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FULL COMMUNICATION

The Polar High Molecular Weight Fraction of the *Agaricus blazei* Murill Extract, AndoSan™, Reduces the Activity of the Tumor-Associated Protease, Legumain, in RAW 264.7 Cells

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ABSTRACT AndoSan™ is an extract of *Agaricus blazei* Murill (AbM; 82.4%), *Hericium erinaceum* (14.7%), and *Grifola frondosa* (2.9%). The main ingredient of AndoSan, AbM, is rich in different forms of β-glucans. Since these exhibit potent antitumor activity and have immunomodulatory effects, the stimulatory effect of AndoSan on the production of different cytokines, chemokines, and leukocyte growth factors has predominantly been attributed to β-glucans. AndoSan has been claimed to consist of 90% carbohydrate, of which 2.8% is β-glucans, but in this study, we show that the carbohydrate content is only 2% of the dry weight, corresponding to 0.09% β-glucan per mL of AndoSan. Fractionation of AndoSan, followed by carbohydrate analysis and HPLC analysis revealed that most of the glucose was concentrated in the polar high molecular weight fraction of AndoSan (ethanol insoluble water extract [EIWE]-A) and that this extract was able to significantly inhibit the activity of the tumor-associated protease, legumain, in RAW 264.7 cells. Legumain is synthesized as a zymogen and undergoes pH-dependent autoactivation of the proform to reach an enzymatically active form. In this study, we demonstrate that both the polar and nonpolar AndoSan fractions are able to inhibit the autoactivation of prolegumain, and that the polar fractions of AndoSan are the most potent inhibitors of the active form of the enzyme.

KEY WORDS: • Agaricus brasiliensis • *legumain* • *RAW 264.7*

INTRODUCTION

THE *AGARICUS BLAZEI* MURILL (AbM) mushroom, a member of the *Bacidiomycota* phylum, grows in the wild in the coastal Piedade area outside of São Paulo, Brazil, and has been part of the regular diet in this region. Interestingly, the frequency of serious diseases such as cancer, atherosclerosis, hepatitis, hyperlipidemia, and diabetes was lower in Piedade than in the neighboring regions, supposedly due to the AbM intake.[1] Because of its alleged health effects, the mushroom was brought to Japan in the mid 60's and subjected to medical research. AbM was found to be particularly rich in different forms of β-glucans, such as β-(1→3)-, β-(1→4)-, and β-(1→6)-D-glucans.[2,3] These glucans, which are an integral part of the cell wall of mushrooms, exhibit potent antitumor activity in mouse models and cancer cell cultures,[4–6] and have immunomodulatory effects on monocytes, macrophages, and natural killer (NK) cells.[7–9]

The stimulatory effect of AndoSan™ (an extract of AbM [82.4%], *Hericium erinaceum* [14.7%], and *Grifola frondosa* [2.9%] on the production of IL-1β, IL-6, IL-8, TNF-α, G-CSF, and MIP-1β in monocyte-derived dendritic cells was demonstrated by Førland *et al.*[10] Furthermore, it has been shown that AndoSan stimulation of whole blood *ex vivo* induced release of 17 different cytokines, chemokines, and leukocyte growth factors.[11] *In vivo* studies have demonstrated an immunological stabilizing and anti-inflammatory effect of AndoSan both in healthy volunteers[11] and in patients with inflammatory bowel disease,[12] when given orally. In addition, studies in a mouse model for allergy demonstrated that this particular AbM extract may prevent allergy development and be used as a therapeutical substance against established allergy.[13] Although AndoSan is well studied with regard to bioactivity, little is known about the composition of the extract and which parts of the extract that is linked to the observed activities. In the present study, we extracted lyophilized AndoSan according to increasing polarity and subjected the isolated fractions to a macrophage cell line for the determination of its effect on lysosomal legumain activity.

Legumain (asparaginyl endopeptidase) is a proteolytic enzyme that is prominently expressed in mammalian tissues such as kidney, placenta, and spleen.[14] Legumain knockout mice are born healthy and fertile, but show reduced body weight, extramedullary hematopoiesis in the spleen, and aberrant endolysosomes with the development of kidney

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failure.[15,16] Several studies have reported high levels of legumain in solid tumors, which has been correlated to enhanced tumor invasion and metastasis.[17,18] This may partially be explained by the contribution of legumain in the activation of proMMP-2 and processing of cathepsins.[19,20] Also, legumain has been observed on the cell surface of tumor cells[14] and shown to degrade fibronectin, a major component of extracellular matrix. This suggests that legumain itself may play a direct role in extracellular matrix turnover in various pathological conditions such as tumor growth and metastasis, as well as in atherosclerosis.[21] Other known biological functions of legumain include processing of exogenous antigens within the class II MHC pathway,[22] maturation in Toll-like receptor signaling,[23] and autophagic-lysosomal processing of hepatocellular proteins.[24]

Legumain was first discovered in beans[25] before the mammalian version was described by Chen *et al*.[26] The autoactivation of the 433 amino acid prolegumain is highly pH-dependent and occurs normally at pH 3.5 to 4.5. The active enzyme has a strong specificity for hydrolysis of peptides C terminally to asparagine (Asn), but under certain conditions, hydrolysis after aspartic acid (Asp) also occurs.[27,28] We recently showed that at pH 4.0 the autoactivation is accelerated by glycosaminoglycans (GAGs), as well as the polyanionic polysaccharide, alginate, and in the presence of certain GAGs and alginates the autoactivation also occurs at pH 5.0 and 5.5.[29,30] The most potent endogenous inhibitors of legumain are cystatin E/M and cystatin C,[31,32] whereas the classic chemical inhibitor of cysteine proteases, E64, does not affect legumain activity.[26]

Even though animal models have demonstrated antitumor and anti-infective properties of β-glucans from AbM fruiting bodies,[9] little is known about the nature of the active components present in the extract prepared from mushroom mycelium. In this study, we show that the polar high molecular weight fraction of AndoSan has an inhibitory effect on legumain activity in RAW 264.7 cells.

MATERIALS AND METHODS

Extraction

AndoSan was kindly provided by ImmunoPharma AS (Oslo, Norway). The brownish-colored product was a fermented extract that was composed of 82.4% AbM (jap.: Himematsutake), 14.7% from *H. erinaceum* (Yamabushitake),[33] and 2.9% from *G. frondosa* (Maitake).[34] The lipopolysaccharide (LPS) content of AndoSan was found, using the Limulus amebocyte lysate test (COA-MATIC Chroma-LAL; Chromogenix, Falmouth, MA, USA) with a detection limit 0.005 EU/mL (1 EU = 0.1 ng/mL) to be a minuscule concentration of < 0.05 pg/mL.[11] Lyophilized AndoSan (30 g) was extracted (see flow diagram Fig. 1) with dichloromethane (3 × 200 mL) by gentle stirring for 3 h at ambient temperature and filtered for recovering the dichloromethane insoluble material (DIM). The dichloromethane soluble material was designated DSM. DIM was treated with methanol (5 × 200 mL), and both DSM and the methanol soluble material (MSM) were concentrated under reduced pressure and dried in air.

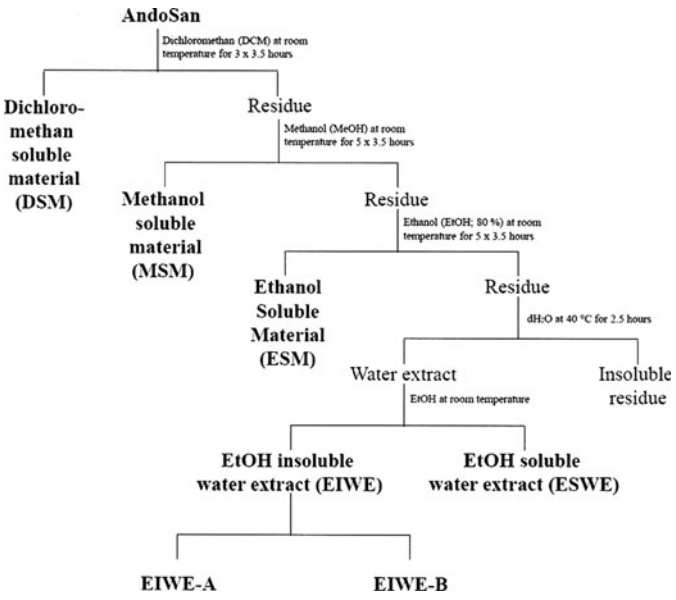

FIG. 1. Flowchart of the experimental setup.

The remaining methanol insoluble material was then treated with 80% ethanol (6 × 200 mL), concentrated under reduced pressure, air-dried and designated ethanol soluble material (ESM). The remaining ethanol insoluble material was dissolved in 500 mL dH_2O and incubated at 40°C for 2.5 h. High molecular components were then precipitated by adding 2 × sample volume 96% ethanol and left at 4°C overnight, then separated by centrifugation at 1750 *g* for 30 min. The precipitate and supernatant were designated ethanol insoluble water extract (EIWE) and ethanol soluble water extract (ESWE), respectively. EIWE was washed twice with ethanol, and ESWE and EIWE were dried free of ethanol before they were dissolved in 200 mL dH_2O and lyophilized. EIWE was further fractionated by size. Two fractions were obtained by size exclusion chromatography (SEC), as described below, and designated EIWE-A and EIWE-B.

Size exclusion chromatography

SEC was performed using a Sephacryl S100 HR 26/100 column (molecular weight [MW] fractionation range 1–100 kDa for globular proteins; Amersham Pharmacia Biotech Inc.) coupled to a P-50 Pump (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA), LKB Superfrac fraction collector (Amersham Pharmacia Biotech Inc.), and an RID-6A refractive index detector controlled by Chromeleon version 7.0 software. Samples (5 mL, dry powder dissolved in dH_2O to 4 mg/mL concentration) were injected, and the column was eluted with dH_2O (filtered through 45 μm filter, gassed with helium; 0.5 mL/min), at ambient temperature.

Size exclusion high-performance liquid chromatography

Size exclusion high-performance liquid chromatography (SEC-HPLC) was performed using a TSKgel G3000 PW_{XL} column (TOSHO Co., Inc., Tokyo, Japan) and a TSKgel PW_{XL} guard coupled to a PWLaChrom Elite L-2130 Pump

(Hitachi High Technologies America, Inc., Pleasanton, CA, USA), L-2200 autosampler and an L-2490 refractive index detector controlled with the EZChrom Elite software. The column was eluted with 50 mM Na_2SO_4 (0.5 mL/min) at ambient temperature, and samples (95 μL, dry powder dissolved in 50 mM Na_2SO_4 to concentration 1 mg/mL) were injected. Relative average molecular weights (MW) were estimated using oat β-glucan standards in the range MW 35.6–70.6 kDa (Megazyme International Ireland Ltd, Bray, Ireland) and Polymer Standard Service software (PSS WinGPC scientific V 6.20 GmbH, Mainz, Germany).

Carbohydrate characterization

The monosaccharide composition and carbohydrate content was determined by gas chromatography of the trimethylsilylated (TMS) derivatives of the methyl-glycosides obtained by methanolysis of the polymers using 3 M HCl in anhydrous methanol at 80°C for 24 h.[35] The TMS derivatives were analyzed by capillary gas chromatography on a Focus GC (Thermo Scientific, Milan, Italy). Mannitol was included as internal standard throughout the total procedure.

Linkage elucidation was performed by methylation analysis carried out after the method of Ciucanu and Kerek.[36] The methylation procedure was followed by GC-MS analysis of the derived partially methylated alditol acetates using a GCMS-QP2010 (Shimadzu, Kyoto, Japan) attached to a Restek Rxi-5MS (30 m; 0.25 mm i.d.; 0.25 μm film) column. The injector temperature was 280°C, the ion source temperature 200°C, and the interface temperature 300°C. The column temperature was 80°C when injected, then increased with 10°C/min to 140°C, followed by 4°C/min to 210°C, and then 20°C/min to 300°C. Helium was the carrier gas (pressure control: 80 kPa). The compound at each peak was characterized by an interpretation of the characteristic mass spectra and retention times in relation to standard sugar derivatives. Effective carbon-response factors were applied for quantification.[37]

Cell culturing and stimulation

The murine macrophage-like cell line, RAW 264.7 (American Type Culture Collection [ATCC], Rockville, MD, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Paisley, United Kingdom) supplemented with 10% fetal bovine serum gold, 1 mM sodium pyruvate (PAA Laboratories GmbH, Pasching, Austria), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cells were maintained at 37°C with 5% CO_2 in a humidified incubator. The cells were seeded in six-well plates at a density of 5×10^5 cells per well (2.5×10^5 cells/mL) 24 h before stimulation with unfractionated AndoSan, MSM, ESM, ESWE, EIWE, EIWE-A, or EIWE-B) with or without Polymyxin B (10 μg/mL) at various concentrations in serum-free media and harvested after 48 h.

Harvesting of cell lysates

Cell lysates were obtained by washing adherent cells in 1 × phosphate buffered saline before adding lysis buffer (100 mM sodium citrate, 1 mM disodium-EDTA, 1% n-octyl-β-D-glucopyranoside, pH 5.8). After three cycles of freezing (− 70°C) and thawing (30°C), the cell lysates were centrifuged at 10,000 *g* for 10 min, and the supernatants were frozen at − 70°C or applied directly to enzyme activity analyses. Total protein concentrations in cell lysates were measured by the procedure described by Bradford[38] and performed according to the manufacturer (Bio-Rad Laboratories, Hercules, CA, USA) in a microplate reader, Wallac Victor 3 (PerkinElmer, Waltham, MA, USA), measuring absorbance at 595 nm. Bovine serum albumin (0–400 μg/mL; Sigma-Aldrich) was used to establish a standard curve for the calculation of total protein concentrations in cell lysates. All measurements were performed in duplicate.

Active legumain and preparation and autoactivation of prolegumain

Active legumain isolated from bovine kidney[39] was kindly provided by Harald Thidemann Johansen, School of Pharmacy. Prolegumain (200 ng/mL) was obtained from conditioned media of HEK 293 cells stably transfected with full-length cDNA for human legumain in pcDNA3.1 vector (designated M38L cells) as described.[29,40] The concentration of secreted prolegumain was determined by enzyme-linked immunosorbent assay (ELISA) as described below. PD-10 Desalting Columns prepacked with Sephadex™ G-25 (GE HealthCare, Little Chalfont, United Kingdom) were used for buffer exchange of conditioned serum-free DMEM medium from M38L cells containing secreted prolegumain (200 ng/mL) to sodium acetate buffer pH 4.0 (200 mM sodium acetate and 4 mM Na-EDTA). Unfractionated AndoSan, MSM, ESM, ESWE, EIWE, EIWE-A, or EIWE-B were dissolved in 0.9% NaCl and active legumain or prolegumain was incubated at room temperature or 37°C, respectively, in the presence of the different fractions or 0.9% NaCl (control) before legumain activity measurements.

Enzyme-linked immunosorbent assay

Established ELISA procedures were performed to determine the concentrations of prolegumain (DY4769; R&D Systems, Minneapolis, MN USA) in conditioned media from M38L cells.

Legumain activity assay

The proteolytic activity of legumain was measured by cleavage of the legumain-specific fluorogenic substrate Z-Ala-Ala-Asn-AMC (Bachem AG, Bubendorf, Switzerland), as described.[41] In brief, samples (20 μL) were added to black 96-well microplates (Corning Life Science, Lowell, MA, USA), followed by 100 μL of legumain assay buffer pH 5.8 (1 mM DTT, 39.5 mM citric acid, 121 mM Na_2HPO_4, 1 mM Na_2EDTA, 0.01% CHAPS; Sigma Aldrich), and 50 μL of the substrate solution (final concentration 10 μM). Immediately after the addition of substrate, kinetic measurements based on the increase in fluorescence over 30 min was performed at 30°C in a plate reader Wallac Victor 3 (PerkinElmer, Waltham, Massachusetts, USA). Filters were 360 nm for excitation and 460 nm for emission.

Quenching refers to any process that decreases the fluorescence intensity of a given substance. A variety of processes can result in quenching and due to the brownish color of the material, the effect of quenching was taken under consideration. To correct for potential quenching by the colored extracts, a fluorescent group, N-AMC (Bachem AG), was added to the samples of unfractionated AndoSan or the different fractions before the fluorescence was measured in a plate reader Wallac Victor 3 (PerkinElmer).

Statistical analysis

Analysis of significant differences was performed by paired *t*-test using Minitab Version 16 Statistical Software (Minitab, Inc., State College, PA, USA). Differences were considered significant when $P < .05$. Spearman's rank order correlation was used to evaluate dose- or time-dependency and $P < .05$ was considered significant.

RESULTS

AndoSan fractionation

AndoSan was lyophilized with a yield of 4.5 mg dry material/mL. The lyophilized material appeared as a brown, finely powdered product, and it was extracted with solvents with increasing polarity. ESWE had the largest yield (31.5%) followed by ESM (24.5%), MSM (17.0%), EIWE (16.0%), and DSM (0.5%). The insoluble residue constituted 10.5%. The low yield of DCME prevented further experimental focus on this extract.

EIWE was further fractionated by size. Two fractions were obtained by SEC (Fig. 2A), and designated EIWE-A and EIWE-B. SEC-HPLC analysis of EIWE-A and EIWE-B (Fig. 2B) showed that both fractions contained components eluted after 17.6 min, which is in the lower molecular weight region of the column. Also, a small additional peak was detected at 12.2 min in EIWE-A, indicating the presence of higher molecular weight compounds present in this fraction. Relative average molecular weight (MW) was estimated to 50 kDa using oat β-glucan standards.

Carbohydrate analysis

As mentioned, β-glucans are assumed to be active components in AbM, the main mushroom from which AndoSan is extracted, and in the present study we focused on the analysis of carbohydrates in the different fractions of AndoSan. The carbohydrate contents and monosaccharide compositions are shown in Table 1. The lyophilized AndoSan contained 2% carbohydrate, corresponding to 0.09% mg β-glucan per mL

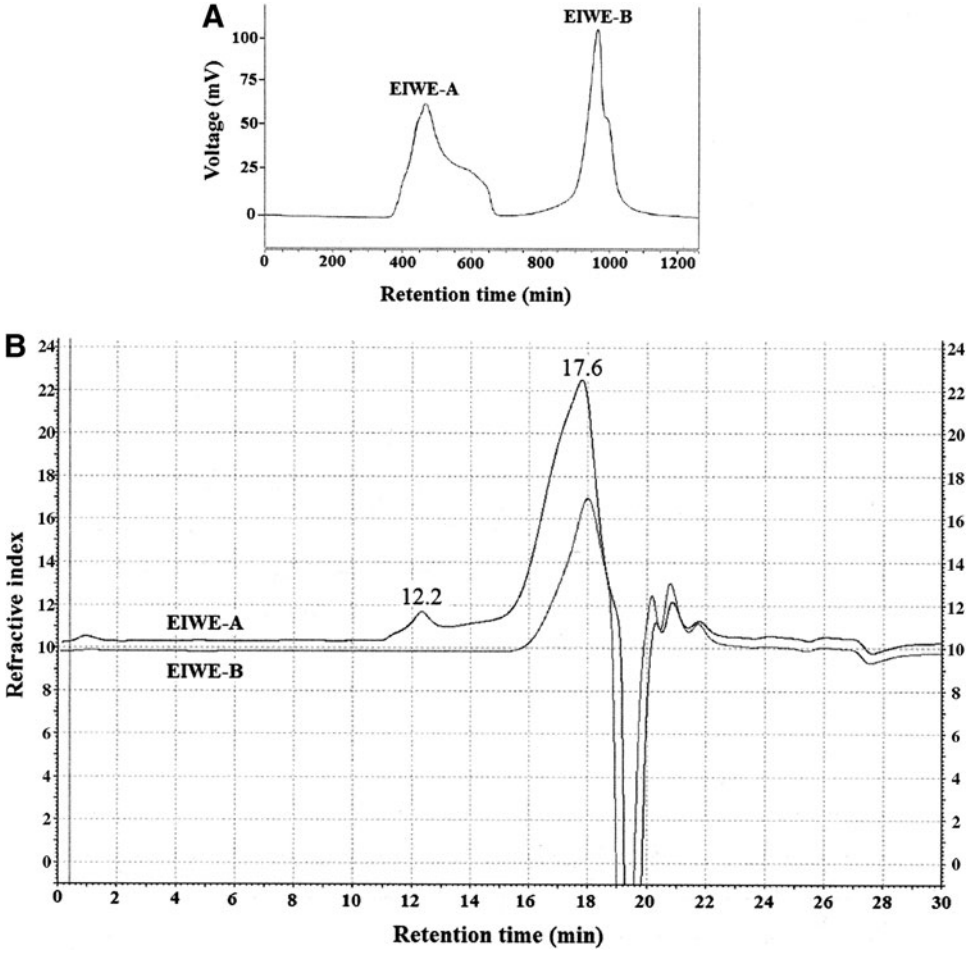

FIG. 2. Size exclusion chromatography (SEC) and size exclusion high-performance liquid chromatography (SEC-HPLC). **(A)** Separation of EIWE fractions by SEC using Sepharyl S-100 HR column. **(B)** SEC-HPLC analysis of EIWE-A and EIWE-B. EIWE, ethanol insoluble water extract.

Table 1. The Carbohydrate Content and Percentage Distribution of Monosaccharides in the Carbohydrates Detected in the Extracts

Name of fraction	*Unfractionated AndoSan™*	*MSM*	*ESM*	*ESWE*	*EIWE*	*EIWE-A*	*EIWE-B*
Carbohydrate content	2.0	5.7	1.6	1.7	6.3	9.7	0.4
Arabinose	19.4	11.1	31.4	19.4	10.6	9.2	Trace
Galactose	10.0	16.6	2.4	8.5	17.3	16.7	Trace
Galacturonic acid	8.3	13.2	0.3	Trace	11.6	12.6	Trace
Glucose	23.0	21.8	31.3	17.9	22.7	24.7	6.4
Glucuronic acid	Trace	2.1	8.3	0.6	3.0	n.d	n.d
Mannose	6.8	9.8	1.1	7.4	9.5	11.4	Trace
Rhamnose	6.8	6.6	6.1	8.0	6.2	5.4	n.d
Ribose	n.d	n.d.	Trace	Trace	n.d	n.d	n.d
Xylose	25.6	18.7	19.0	38.2	19.1	20.0	93.6

EIWE, ethanol insoluble water extract; ESM, ethanol soluble material; ESWE, ethanol soluble water extract; MSM, methanol soluble material; n.d., not detectable.

AndoSan, whereas MSM, ESM, ESWE, and EIWE contained 5.7%, 1.6%, 1.7%, and 6.3% carbohydrate, respectively. Due to limited solubility in methanol and ethanol, the carbohydrates detected in MSM and ESM were monosaccharides and disaccharides rather than polymers, and likewise in the ethanol soluble water extract (ESWE). The carbohydrates present in EIWE, on the other hand, were constituents of polymers (Table 2). EIWE contained the highest amount of glucose (23%). The carbohydrate contents of fractions EIWE-A and EIWE-B, were 9.7% and 0.4%, respectively (Table 1). Of total carbohydrate contents, EIWE-A contained 25% glucose, and HPLC analysis revealed that high molecular weight components were present in EIWE-A, rather than EIWE-B (Fig. 2B). Due to the high content of glucose in EIWE-A, glycosidic linkage analysis was performed on this fraction. Glucose appeared mainly as terminally-linked units (17% of total carbohydrate), but also 1→4-, 1→3-, 1→6-, and 1→3,6-linked glucose units were found, suggesting the presence of glucans (Table 2). Terminal xylose constituted about 11%, whereas the content of 1→3-linked xylose was 9% in addition to branching in O-2 and O-4. Terminally linked mannose was found in addition to galactose residues that were 1→6 and 1→2,6-linked, terminally linked, and 1→3-linked, indicating the presence of galactans. There were also detected a considerable content of terminal arabinose (9%), in addition to small amounts of terminal, 1→2-linked and 1→2,3-linked rhamnose.

Table 2. Binding Pattern of Carbohydrates in EIWE-A

	% of total carbohydrates
T-Ara*f*	9.2
T-Fuc*p*	Trace
1→3 Fuc*p*	Trace
T-Glc*p*	13.3
1→3 Glc*p*	2.0
1→4 Glc*p*	5.7
1→6 Glc*p*	3.3
1→3,6 Glc*p*	Trace
1→4,6 Glcp	Trace
T Gal*p*	8.3
1→3 Gal*p*	8.4
1→6 Gal*p*	Trace
1→2,6 Ga*lp*	Trace
1→2,3 Gal*p*	Trace
T Man*p*	11.4
T Rha*p*	2.0
1→2 Rha*p*	3.4
1→2,3 Rha*p*	Trace
T Xyl*p*	11.0
1→3 Xyl*p*	9.0
1→2,3 Xyl*p*	Trace
1→2,4 Xyl*p*	Trace

f, furanose; *p*, pyranose.

Effects of various fractions of AndoSan on legumain activity in RAW 264.7 cells

RAW 264.7 cells were incubated for 48 h in the presence of various concentrations (0.125, 0.25, 0.5, or 1.0 mg/mL) of unfractionated AndoSan, MSM, ESM, ESWE, or EIWE. Incubation with unfractionated AndoSan significantly reduced the legumain activity in a dose-dependent manner (rho = −0.778, $P < .01$) and by up to 80% of the control at the highest concentration ($P < .01$). ESWE at 1 mg/mL and EIWE at 0.25, 0.5, and 1 mg/mL also reduced the legumain activity ($P \leq .018$), but to a lesser extent than unfractionated AndoSan. ESWE showed weaker dose-dependent correlations (rho = −0.446 $P = .0138$) than AndoSan, whereas EIWE did not show statistically significant dose-dependent activity (rho = 0.0438, $P = .816$). Incubation with 0.5 mg/mL MSM resulted in a slightly increased legumain activity, compared to control ($P < .01$), whereas ESM and the remaining concentrations of the other fractions had no significant effect on the legumain activity level (Fig. 3A). The extract fraction exhibiting the most potent inhibition of legumain activity was EIWE, which was further fractionated into EIWE-A and EIWE-B, as described above. RAW 264.7 cells were incubated for 48 h in the presence of various concentrations (0.125, 0.25, or 0.5 mg/mL) of EIWE, EIWE-A, or EIWE-B. Incubation with EIWE-A gave significant dose-dependent reductions (rho = −0.782, $P < .01$) in legumain activity and significant reductions in legumain activity compared to the control ($P < .05$). Moreover, EIWE-B

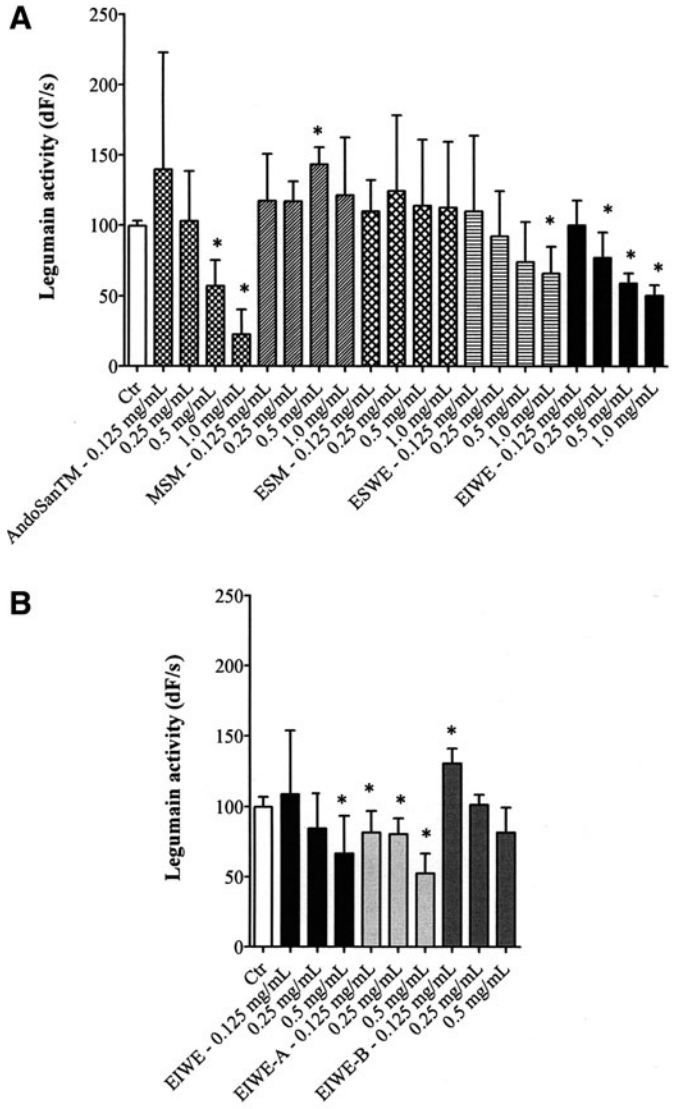

FIG. 3. Effects of various extracts of AndoSan™ on legumain activity in RAW 264.7 cells. RAW 264.7 cells were incubated for 48 h in the absence or presence of various concentrations of **(A)** unfractionated AndoSan, MSM, ESM, ESWE, or EIWE (0.125, 0.25, 0.5, or 1.0 mg/mL) and **(B)** EIWE, EIWE-A, or EIWE-B (0.125, 0.25, or 0.5 mg/mL). Legumain activity [change in fluorescence per second (dF/s)] in the various lysates was measured using a fluorogenic peptide substrate as described in the Experimental section and shown in percent of control. Parallel incubations were performed for each polysaccharide, and samples were run in duplicates. The data are given as mean ± SD of three separate experiments. Activity bars denoted * are significantly different from the control ($P < .05$) according to the paired *t*-test. ESM, ethanol soluble material; ESWE, ethanol soluble water extract; MSM, methanol soluble material.

did not show significant dose-dependent reduction in legumain activity (rho = −0.380, $P = .0663$), and measured effects were not significantly lower than the control; on the contrary, incubation with 0.125 mg/mL EIWE-B resulted in a slight increase in legumain activity ($P < .01$; Fig. 3B).

According to previous observations, LPS inhibits legumain activity in RAW 264.7 cells,[29] thus, all fractions were checked for LPS contamination by adding polymyxin B (10 μg/mL) to the cells in addition to the different fractions. No change in legumain activity was observed after polymyxin B treatment, indicating that the inhibiting effect was due to active components in AndoSan other than LPS (not shown).

Inhibition of active legumain by unfractionated and fractionated AndoSan

Legumain activity was measured after 30 min incubation of legumain in the presence of increasing concentrations (1.5, 3, 6, 12.5, 25, 50, 100, and 200 μg/mL in incubates) of unfractionated AndoSan, MSM (not shown), ESM (not shown), ESWE, EIWE, EIWE-A, or EIWE-B (Fig. 4). A reduction in legumain activity was observed for EIWE, EIWE-A, and EIWE-B at concentrations increasing from 6.25 to 200 μg/mL (and a maximal 69%, 53%, and 45% reduction, respectively, was observed at 200 μg/mL, compared to control. Whereas ESWE reduced the legumain activity with only 18% at 200 μg/mL, incubation with 200 μg/mL unfractionated AndoSan, ESM, or MSM resulted in a 34%, 19%, and 13% inhibition of legumain activity,

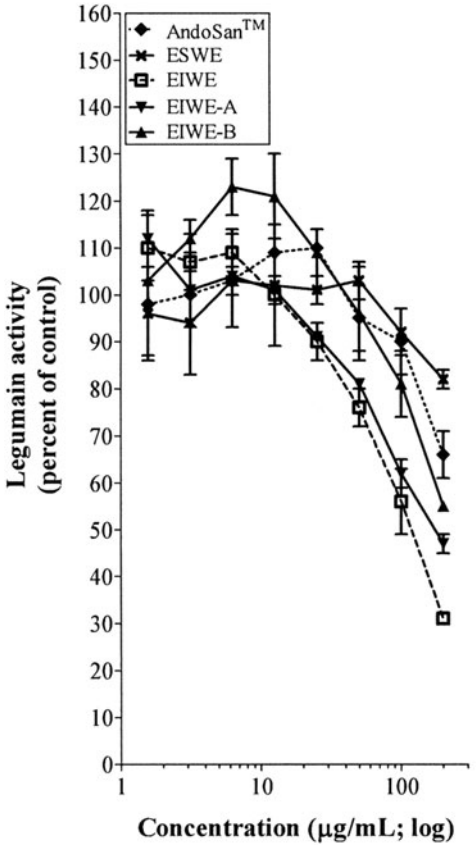

FIG. 4. Inhibition of active legumain by AndoSan. Active legumain was incubated for 30 min at pH 5.5 in the presence of increasing concentrations (1.5, 3, 6, 12.5, 25, 50, 100, 200 μg/mL in incubate) of AndoSan, MSM, ESM, ESWE, EIWE, EIWE-A, or EIWE-B. Legumain activity (dF/s) in the various incubates is shown in percent of control (absence of AndoSan). Samples were run in duplicates and results from one representative experiment are shown ($n = 2$).

respectively. ESWE, MSM, and ESM concentrations below 50 μg/mL and unfractionated AndoSan concentrations below 25 μg/mL had no effect on the legumain activity. Substitution of Z-Ala-Ala-Asn-AMC with a fluorescent group (N-AMC) demonstrated that the colored extracts did not provide quenching (not shown).

Inhibition of prolegumain autoactivation by unfractionated and fractionated AndoSan

Legumain activity was measured after 4 h incubation of prolegumain at pH 4.0 and 37°C in the presence of increasing concentrations (1.5, 3, 6, 12.5, 25, 50, 100, and 200 μg/mL in incubates) of unfractionated AndoSan, MSM, ESM, ESWE, EIWE, EIWE-A, or EIWE-B. A maximal 94%, 90%, and 83% reduction in legumain activity was registered for EIWE-B, EIWE, and EIWE-A, respectively at 25 μg/mL, compared to control. Further increase in EIWE and EIWE-B concentrations had no additional effect on the legumain activity level, whereas EIWE-A concentrations above 25 μg/mL resulted in a slight increase in legumain activity. A dose-dependent reduction in legumain activity was observed for ESWE, unfractionated AndoSan, ESM (not shown), and MSM (not shown) (97%, 93%, 84%, and 76% reduction, respectively, at 200 μg/mL, compared to control; Fig. 5 A). Furthermore, unfractionated AndoSan, MSM, ESM, ESWE, EIWE, EIWE-A, and EIWE-B (200 μg/mL in incubates) were found to directly inhibit prolegumain autoactivation in a time-dependent manner at pH 4.0 and 37°C (Fig. 5B). One hour incubation of prolegumain with unfractionated AndoSan, ESWE, ESM (not shown), EIWE-B, or EIWE resulted in a lower legumain activity (100%, 100%, 87%, 84%, and 49% reduction, respectively) compared to control. Furthermore, 2 h incubation with MSM (not shown) and 3 h incubation with EIWE-A resulted in a significantly lower ($P < .01$) legumain activity (51% and 37% reduction, respectively) compared to control. Further incubation up to 6 h resulted in minor additional effects on the activation levels compared to control.

DISCUSSION

Extracts of AbM is thought to exert their antitumor and anti-infective effects mainly through modulation of the immune system, and by promoting proinflammatory responses through macrophages, NK-cells, and neutrophils.[7,42–45] In this study, we show that in RAW 264.7 cells, the activity of the cancer-related cysteine protease legumain is reduced by the AbM-based extract AndoSan. By extracting AndoSan with solvents of increasing polarity, the fractions exhibiting the highest biological activities were identified. The precipitated water extract, EIWE-A, in which high molecular weight compounds such as carbohydrates and proteins were retained, was the most potent inhibitor of legumain activity. On the other hand, the alcohol extracts MSM and ESM and the ESWE showed no inhibiting effect.

Lyophilized AndoSan contained 2% carbohydrates, and was composed of glucose (23%), xylose (26%), arabinose (19%), galactose (10%), galacturonic acid (8%) mannose (7%), and rhamnose (7%). The monosaccharides detected correspond well with the analysis previously reported on the AbM mushroom,[46] and in addition, galacturonic acid was detected. The carbohydrates detected in AndoSan were mono- and oligosaccharides, which were extracted into MSM, ESM,

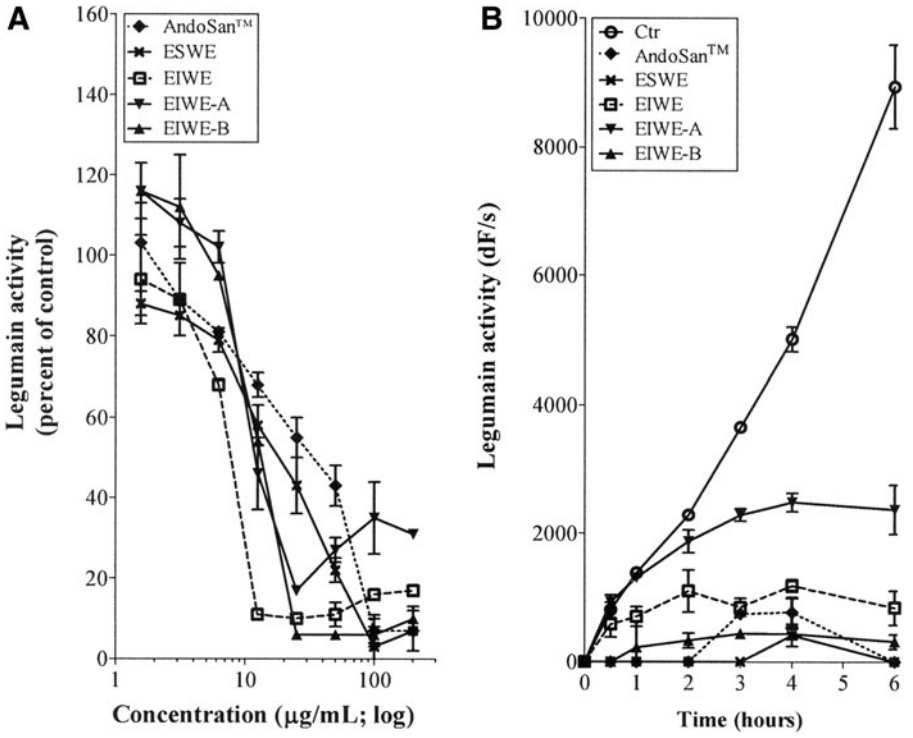

FIG. 5. Autoactivation of prolegumain is inhibited by AndoSan. **(A)** Prolegumain (200 ng/mL) was incubated at pH 4.0 and 37°C for 4 h in the absence (control) or presence of increasing concentrations (1.5, 3, 6, 12.5, 25, 50, 100, 200 μg/mL in incubate) of unfractionated AndoSan, ESWE, EIWE, EIWE-A, or EIWE-B. Legumain activity (dF/s) in the various incubates is shown in percent of control. Samples were run in duplicates and results from one representative experiment are shown ($n = 3$). **(B)** Prolegumain was incubated for up to 6 h at pH 4.0 at 37°C in the absence (control) or presence of unfractionated AndoSan, ESWE, EIWE, EIWE-A, or EIWE-B (200 μg/mL in incubate). Legumain activity (dF/s) in the various incubates is shown. Samples were run in duplicates and results from one representative experiment are shown ($n = 3$).

and partly also into the water extract, in addition to small amounts of larger polymers that were retained in the ethanol-precipitated 50 kDa extract EIWE-A.

As for AndoSan, the polymeric fraction EIWE-A appeared brownish in color, and ^{1}H-NMR analysis (not shown) indicated carbohydrates as the main component in addition to aliphatic structures, traces of aromatic compounds, and alkenes. The unidentified coextracted components in EIWE-A may be attributed to Maillard components as a result of the production process of AndoSan. Methanolysis showed that EIWE-A contained 10% carbohydrate of which 25% was glucose (Table 1). Methylation analysis revealed presence of 1→4-, 1→3-, and 1→6-linked glucose in addition to 1→3,6-, and terminally (1→)-linked residues. Previous studies have shown that AbM contains a 1→4 α-glucan,[7] a 1→4/1→6 α-glucan, a 1→6 β-glucan,[7,47,48] and 1→3 β-glucan,[49] and the linkages detected in EIWE-A are assumably fragments from these types of glucans, only found in lower amounts and molecular size than in the original mushroom due to degradation during the fermentation process. High amounts of terminally linked units indicate high degree of branching and/or low molecular weight. Additional carbohydrate polymers present may also contribute to the observed activity of legumain. The galacturonic acid and rhamnose residues detected may be parts of a pectin type polysaccharide, and arabinose and 1→3-linked galactans are often found as side chains in these structures.[50] A 1→3-linked xylan with side chains in position 2 and 3 was also detected in EIWE-A. Trace amounts of a 1→6-linked galactan with side chains in position 2 and terminally linked mannose can be attributed to the mannogalactan previously reported for AbM.[48]

Several studies have reported that AbM is rich in biological response modulators such as β-glucans,[48,51] thus, the inhibiting effect of AndoSan on legumain activity may partially be caused by β-glucans and recognized by pattern recognizing receptors such as the lectin-binding site for β-glucan in complement receptor 3 (CR3),[52,53] Toll-like receptor 2 (TLR-2),[54] and dectin-1 receptor[55,56] on RAW 264.7 cells. However, in the present study, we show that the carbohydrate content in AndoSan is only 2% of the dry weight and contains ∼0.1% glucans, which is in stark contrast to previous reports, which state that dry powder of AndoSan consists of 90% carbohydrate of which 2.8% is β-glucan.[11,13] The original mushroom fruiting body may contain 90% carbohydrate, but the AndoSan preparation is made of a mycelium extract. In addition, it should be noted that polysaccharides are prone to be broken down during the fermenting process, that is part of the AndoSan production, resulting in minor amounts of polysaccharides and proteins in the final extract, in addition to non-identifiable fermentation products. Thus, according to our results, the beneficial biological properties of AndoSan cannot be attributed to β-glucans alone. Other glycans detected may also contribute. Unidentified components present in EIWE-A corresponds to the low molecular weight SEC-HPLC peak observed in both fractions EIWE-A and EIWE-B (Fig. 2B). Since only EIWE-A, and not EIWE-B, showed significant inhibitory effect on RAW 264.7 cells, we conclude that the 50 kDa fraction of EIWE-A is responsible for the observed effect, which points toward polysaccharides and/or proteins. Trace amounts of protein components present need to be taken further into consideration. A few legumain inhibitors have been isolated from fungi, for example, clitocypin (MW 16.8 kDa) from *Clitocybe nebularis*[57] and macrocypin (MW 25 kDa) from *Macrolepiota procera*.[58] These mushrooms belong to the same order as AbM, namely the *Agaricales* and, therefore, AndoSan might contain cysteine protease inhibitors. This has so far not been confirmed, but is further studied in an ongoing project.

Legumain is predominantly a lysosomal enzyme. For active components to interact with the enzyme directly in the lysosomes, they may enter the cell through phagocytosis and/or macropinocytosis. Macropinocytosis, a type of endocytosis, is the invagination of the cell membrane to form and fill a pocket in a nonspecific manner, which then forms a vesicle that is 0.5–5.0 μm in diameter. The vesicle then travels into the cytosol and fuses with other vesicles such as endosomes and lysosomes.[59] Inside the lysosomes, the active components may interact directly with prolegumain or legumain. In this study, we show that both the autoactivation of prolegumain as well as the active form of the enzyme are inhibited by AndoSan. Both unfractionated AndoSan and all the different AndoSan fractions showed inhibiting effects on prolegumain autoactivation. Unfractionated AndoSan, EIWE, and EIWE-A were the most potent inhibitors of the active form of the enzyme. Based on these observations we conclude that the components inhibiting autoactivation of prolegumain were present in all fractions, whereas only AndoSan, EIWE, and EIWE-A contained carbohydrate polymers that inhibited active legumain directly at low concentrations. AndoSan and EIWE-A were also able to significantly inhibit legumain activity in the RAW 264.7 cells. The fact that the nonpolar fractions had no influence on legumain activity in RAW 264.7 cells indicates that the main mechanism by which AndoSan conducts its effect on these cells is by inhibiting the active form of the enzyme, rather than by inhibiting prolegumain autoactivation. Future studies with additional separation and compositional analysis may be performed to identify bioactive components in the 50 kDa fraction EIWE-A.

In conclusion, the carbohydrate contents of AndoSan is 2% of the dry weight, corresponding to 0.09% β-glucan per mL AndoSan, and most of the glucose is found in the most polar high molecular weight (∼50 kDa) fraction of AndoSan; EIWE-A. EIWE-A significantly inhibited the activity of the lysosomal protease legumain in RAW 264.7 macrophages. Both the polar and nonpolar fractions were able to inhibit prolegumain autoactivation, whereas the active form of the enzyme was most potently inhibited by the polar fractions EIWE, EIWE-A, and EIWE-B.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest, except for GH who is shareholder of ImmunoPharma AS.

REFERENCES

1. Wasser SP, Weis AL: Therapeutic effects of substances occurring in higher Basidiomycetes mushrooms: a modern perspective. *Crit Rev Immunol* 1999;19:65–96.
2. Kawagishi H, Inagaki R, Kanao T, *et al.*: Fractionation and antitumor activity of the water-insoluble residue of *Agaricus blazei* fruiting bodies. *Carbohydr Res* 1989;186:267–273.
3. Firenzuoli F, Gori L, Lombardo G: The medicinal mushroom *Agaricus blazei* murrill: review of literature and pharmacotoxicological problems. *Evid Based Complement Alternat Med* 2008;5:3–15.
4. Ebina T, Fujimiya Y: Antitumor effect of a peptide-glucan preparation extracted from *Agaricus blazei* in a double-grafted tumor system in mice. *Biotherapy* 1998;11:259–265.
5. Itoh H, Ito H, Amano H, Noda H: Inhibitory action of a (1— >6) -beta-D-glucan-protein complex (F III-2-b) isolated from *Agaricus blazei* Murill (''himematsutake'') on Meth A fibrosarcoma-bearing mice and its antitumor mechanism. *Jpn J Pharmacol* 1994;66:265–271.
6. Takaku T, Kimura Y, Okuda H: Isolation of an antitumor compound from *Agaricus blazei* Murill and its mechanism of action. *J Nutr* 2001;131:1409–1413.
7. Fujimiya Y, Suzuki Y, Oshiman K, *et al.*: Selective tumoricidal effect of soluble proteoglucan extracted from the basidiomycete, *Agaricus blazei* Murill, mediated via natural killer cell activation and apoptosis. *Cancer Immunol Immunother* 1998;46:147–159.
8. Hetland G, Sandven P: beta-1,3-Glucan reduces growth of Mycobacterium tuberculosis in macrophage cultures. *FEMS Immunol Med Microbiol* 2002;33:41–45.
9. Hetland G, Johnson E, Lyberg T, Bernardshaw S, Tryggestad AM, Grinde B: Effects of the medicinal mushroom *Agaricus blazei* Murill on immunity, infection and cancer. *Scand J Immunol* 2008;68:363–370.
10. Førland DT, Johnson E, Tryggestad AM, Lyberg T, Hetland G: An extract based on the medicinal mushroom *Agaricus blazei* Murill stimulates monocyte-derived dendritic cells to cytokine and chemokine production *in vitro. Cytokine* 2010;49:245–250.
11. Johnson E, Førland DT, Sætre L, Bernardshaw SV, Lyberg T, Hetland G: Effect of an extract based on the medicinal mushroom *Agaricus blazei* murill on release of cytokines, chemokines and leukocyte growth factors in human blood *ex vivo* and *in vivo. Scand J Immunol* 2009;69:242–250.
12. Førland DT, Johnson E, Sætre L, Lyberg T, Lygren I, Hetland G: Effect of an extract based on the medicinal mushroom *Agaricus blazei* Murill on expression of cytokines and calprotectin in patients with ulcerative colitis and Crohn's disease. *Scand J Immunol* 2011;73:66–75.
13. Ellertsen LK, Hetland G: An extract of the medicinal mushroom *Agaricus blazei* Murill can protect against allergy. *Clin Mol Allergy* 2009;7:6.
14. Liu C, Sun C, Huang H, Janda K, Edgington T: Overexpression of legumain in tumors is significant for invasion/metastasis and a candidate enzymatic target for prodrug therapy. *Cancer Res* 2003; 63:2957–2964.
15. Shirahama-Noda K, Yamamoto A, Sugihara K, *et al.*: Biosynthetic processing of cathepsins and lysosomal degradation are abolished in asparaginyl endopeptidase-deficient mice. *J Biol Chem* 2003;278:33194–33199.
16. Miller G, Matthews SP, Reinheckel T, Fleming S, Watts C: Asparagine endopeptidase is required for normal kidney physiology and homeostasis. *FASEB J* 2011;25:1606–1617.
17. Murthy RV, Arbman G, Gao J, Roodman GD, Sun XF: Legumain expression in relation to clinicopathologic and biological variables in colorectal cancer. *Clin Cancer Res* 2005;11:2293–2299.
18. Gawenda J, Traub F, Luck HJ, Kreipe H, von Wasielewski R: Legumain expression as a prognostic factor in breast cancer patients. *Breast Cancer Res Treat* 2007;102:1–6.
19. Chen JM, Fortunato M, Stevens RA, Barrett AJ: Activation of progelatinase A by mammalian legumain, a recently discovered cysteine proteinase. *Biol Chem* 2001;382:777–783.
20. Mai J, Finley RL Jr., Waisman DM, Sloane BF: Human procathepsin B interacts with the annexin II tetramer on the surface of tumor cells. *J Biol Chem* 2000;275:12806–12812.
21. Morita Y, Araki H, Sugimoto T, *et al.*: Legumain/asparaginyl endopeptidase controls extracellular matrix remodeling through the degradation of fibronectin in mouse renal proximal tubular cells. *FEBS Lett* 2007;581:1417–1424.
22. Manoury B, Hewitt EW, Morrice N, Dando PM, Barrett AJ, Watts C: An asparaginyl endopeptidase processes a microbial antigen for class II MHC presentation. *Nature* 1998;396:695–699.
23. Sepulveda FE, Maschalidi S, Colisson R, *et al.*: Critical role for asparagine endopeptidase in endocytic Toll-like receptor signaling in dendritic cells. *Immunity* 2009;31:737–748.
24. Øverbye A, Sætre F, Hagen LK, Johansen HT, Seglen PO: Autophagic activity measured in whole rat hepatocytes as the accumulation of a novel BHMT fragment (p10), generated in amphisomes by the asparaginyl proteinase, legumain. *Autophagy* 2011;7:1011–1027.
25. Kembhavi AA, Buttle DJ, Knight CG, Barrett AJ: The two cysteine endopeptidases of legume seeds: purification and characterization by use of specific fluorometric assays. *Arch Biochem Biophys* 1993;303:208–213.
26. Chen JM, Dando PM, Rawlings ND, *et al.*: Cloning, isolation, and characterization of mammalian legumain, an asparaginyl endopeptidase. *J Biol Chem* 1997;272:8090–8098.
27. Li DN, Matthews SP, Antoniou AN, Mazzeo D, Watts C: Multistep autoactivation of asparaginyl endopeptidase *in vitro* and *in vivo. J Biol Chem* 2003;278:38980–38990.
28. Halfon S, Patel S, Vega F, Zurawski S, Zurawski G: Autocatalytic activation of human legumain at aspartic acid residues. *FEBS Lett* 1998;438:114–118.
29. Berven L, Johansen HT, Solberg R, Kolset SO, Samuelsen ABC: Autoactivation of prolegumain is accelerated by glycosaminoglycans. *Biochimie* 2013;95:772–781.
30. Berven L, Solberg R, Truong HHT, *et al.*: Alginates induce legumain activity in RAW 264.7 cells and accelerate autoactivation of prolegumain. *Bioactive Carbohydr Dietary Fibre* 2013; 2:30–44.

31. Cheng T, Hitomi K, van Vlijmen-Willems IM, *et al.*: Cystatin M/E is a high affinity inhibitor of cathepsin V and cathepsin L by a reactive site that is distinct from the legumain-binding site. A novel clue for the role of cystatin M/E in epidermal cornification. *J Biol Chem* 2006;281:15893–15899.
32. Alvarez-Fernandez M, Barrett AJ, Gerhartz B, Dando PM, Ni J, Abrahamson M: Inhibition of mammalian legumain by some cystatins is due to a novel second reactive site. *J Biol Chem* 1999;274:19195–19203.
33. Adachi Y, Okazaki M, Ohno N, Yadomae T: Enhancement of cytokine production by macrophages stimulated with (1— >3)-beta-D-glucan, grifolan (GRN), isolated from *Grifola frondosa. Biol Pharm Bull* 1994;17:1554–1560.
34. Lee EW, Shizuki K, Hosokawa S, *et al.*: Two novel diterpenoids, erinacines H and I from the mycelia of *Hericium erinaceum. Biosci Biotechnol Biochem* 2000;64:2402–2405.
35. Chambers RE, Clamp JR: An assessment of methanolysis and other factors used in the analysis of carbohydrate-containing materials. *Biochem J* 1971;125:1009–1018.
36. Ciucanu I, Kerek F: A simple and rapid method for the permethylation of carbohydrates. *Carbohydr Res* 1984;131:209–217.
37. Sweet DP, Shapiro RH, Albersheim P: Quantitative analysis by various G.L.C. response-factor theories for partially methylated and partially ethylated alditol acetates. *Carbohydr Res* 1975;40:217–225.
38. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–254.
39. Yamane T, Takeuchi K, Yamamoto Y, *et al.*: Legumain from bovine kidney: its purification, molecular cloning, immunohistochemical localization and degradation of annexin II and vitamin D-binding protein. *Biochim Biophys Acta* 2002;1596:108–120.
40. Smith R, Johansen HT, Nilsen H, *et al.*: Intra- and extracellular regulation of activity and processing of legumain by cystatin E/M. *Biochimie* 2012;94:2590–2599.
41. Johansen HT, Knight CG, Barrett AJ: Colorimetric and fluorimetric microplate assays for legumain and a staining reaction for detection of the enzyme after electrophoresis. *Anal Biochem* 1999;273:278–283.
42. Bernardshaw S, Johnson E, Hetland G: An extract of the mushroom *Agaricus blazei* Murill administered orally protects against systemic Streptococcus pneumoniae infection in mice. *Scand J Immunol* 2005;62:393–398.
43. Bernardshaw S, Hetland G, Grinde B, Johnson E: An extract of the mushroom *Agaricus blazei* Murill protects against lethal septicemia in a mouse model of fecal peritonitis. *Shock* 2006; 25:420–425.
44. Fujimiya Y, Suzuki Y, Katakura R, Ebina T: Tumor-specific cytocidal and immunopotentiating effects of relatively low molecular weight products derived from the basidiomycete, *Agaricus blazei* Murill. *Anticancer Res* 1999;19:113–118.
45. Mizuno M, Minato K, Ito H, Kawade M, Terai H, Tsuchida H: Anti-tumor polysaccharide from the mycelium of liquid-cultured *Agaricus blazei* mill. *Biochem Mol Biol Int* 1999;47:707–714.
46. Kozarski M, Klaus A, Niksic M, Jakovljevic D, Helsper JPFG, Van Griensven LJ: Antioxidative and immunomodulating activities of polysaccharide extracts of the medicinal mushrooms *Agaricus brasiliensis, Ganoderma lucidum* and *Phellinus linteus. Food Chem* 2011;129:1667–1675.
47. Smiderle FR, Ruthes AC, van Arkel J, *et al.*: Polysaccharides from Agaricus bisporus and Agaricus brasiliensis show similarities in their structures and their immunomodulatory effects on human monocytic THP-1 cells. *BMC Complement Altern Med* 2011;11:58.
48. Smiderle FR, Alquini G, Tadra-Sfeir MZ, Iacomini M, Wichers HJ, Van Griensven LJ: Agaricus bisporus and Agaricus brasiliensis (1— >6)-beta-D-glucans show immunostimulatory activity on human THP-1 derived macrophages. *Carbohydr Polym* 2013;94:91–99.
49. Yu CH, Kan SF, Shu CH, Lu TJ, Sun-Hwang L, Wang PS: Inhibitory mechanisms of *Agaricus blazei* Murill on the growth of prostate cancer *in vitro* and *in vivo. J Nutr Biochem* 2009; 20:753–764.
50. Voragen AG, Coenen GJ, Verhoef RP, Schols HA: Pectin, a versatile polysaccharide present in plant cell walls. *Struct Chem* 2009;20:263–275.
51. Ohno N, Furukawa M, Miura NN, Adachi Y, Motoi M, Yadomae T: Antitumor beta glucan from the cultured fruit body of *Agaricus blazei. Biol Pharm Bull* 2001;24:820–828.
52. Czop JK, Valiante NM, Janusz MJ: Phagocytosis of particulate activators of the human alternative complement pathway through monocyte beta-glucan receptors. *Prog Clin Biol Res* 1989;297: 287–296.
53. Vetvicka V, Thornton BP, Ross GD: Soluble beta-glucan polysaccharide binding to the lectin site of neutrophil or natural killer cell complement receptor type 3 (CD11b/CD18) generates a primed state of the receptor capable of mediating cytotoxicity of iC3b-opsonized target cells. *J Clin Invest* 1996;98:50–61.
54. Roeder A, Kirschning CJ, Rupec RA, Schaller M, Weindl G, Korting HC: Toll-like receptors as key mediators in innate antifungal immunity. *Med Mycol* 2004;42:485–498.
55. Brown GD, Taylor PR, Reid DM, *et al.*: Dectin-1 is a major beta-glucan receptor on macrophages. *J Exp Med* 2002;196: 407–412.
56. Gantner BN, Simmons RM, Canavera SJ, Akira S, Underhill DM: Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J Exp Med* 2003;197:1107–1117.
57. Sabotic J, Galesa K, Popovic T, Leonardi A, Brzin J: Comparison of natural and recombinant clitocypins, the fungal cysteine protease inhibitors. *Protein Expr Purif* 2007;53:104–111.
58. Sabotic J, Popovic T, Puizdar V, Brzin J: Macrocypins, a family of cysteine protease inhibitors from the basidiomycete *Macrolepiota procera. FEBS J* 2009;276:4334–4345.
59. Falcone S, Cocucci E, Podini P, Kirchhausen T, Clementi E, Meldolesi J: Macropinocytosis: regulated coordination of endocytic and exocytic membrane traffic events. *J Cell Sci* 2006; 119(Pt 22):4758–4769.