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Studies on the Immuno-Modulating and Antitumor Activities of *Ganoderma lucidum* (Reishi) Polysaccharides: Functional and Proteomic Analyses of a Fucose-Containing Glycoprotein Fraction Responsible for the Activities

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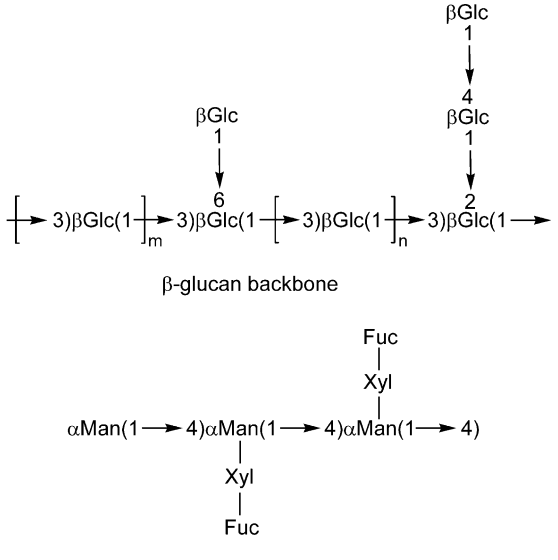
Abstract—A fucose-containing glycoprotein fraction which stimulates spleen cell proliferation and cytokine expression has been identified from the water-soluble extract of *Ganoderma lucidum*. Proteomic analysis of mouse spleen cells treated with this glycoprotein fraction showed ~50% change of the proteome. Further studies on the activities of this glycoprotein fraction through selective proteolysis and glycosidic cleavage indicate that a fucose containing polysaccharide fraction is responsible for stimulating the expression of cytokines, especially IL-1, IL-2 and INF-γ.

The water-soluble extract of *Ganoderma lucidum* (Reishi or Ling-Zhi) has been used in traditional Chinese medicine (TCM) as anti-tumor and immnuo-modulating agent. It also exhibits liver protective, hypoglycemic and platelet aggregation-inhibiting activities.[1] The active constituents responsible for each of these activities have been qualitatively described, but the molecular basis of their action has not been elucidated. Of particular significance among these functions is its immnuo-modulating and anti-tumor activities.[2,3] Previous studies have shown that the water soluble, polysaccharide components of Reishi exhibit the anti-tumor activity[2] and are able to stimulate the expression of CD_4 and T-cell counts during or after chemotherapy. The saccharides were known to contain either a polysaccharide backbone with β-1,3-linkages[3,4] or a polymannose backbone with α-1,4-linkages,[5] both with side chains of unknown structure (Scheme 1). The real carbohydrate epitope responsible for the anti-tumor activity and its receptor have not been identified, however, though the receptor CR3 has been shown to bind the β-glucan polysaccharide with undefined side chains.[3b]

As part of our interest in understanding the function of Reishi polysaccharides, we report here the identification from the water soluble TCM of a glycoprotein fraction



Scheme 1. Reported backbones of Reishi polysaccharides.

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which is shown to stimulate spleen-cell proliferation and expression of such cytokines as interleukins I and II, and interferon γ. Proteomic analysis of mouse speen cells treated with this glycoprotein fraction showed ~50% change in the proteome. The composition of this glycoprotein fraction has been determined, and the saccharide moiety has been confirmed to be responsible for the activities. Furthermore, the presence of fucose in the saccharide fraction is required for the activities.

Results and Discussion

The carbohydrate composition analyses of crude water soluble Reishi extract indicated that glucose and mannose exist as the major components together with smaller amounts of other sugars, including fucose, *N*-acetylglucosamine, xylose and rhamnose (Table 1). The crude extract contains 15.6% proteins, the amino acid analysis of which was shown in Table 2. This crude extract is currently commercially available as TCM and used as immuno-modulating and anti-tumor agents.

To identify the active components in this crude extract, gel filtration chromatography of the crude extract was carried out by using a Sephacryl S-500 column eluted with 0.1 N Tris buffer (pH 7.0), and the sugar content of each fraction was determined by anthrone analysis. Five fractions were obtained and the main fraction was designated as fraction 3 (Fig. 1). The colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for cell proliferation was then carried out to evaluate Con A-stimulated mouse spleen cells in the presence of various concentrations of the crude Reishi extract and fractions 1–5. In comparison with the control experiment (without treatment of samples), the cell proliferation activity was significantly enhanced with fraction 3 and slightly enhanced with fraction 2. The optimal concentration of fraction 3 was between 0.01 and 0.1 μg/mL and that of crude Reshi extract was between 0.1 and 1.0 μg/mL, as shown in Figure 2. Carbohydrate

Table 1. Carbohydrate compositions of crude Reishi extract

Sugar components	Percentage (%)
D-Glucose	58.0
D-Mannose	15.5
L-Fucose	9.7
D-Galactose	9.3
D-Xylose	5.4
D-GlcNAc	1.0
L-Rhamnose	0.5

Table 2. Amino acid analysis of Reishi extract

Amino acid	Relative abundance
Asp	117
Thr	66
Ser	54
Glu	120
Pro	60
Gly	108
Ala	100
Val	61
Met	6
Ile	36
Leu	55
Tyr	16
Phe	28
His	12
Lys	21
Arg	22

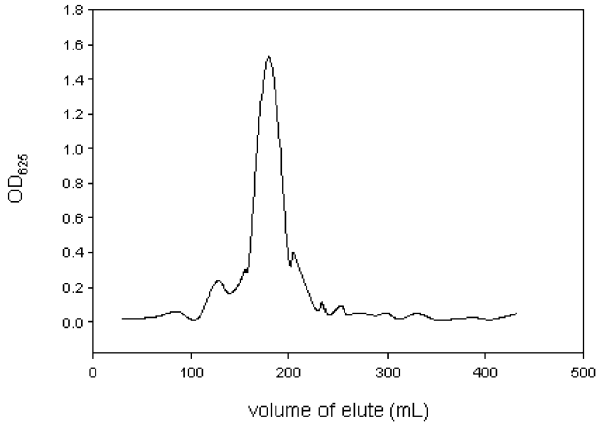


Figure 1. Gel filtration chromatography of Reishi extract using Sephacryl S-500 column (fraction 1: 100–130 mL; fraction 2: 130–155 mL; fraction 3: 155–205 mL; fraction 4: 205–220 mL; fraction 5: 220–255 mL).

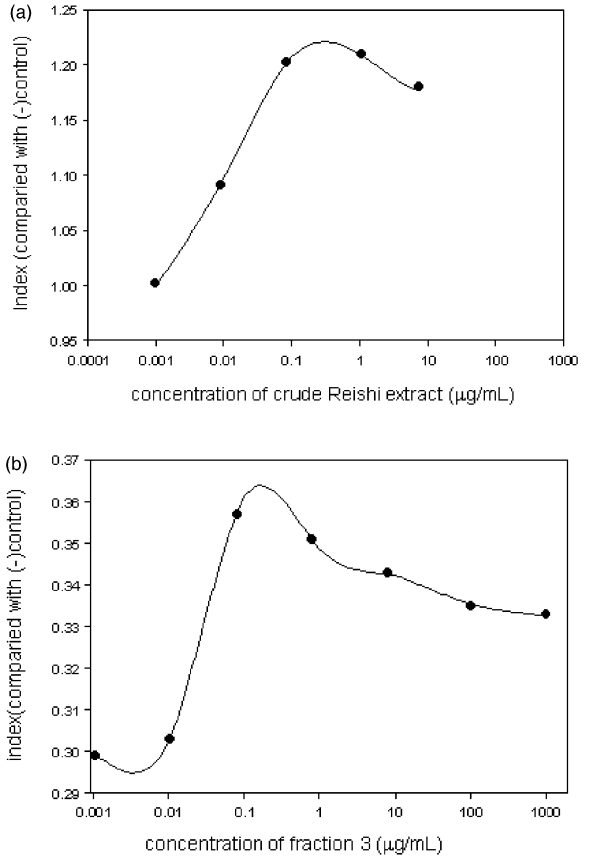


Figure 2. Colorimetric assay (MTT) for Con A-stimulated cell proliferation: (A) crude Reishi extract; (B) fraction 3.

analysis of fraction 3 was further carried out, and it was found that the relative composition is similar to that of the crude extract (Table 3), except that the galactose content increases.

RT-PCR experiments were next used to identify the cytokines expressed in mouse spleen cells after treatment with fraction 3 and the crude extract. Among six cytokines tested—IL-1, IL-2, IFN-γ, TNF-α, IL-4 and IL-6—the first three were observed to express significantly in the presence of fraction 3 (10 μg/mL) when compared to the expression of a house keeping gene (hypoxanthine phosphoribosyltransferase), as shown in Figure 3. On the other hand, none of the cytokines was detected at the same concentration of crude Reishi extract. This result suggests that the active component of Reishi is highly located in fraction 3, and the activity is perhaps related to the activation of immune cells. In addition, the high-level expression of IFN-γ induced by fraction 3 suggests that fraction 3 may activate NK cells.

Moreover, treatment of mouse spleen cells with fraction 3 (10 μg/mL) resulted in a significant change of the proteome. Figure 4a (no treatment, control) and Figure 4b (treatment with fraction 3) show 623 and 568 detectable spots in the 2-D electrophoresis, respectively. Further analysis indicated that ~191 spots disappear after the treatment (Fig. 4c) and 137 spots appear after the treatment (Fig. 4d). The data-imaging comparison demonstrated that there is ~50% change in the proteome. Overall, there are 431 spots matched before and after treatment, and 137 spots (24.1%) not matched.

To further understand the composition and activity of fraction 3, it was treated with protease K to partially destroy the protein component. The result showed that proliferation of Con A-stimulated spleen cells remained the same. Glycolytic cleavage by α1,2-fucosidase, however, abolished the activity of fraction 3 completely (based on MTT assay). In contrast, the activity of fraction 3 was slightly reduced after treatment with α1,3/4-fucosidase. This experiment establishes that the active component is a polysaccharide or glycopeptide fraction containing terminal fucose residues with α1,2-fucosidic linkages. Overall, as shown in Scheme 2, the main active component is a glycoprotein fraction containing essential terminal fucose residues with α1,2-linkages. The protein moiety is not required for the activity.

Although the work described here represents a preliminary study, it provides new information regarding the active principles of Ganoderma currently used in humans. The identification of a glycoconjugate fraction, which contains fucose residues in the α-1,2-linkage essential for the activities provides a new direction for future study. Whether the activities originate from a single fuco-conjugate molecule or its mixture remains to be investigated. Work is in progress to determine the minimal epitope of the fuco-saccharide and to identify the receptor for structure–activity relationship study.

Table 3. Carbohydrate compositions of fraction 3

Sugar components	Percentage (%)
D-Glucose	58.1
D-Mannose	15.1
L-Fucose	7.1
D-Galactose	13.5
D-Xylose	3.1
D-GlcNAc	1.2
L-Rhamnose	0.7

Experimental

Materials

Crude Reishi extract (prepared via alkaline extraction (0.1 N NaOH), neutralization and ethanol precipitation) was obtained from Pharmanex Co. (CA, USA). Immobiline DryStrip [pH 3–10 NL (nonlinear), 18 cm] and IPG buffer (pH 3–10 NL) were purchased from Amershan Pharmacia Biotech (Uppsala, Sweden). CHAPS, Tris buffer, agarose, iodoacetamide and α-cyano-4-hydroxycinnamic acid were from Sigma Co. (St. Louis, MO, USA); dithioerythreitol (DTE) was from Merck Co. (Darmstast, Germany); acrylamide, ammonium persulfate (APS) and TEMED were from Bio-Rad (Hercules, CA, USA); sodium dodecyl sulfate (SDS) and glycine were from Fluka (Buchs, Switzerland); sequencing grade trypsin was from Promega (Madison, WI, USA).

Purification of Reishi extract

Twenty-eight mg of the crude extract were dissolved in 2 mL of Tris buffer (pH 7.0, 0.1 N) and centrifuged to remove the insoluble materials (7 mg). The supernatant was purified by gel filtration chromatography using a Sephacryl S-500 column (100×1.6 cm) with 0.1 N Tris buffer (pH 7.0) as the eluent. The flow rate was set at 0.5 mL/min, and 7.5 mL per tube was collected. After the chromatography, each fraction was subjected to anthrone analysis to detect sugar components. Five fractions were collected (fractions 1–5), each dialyzed to remove excessive salt and lyophilized to give 1.0, 6.2, 5.3, 2.1, and less than 1 mg, respectively.

Anthrone colorimetric method[6]

Each 1.5 mL of anthrone (9,10-dihydro-9-oxoanthracene) solution (0.2 g anthrone dissolved in 100 mL of concd sulfuric acid) in a series of test tubes immersed in an ice water bath was carefully overlayed with 1.5 mL of sample (20–40 μg/mL of D-glucose or equivalent). After all additions had been made, the tubes were shaken rapidly and then replaced in an ice water bath. The tubes were heated for 5 min in a boiling water bath and then cooled; the optical densities were read within an hour at 625 nm against distilled water. Standards, reagent blanks and unknowns were run in triplicate because of likely contamination by other carbohydrate sources. Calculations were made on the basis that the optical densities are directly propotional to the carbohydrate concentration.

Mitogen-induced proliferation of spleen cells and colorimetric MTT assay[7]

Whole spleen cells were harvested from BALB/c male mice (6 weeks old), suspended in RPMI-1640 medium containing 10% FCS (fetal calf serum), and centrifuged to remove the supernatant. The collected precipitated cells were first suspended in 1 mL of RBC lysis buffer (8% NH_4Cl), then 14 mL more of the same lysis buffer were added to destroy red blood cells. After 1 min, the solution was diluted with 15 mL RPMI-1640 medium to stop the reaction, centrifuged to collect the cells, and adjusted the cell final concentration to 2×10^6 cells/mL with RPMI-1640 medium. Concanavalin A (Con A, final concn: 1 μg/mL) was added to the resulting mixture. The cells were incubated with or without a Reishi extract (or partially purified fraction) in 96-well ELISA plates at 37 °C with 5% CO_2 for 72 h. The cell proliferation was measured based on the MTT assay.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was dissolved in phosphate buffered saline (PBS) at 5 mg/mL and filtered to sterilize and remove a small amount of insoluble residue present in some batches of MTT. At the times indicated below, the MTT solution (25 μL) was added to each well, and plates were incubated at 37 °C for 4 h. Acid-isopropanol (100 μL of 0.04 N HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on a Perkin-Elmer ELISA reader (HTS 7000 plus), using a test wavelength of 570 nm, a reference wavelength of 620 nm. Plates were normally read within 1 h after the addition of isopropanol.

Reverse transcription (RT) and polymerase chain reaction (PCR)[8]

Mouse spleen cells were aseptically removed from healthy mice (BALB/c male mice, 6 weeks old), adjusted to an ideal cell concentration (4×10^6 cells/mL) and incubated in RPMI-1640 medium containing 10% of FCS (fetal calf serum) at 37 °C with 5% CO_2. After 6 h, the

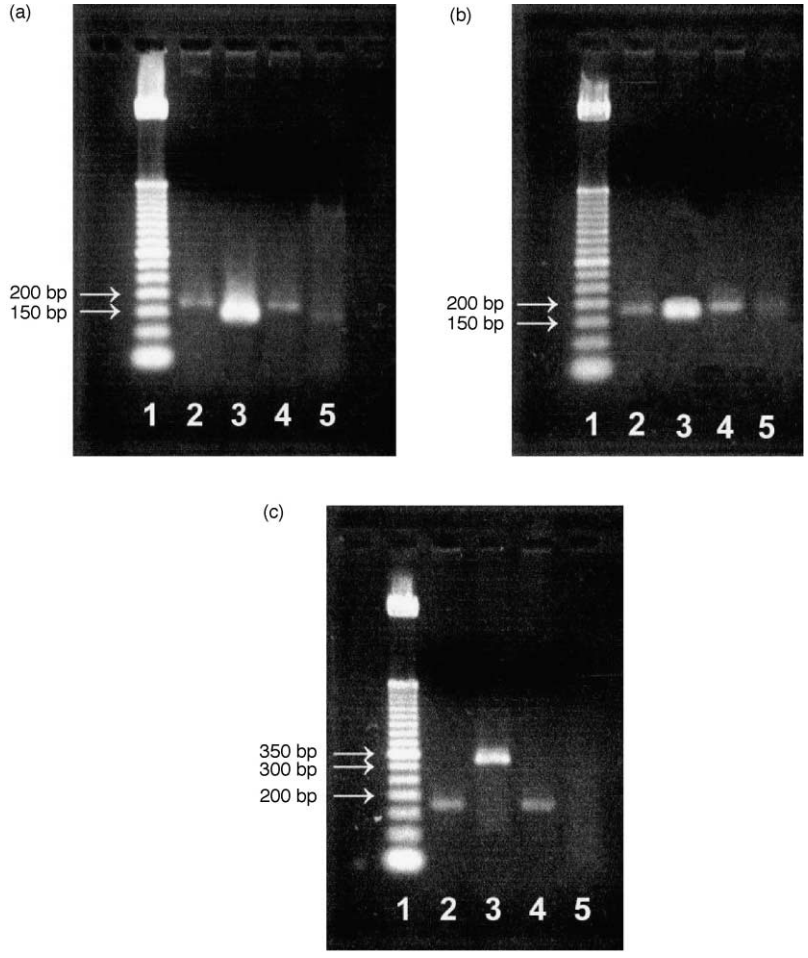


Figure 3. Gel electrophoresis of the RT-PCR experiments to detect the expression of (a) IL-1, (b) IL-2, and (c) IFN-γ when the spleen cells were incubated with fraction-3. (a) Column 1, molecular weight marker (50 bp ladder); column 2, house keeping gene (200 bp) in the presence of fraction 3; column 3, IL-1 expression (152 bp) in the presence of fraction 3; column 4, house keeping gene in the absence of fraction 3; column 5, no obvious IL-1 expression in the absence of fraction 3. (b) Column 1, molecular weight marker (50 bp ladder); column 2, house keeping gene (200 bp) in the presence of fraction 3; column 3, IL-2 expression (167 bp) in the presence of fraction 3; column 4, house keeping gene in the absence of fraction 3; column 5, no obvious IL-2 expression in the absence of fraction 3. (c) Column 1, molecular weight marker (50 bp ladder); column 2, house keeping gene (200 bp) in the presence of fraction 3; column 3, IFN-γ expression (336 bp) in the presence of fraction 3; column 4, house keeping gene in the absence of fraction 3; column 5, no obvious IFN-γ expression in the absence of fraction 3.

cells were subjected to RNA extraction using Qiagen RNAeasy mini kit to obtain ~1 μg of the desired RNA. Reverse transcription (RT) was performed using the Thermoscript R/T PCR System, and the Thermoscript system protocol I, from Gibco BRL. The reaction was carried out as follows: 8 μL of RNA, 2 μL of primer [Oligo(dT_{20})], 2 μL of 10 mM dNTP Mix, and DEPC H_2O (0.1% diethylpyrocarbonate-treated H_2O) was added to each tube, which was then incubated at 65 °C for 5 min and immediately put on ice. The following solutions were added to each tube as a 8 μL mixture: 4 μL of 5×cDNA buffer, 1 μL of 0.1 M dithiothreitol (DTT), 1 μL of RNaseOut (a ribonuclease inhibitor) and 1 μL of Thermoscript R/T, and 1 μL of DEPC water. The mixture was incubated at room temperature for 10 min and then 55 °C for 30 min to allow first strand of cDNA synthesis. Enzyme activity was terminated by incubating the reactions at 85 °C for 5 min and the tubes were then placed on ice for 10 min. The samples were stored at −20 °C until used for PCR.

Each sample (3 μL) was added to each reaction tube and the following reagents were added as a 47 μL mix: 5 μL of 10×PCR buffer, 4 μL of 10 mM dNTP Mix, 2 μL of each primer (10 OD/mL, sense and anti-sense), 33 μL of DEPC H_2O, and 1 μL of ProZyme® (DNA polymerase, from PROtech Technology). The reaction tubes were placed in a Strategene PCR Robocycler (Gradient 96) and run under the following condition: 1 cycle at 92 °C for 2 min (initial deneturation), then 30 consecutive cycles of 91 °C for 10 s (denaturation), 59 °C for 25 s (primer annealing) and 72 °C for 25 s (primer extension). The reactions were analyzed by gel electrophoresis.

Sugar composition analysis—TMS method

For monosaccharide analysis, the polysaccharide extracts/fractions were methanolyzed with 0.5 M methanolic-HCl (Supelco) at 80 °C for 16 h, re-*N*-acetylated with 500 μL of methanol, 10 μL of pyridine and 50 μL of acetic anhydride, and then treated with the Sylon HTP® trimethylsilylating reagent (Supelco) for 20 min at room temperature, dried and redissolved in hexane. GC–MS analysis of the trimethylsilylated derivatives was carried out using a Hewlett-Packard (HP) Gas Chromatograph 6890 connected to a HP 5973 Mass Selective Detector. Samples were dissolved in hexane prior to splitless injection into a HP-5MS fused silica capillary column (30 m×0.25 mm I.D., HP). The column head pressure was maintained at around 8.2 psi

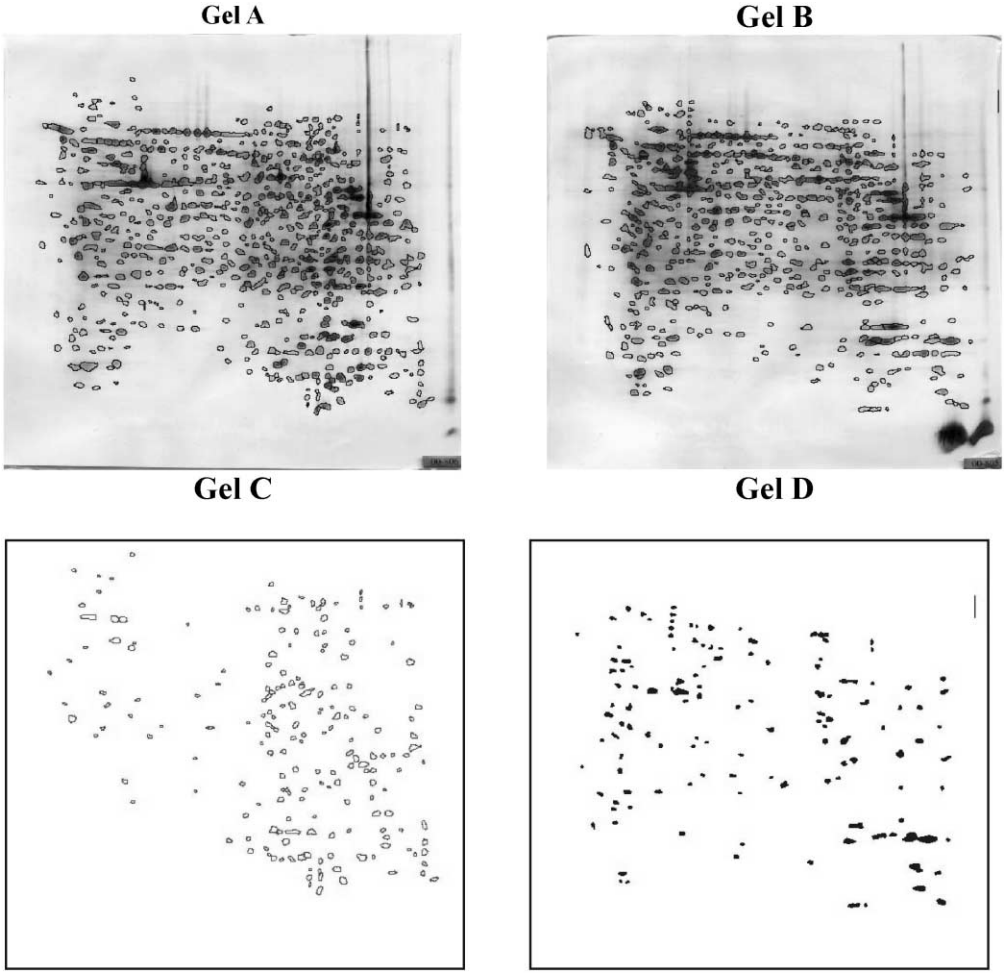
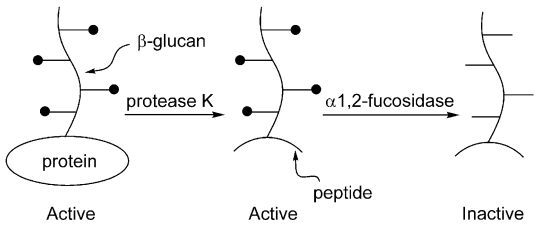


Figure 4. Proteomic analysis of the mouse spleen cells treated with fraction 3. Gel A: cells with no treatment; gel B: cells treated with fraction 3; gel C: disappearing spots when gel A is compared with gel B; gel D: appearing spots when gel A is compared with gel B.



Scheme 2. The proposed model to interpret the relationship between enzymatic treatment and bioactivity.

to give a constant flow rate of 1 mL/min using helium as carrier gas. Initial oven temperature was held at 60 °C for 1 min, increased to 140 °C at 25 C/min, to 250 °C at 5 °C/min, and then increased to 300 °C at 10 °C/min.

Amino acid composition analysis

The analysis was carried out based on a well-established method.[9] A sample of crude Reishi extract (6 mg) was dissolved in 1 mL solution of 6 M HCl and TFA (4/1), and heated at 140 °C for 3 h. The mixture was concentrated to give a dry residue and dissolved in 100 μL citrate buffer. A small aliquot (4 μL) was withdrawn and subjected to composition analysis by amino acid analyzer (Jeol JLC-6AH).

Sample preparation for proteomic studies

Reishi extract-treated mouse spleen cells were lysed in 350 μL of lysis buffer containing 8 M Urea, 2% CHAPS, 65 mM DTE, 2% v/v isocratic pH gradient (IPG) buffer pH 3–10 NL (non-linear), and a trace of bromophenol blue. The sample was centrifuged for 10 min at 13,000 rpm. The total protein concentration in the sample was measured using Bio-Rad protein concentration assay kit. Samples equal to 500 μg of proteins were loaded on immobilized pH gradient strips (pH 3–10 NL, 18 cm) for 2-dimensional electrophoresis.

2-Dimensional electrophoresis and image processing

The separations were performed as described by Hochstrasser et al.[10] The isoelectric focusing was carried out in an IPGPhor apparatus (Amersham Pharmacia Biotech). The second dimension was done in 10–15% polyacrylamide gradient gels using the Protean II ×L 2D multi cell (Bio-Rad). Protein spots were stained with fluorescence dye Sypro Ruby™ (Molecular Probes).

Sypro Ruby-stained gels were scanned with fluorescence laser scanner (Bio-Rad) generating 10 Mb image. The images were analyzed with ImageMaster™ software (Amersham Pharmacia Biotech). For each gel the spots were detected and quantified automatically, using default spot detection parameters. Manual spot editing was performed in agreement with the visual inspection of the gels. The relative volume was calculated in order to correct any differences in protein loading and gel staining.

MALDI-TOF MS analysis

Sypro Ruby-stained protein spots were cut from the gel and washed with 200 μL of 50 mM amoniun bicarbonate, pH 8.5, buffer in 50% CH_3CN. Following dehydration in CH_3CN and speed vacuum centrifugation, the gel pieces were swollen in a digestion buffer containing 100 mM amoniun bicarbonate, pH 8.5, 1 mM $CaCl_2$, 10% CH_3CN and 50 ng of sequencing grade trypsin. The resulting peptides were extracted with 50% CH_3CN/5% TFA after overnight digestion. A 1 μL aliquot of peptide mixture was deposited on the MALDI target 96-well plate and after a few seconds 1 μL of a matrix solution (α-cyano-4-hydroxycinnamic acid in 50% CH_3CN/0.1% TFA) was added. The mixture was allowed to dry at ambient temperature. Positive-ion mass spectrum was measured on a MALDI reflection time-of-flight mass spectrometer MALDI (Micromass UK, Manchester, UK) equipped with a nitrogen laser. The reported spectra were accumulated from 50 to 100 laser shots.

General procedure of fucosidase treatment

A sample of 10 mg of Reishi extract or fraction 3 in 50 mM citrate buffer (pH 6.0) was treated with α1,2- or α1,3/4-fucosidase (5 Unit) at 37 °C for a period of time (2–12 h). The mixture was heated in boiling water for 5 min to destroy the enzyme activity, dialyzed against H_2O at 4 °C, and lyophilized to give a dry powder for activity studies.

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