteration of conformation during formolysis.

Antitumour activity of the fragments of formolysis of LELFD. — The native and helix conformers of LELFD possess marked antitumour activity in murine tumour systems[1]. Each fraction from LELFD-native showed significant activity against sarcoma 180, and the inhibition ratio was essentially the same as that of the LELFD. Of the fractions from LELFD-helix, only 1.5 ppt (mol. wt. 34,000) showed significant activity on i.p administration, as did the 1.5 ppt and 2.5 ppt (mol. wt. 21,000) by i.v. administration. These results suggested that the minimal effective molecular weight is 34,000 for i.p and 21,000 for i.v. administrations. Similar observations have been reported for curdlan[8]. The antitumour activity of these fractions showed a correlation of the activity with the ordered structure (see above) in accord with previous findings[15,16].

The minimal effective molecular weights of the antitumour (1→3)-β-D-glucans in curdlan[8], lentinan[7], and schizophyllan (SPG)[9] were reported to be 2,600 (single strand state), 6,260 (single strand), and 50,000 (triple strand), respectively. The effective mol. wt. of LELFD-helix was 34,000 (single strand state) by i.p. administration, and this value was higher than that of SPG which has same primary structure. This difference probably reflects different proportions of ordered structure.

Since the transition LELFD-native→LELFD-helix is effected by dissolution in 8M urea and the reverse transition by heating an aqueous gel (100 mg/mL) at 150° for 10 min and then cooling, only changes in conformation are involved and the different behavior on formolysis probably reflects differences in inter- and intramolecular associations. A related phenomenon is the annealing of curdlan, (1→3)-β-D-glucan[17], by which the proportion of triple helix is increased and the molecular framework reinforced. Takahashi *et al.*[18] reported that heat-treated (120°) curdlan gel was more resistant to degradation with (1→3)-β-D-glucanase (Zymolyase) than alkali-treated gel, and also proposed that the former would be rigidly cross-linked by hydrophobic bonds rather than hydrogen bonds[18].

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