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Studies on the characteristic and activity of low-molecular fragments from zymosan

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ABSTRACT

Zymosan was hydrolysed with HCl and fractionated by ultrafiltration and dialysis to obtain water-soluble fragments A, B and C. Physical and chemical analyses showed that these fractions are composed primarily of glucose and have molecular weights of 8 kDa, 5 kDa and 2 kDa, respectively. A glycosidic linkage analysis indicated that they are mainly composed of β-1,3-glucans. Fragment A, which has the highest molecular weight, contains approximately 30% β-1,6-linked glucans, but fragment C is almost entirely composed of linear β-1,3-glucan chains. The anti-chronic atrophic gastritis activity experiments showed that fragment A has significant activity, the activity of zymosan is quite low and the activities of fragments B and C are in between those of fragment A and zymosan.

1. Introduction

Yeast cell walls are primarily composed of mannans and glucans (Magnelli, Cipollo, & Abeijon, 2002). Zymosan is a β-1,3-glucan derived from yeast cell walls (Manners, Masson, & Patterson, 1973) that has been used in food and the feed industry. Its chemical components, structural characteristics, and pharmacological activities have been widely studied, and it has been found that zymosan has immunomodulation (Suzuki & Tanaka, 1990) antitumour (Gu, Takagi, Nakamura, et al., 2005; Khalikova, Zhanaeva, & Korolenko, 2005) and anti-inflammation effects (Williams, Browder, McNamee, et al., 1982). However, zymosan has a large molecular weight (240 kDa) and is insoluble in water, which influences its absorbance and biological effects when administered orally. Additionally, the purity zymosan is not sufficient to meet the demands for newly developed medical products, such as food supplements or drugs.

Chronic atrophic gastritis (CAG) is a long-lasting disease that is related to stomach tumours. Until now, there has been no effective clinical treatment for CAG, although it has usually been treated with pentagastrin, sucralfate, dry yeast and traditional Chinese medicines, such as extracts of *Hericium erinaceu Pers*, that contain mainly polysaccharides and oligosaccharides (Jiang et al., 2007). The carbohydrates contained in *H. erinaceu Pers* have been shown to possess anti-CAG activity (Jiang et al., 2008). Based on the above information, we hydrolysed zymosan to obtain various water-soluble fragments and studied their anti-CAG activities in an attempt to understand the influence of structure and molecular weight on the activity and potential uses of zymosan.

2. Reagents and materials

The yeast glucan G 70 (zymosan), batch number 2008083M, was purchased from the Angel Yeast Company, China. An ultrafiltration system and filtration membranes were acquired from the Millipore Company. Dialysis tubes, standard monosaccharides and BSA were obtained from Sigma. The drugs Wei Lexin and an oral suspension of sucralfate were used as positive controls in the pharmacological experiments and were acquired from the Pharmaceutical Company of Jilin University and a Chinese medical shop, respectively. The other chemicals were obtained from local sources.

3. Experiment

3.1. Preparation of zymosan fragments

A total of 100 g of zymosan was dispersed in a flask containing 2000 mL of 0.2 mol/L HCl, and the dispersion was thoroughly mixed by stirring at 200 rpm with an electric stirrer for 30 min at room temperature. The flask was then put into a water bath and heated to 90 °C over 30 min. After heating at this temperature for 2 h, the flask was placed in cold water and the pH was adjusted to 7.0 using a 20% solution of NaOH. The hydrolysate was centrifuged for 10 min at 3000 rpm, and the precipitate was lyophilised (31.5 g, P). The supernatant was filtered by an ultrafiltration system equipped with a 10 K membrane. The high-molecular-weight fractions (concentrated solution) and low-molecular-weight fractions

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(dilute solution) were collected. The dilute solution was concentrated to a suitable volume and passed through an ultrafiltration system equipped with a 5 K membrane, and the concentrated solution was lyophilised to obtain zymosan fragments with a molecular weight of 8 kDa (A, recovered amount: 18.23 g). The dilute fractions from the second filtration were concentrated to a suitable volume and filtered through the ultrafiltration system equipped with a 3 K membrane, and the concentrated solution was lyophilised to obtain zymosan fragments with a molecular weight of 5 kDa (B, recovered amount: 14.86 g). The dilute fractions from the third filtration were concentrated and dialysed against water with a 1 K dialysis tube to remove any salts from the dilute solution. After dialysis, the inner solution was lyophilised to obtain zymosan fragments with a molecular weight of 2 kDa (C, recovered amount: 8.34 g).

3.2. Analysis of physicochemical properties

The total carbohydrate, uronic acid and protein contents were quantified by the phenol–sulphuric acid (Dubois, Giles, Hamilton, Reberse, & Smith, 1956), m-hydroxydiphenyl (Chaplin, 1986) and Bradford methods (Bradford, 1976), respectively, using glucose (Glc), glucuronic acid (GlcA), and bovine serum albumin (BSA) as standards. The molecular weight of each sample was analysed by HPLC equipped with a refractive index detector on an OH-park column equilibrated with 0.7% sodium sulphate, and calibration curves were obtained by comparison with standard dextrans. The molecular weight of each sample was calculated with GPC software from the National Institute for the Control of Pharmaceutical and Biological Products of China. The sugar components were analysed by converting the sugars into PMP derivatives (Yang et al., 2005), which were detected by HPLC. HPLC was performed using a Shimadzu 2010 instrument equipped with a C-18 column and controlled by a Uniport HP N-2000 data station.

3.3. Gel-permeation chromatography

Samples B and C were subjected to gel-permeation chromatography on a column of Sephadex G-100 (60 cm × 4 cm) to investigate the relationship between the carbohydrates and the proteins. The samples (150 mg) were dissolved in water, applied to the column, eluted with water and collected in 6 mL fractions. The carbohydrate and protein contents were tested using the phenol–sulphuric acid method and by examining their absorbances at a UV wavelength of 280 nm.

3.4. Study on the anti-chronic atrophic gastritis activity of zymogen fragments in rat (Shu, 2002)

3.4.1. Preparation of the antigen-adjuvant

Wistar rats (160–180 g, obtained from Jilin University, College of Pharmacy, certificate of conformity for the SCXK, 2008-0005) were housed in a controlled environment with a 12/12 h light/dark cycle. One of the rats was sacrificed after fasting for 24 h, and its gastric mucosa was removed, mixed with 3 mL of normal saline, and then homogenised. The homogenate was mixed with 3 mL of paraffin to obtain the antigen-adjuvant.

3.4.2. Immunisation of the rats to form the CAG model

A total of 120 wistar rats (220–260 g) were subcutaneously injected with the antigen-adjuvant in the dorsum three times at different points, using 0.5 mL for each injection. This was repeated once 2 weeks later and again after one additional week. Starting with the first injections of the antigen-adjuvant, the rats were fed 2 mL of pig bile (bile:glycerol = 1:1, v/v) every 2 days.

Ten rats were sacrificed on day 50 after immunisation, and their stomachs were removed for histological detection. The results showed that the gastric mucosa was thinning, the number of gastric glands had decreased, and ulcers, blood and a large number of inflammatory cells that had infiltrated under the mucosa could be observed in the sections.

Table 1
Properties of fragments A, B and C.

Sample properties	A	B	C
Carbohydrate content (%)	97.51	92.38	85.82
Uronic acid content (%)	–	–	–
Protein content (%)	1.28	7.50	12.12
Range of molecular weights	5.2–12.1 K	2.2–7.6 K	0.3–3.5 K
Average molecular weight	8458	5785	1846
Sugar components (Glc:Man)	98:2	93:7	98:2

3.4.3. Groups and administration

The remaining 110 rats were randomly separated into 11 groups (10 per group) and given oral administrations for 22 days. In addition to the model control group, the two positive control groups were treated with Wei Lexin (800 mg/kg/day) or sucralfate (100 mg/kg/day). The other groups were treated with zymosan or fractions A, B or C. Two different dosages (400 mg/kg/day and 200 mg/kg/day) were tested for each sample, producing a total of eight treatment groups. Additionally, ten healthy Wistar rats were adopted as a normal group that was given oral administrations of water (4 mL/day) for 22 days. Finally, the rats were sacrificed, and the lesser curvatures from the gastric antrum to the gastric cardia were cut off along with any embedded sections. The anti-CAG effects were estimated by a histopathological observation report.

3.5. Glycosidic linkage analysis of different fragments of zymosan

Samples were methylated once by the Ciucanu method (Ciucanu & Kerek, 1984). The resulting partially methylated alditol acetates were then analysed by GC (Shimadzu GC-2010) and GC–MS (Shimadzu 2010) using a DB-1 capillary column (30 m × 0.25 mm) to detect the positions and amounts of glycosidic linkages in the samples. The injector temperature was 250 °C, and the column temperature was kept at 60 °C for 5 min and then increased to 220 °C at a rate of 5 °C/min. The samples of partially methylated alditol acetates were dissolved in acetone and injected as 1 μL aliquots. The molar ratios were calibrated based on the peak areas and the response factors (Sweet, Shapiro, & Albersh, 1975) of the flame-ionization detector in the GC. Mass spectra were recorded in the positive ion electron ionisation (EI) mode with MSD ChemStation.

FT-IR spectra were acquired using a Bruker (Germany) Vertex 70 FT-IR. The samples were pressed into KBr pellets, and the spectra were recorded in the transmittance mode over a wavelength range of 4000–400 cm^{-1}.

4. Results and discussion

4.1. Chemical and physical properties of the zymosan fragments

The hydrolysis conditions that were screened for the preparation of zymosan fragments included various types and concentrations of acid, temperatures and hydrolysis times. Reasonable yields and properties were observed for various zymosan fragments prepared by the optimised conditions.

The chemical and physical properties of fragments A, B and C are summarised in Table 1.

The samples contained primarily neutral carbohydrates but also included 2–10% protein. To investigate whether the carbohydrates and proteins were linked by chemical bonds, samples B and C were subjected to gel-permeation chromatography on Sephadex G-100. The elution pattern showed that the carbohydrate and

Table 2
Effects of zymosan and its water-soluble fragments on the CAG model animals.

Samples	Dosages (kg/day)	Number of rats	Number of rats with histopathological changes		
			1	2	3
Zymosan	200 mg	10	7	7	6
	400 mg	10	7	6	6
A	200 mg	10	4	3	3
	400 mg	10	2	1	1
B	200 mg	10	6	6	6
	400 mg	10	5	5	5
C	200 mg	10	7	6	6
	400 mg	10	6	5	5
Positive control groups					
Wei Lexin group	800 mg	10	5	4	5
Sucralfate group	100 mg	10	6	7	6
Model control group		10	10	10	10
Normal control group		10	0	0	0

protein curves overlapped well (figure not shown). The fragments were then treated with the Savage method several times, but the protein content was not reduced in the treated samples. This result, combined with the results of the gel-permeation chromatography, indicated that the carbohydrates and proteins were linked by chemical bonds in fragments A, B and C. Uronic acid could not be detected, and the GC trace for A, B and C all showed a single broad peak corresponding to average molecular weights of 8458, 5785 and 1846, respectively, as recorded by the GPC software. All of the samples were primarily composed of glucose along with 2–7% mannose.

4.2. Anti-chronic atrophic gastritis activity in rats

The sections prepared from the stomachs were observed for histopathological changes, and the effects were judged with respect to three changes (Table 2): (1) thinning gastric mucosa, (2) a decreased number of gastric glands, and (3) the observation of ulcers, blood and the infiltration of a large number of inflammatory cells under the mucosa.

All of the rats in the model group had histopathological changes (Fig. 1) in the thickness of the gastric mucosa, the number of gastric glands and the presence of ulcers, blood, and inflammation in the mucosa, which means the model is valid. Half (5/10) and 4/10 of the model rats recovered in the Wei Lexin and sucralfate groups, respectively, which confirms that the two positive control drugs displayed anti-CAG activity. Only 3/10 of the model rats in the zymosan group recovered, regardless of the dosage, but fragment A had a significant effect at the higher dosage (9/10 of the rats recovered). Fragments B and C were also more effective than zymosan and the positive control drugs. These results indicate that water-soluble fragments of zymosan are more effective than zymosan, and both the dosage and the molecular weight of the fragments appear to influence the activity.

4.3. Structural characterisation of the prepared fragments

The results of the glycosidic linkage analysis are shown in Table 3.

Previous structural characterisations of zymosan have reported that zymosan is composed primarily of a linear β-1,3-glucan, although small amounts of a highly branched β-1,6-glucan were also observed. Fragment C, which has the lowest molecular weight, is composed primarily of the linear β-1,3-glucan chains that form the main moiety present in zymosan. However, fragment A, which has the largest molecular weight, is approximately 30% β-1,6-linked glucans that have branching points at the 3 or 4 positions, indicating that the branched β-1,6-glucan moiety in zymosan was present in fragment A. Fraction B should be a mixture of the linear

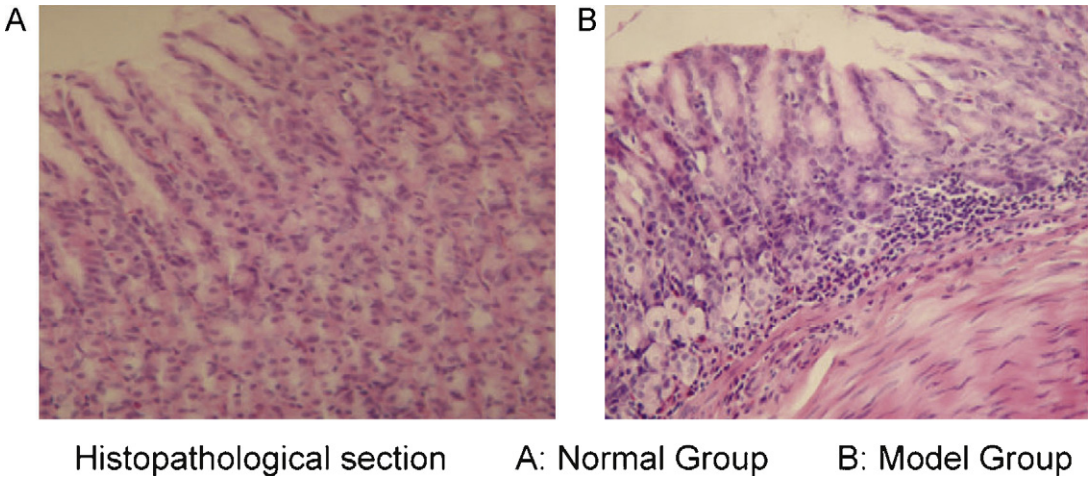

Fig. 1. Histopatholigical section. (A) Normal group; (B) model group.

Table 3
Analysis of the glycosidic linkages in fragments A, B and C.

Glycosyl residue	Fragment	Configuration	Molar ratio (%)		
			A	B	C
Glc	2,3,4,6-M_4	1→	12.13	3.97	4.29
	2,4,6-Me_3	1→3	49.68	72.19	71.66
	2,3,4-Me_3	1→6	18.33	10.45	6.51
	2,3-Me_2	1→4,6	5.24	6.39	6.31
	2,4-Me_2	1→3,6	5.22	1.98	
	2,6-Me_2	1→3,4	1.77		
Man	2,4,6-Me_3	1→3	6.56	5.05	11.23

and branched moieties of zymosan. Small amounts of 1,3-linked mannose were also detected in all three fragments.

4.4. Discussion

Zymosan has a large, complex molecular structure and is insoluble in water. However, it can be hydrolysed to obtain fragments with various molecular weights and higher levels of purity, which are beneficial if zymosan is used in food supplements and medical products. All three fragments exhibited higher anti-CAG activity than zymosan, and sample A displayed a level of activity that was significantly better than the positive control drugs. Thus, the preparation of low-molecular-weight, water-soluble fragments is one way to enhance zymosan's activity. An increased degree of branching in the fragments appears to improve their anti-CAG activity, but the influences of molecular weight and glycosidic linkages on this activity need to be studied further.

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