Search for biologically active compounds in AndoSanTM, a medicinal mushroom extract

Pernille Kristensen Karppinen

Norwegian University of Life Sciences
Department of Chemistry, Biotechnology and Food Science
Master Thesis 60 credits 2012

Foreword

The work with this master thesis was for the most part performed at the University of Oslo, Department of Pharmaceutical Chemistry, but some work has been done at the Norwegian University of Life Sciences, Department of Chemistry, Biotechnology and Food Sciences.
It was carried out in the time period of December 2011 through November 2012. It constitutes 60 of 300 credits of the Master of Technology program in Chemistry and Biotechnology.

My main supervisor at the Department of Pharmaceutical Chemistry Pharmacognosy, Anne Berit C. Samuelsen, deserves many thanks for letting me perform most of the work at the University of Oslo and for fantastic help with everything from laboratory procedures to the process of writing this thesis. I would also like to thank Lise Berven for excellent technical and scientific guidance, and also for having such confidence in me. You have both been amazing.

Also at the University of Oslo, I would like to thank professor Karl Malterud and Helle Wangensteen for great help with NMR and chromatographic methods, and Harald T. Johansen for help with legumain assays. Hoai N.T. Aas and Sutahjini Yogarajah deserve many thanks for help with GC/GC-MS and general laboratory procedures, respectively. I also want to thank Leon Reubsaet for running LC-MS.

At the Norwegian University of Life Sciences I would like to thank Irene Comi for great technical assistance, and supervisors Gerd Vegarud and Tove Gulbrandsen Devold for the collaboration.

I would also like to thank both Hogne Viik at ImmunoPharma and Geir Hetland at Oslo University Hospital for the enthusiasm and constructive input.

Pernille Karppinen
Oslo, December 14th, 2012

Abstract

AndoSan™ is a commercial product currently being subject to research for its ability to repress multiple myeloma, colorectal cancer and inflammatory bowel disease. The product is a fermented aqueous extract of three medicinal mushrooms, where *Agaricus blazei* Murill (AbM) is the main component. Several papers describe the biological effects of AbM and claim that they are caused by fungal β-glucans and proteoglycans, but the exact elucidation of which chemical compounds are responsible for this biological effect is mainly unresolved. Another complicating factor arises with the fact that different parts of the mushroom used in research produce dissimilar extracts and thus makes it difficult to use published literature to state what components AndoSan™ consists of.

The aims of this thesis has therefore been to fractionate this product, test the fractions in a biological system using RAW264.7 cells and initiate the process of identifying biologically active compounds. The bioassay uses legumain, a cysteine protease, to monitor activity. Reduced activity is considered beneficial, due to legumain being overexpressed in several solid tumours. AndoSan™ was divided into five crude fractions based on solubility in different solvents (dichloromethane-, methanol-, ethanol- and ethanol soluble and insoluble water extracts), which were roughly characterized by ^{1}H NMR, SDS-PAGE, TLC, UV absorption and carbohydrate analyses.

The carbohydrate analyses showed that AndoSan™ contains only about 2 % carbohydrate, which is contradictory to what has been stated for pure AbM mushroom (pure mushroom powder contains approximately 90 % carbohydrate). This also indicates that the biological effects of AndoSan™ cannot solely be attributed to β-glucans.

^{1}H NMR was used to state which groups of chemical compounds the different extracts consisted of. The results indicated different compositions of the different extracts, of which the composition of the two alcohol extracts and the two water extracts resembled each other. All extracts contains a mixture of several groups of compounds, and thus further purifications are necessary to provide more detailed descriptions. TLC indicated highly polar components in the water extracts, and SDS-PAGE detected protein in the ethanol insoluble water extract (EIW).

The extracts of AndoSan™ were screened for inhibition of legumain activity in RAW264.7 cells, of which the EIW exhibited the greatest inhibitory activity. Pure AndoSan™ powder and EIW were also screened for their inhibition autoactivation of prolegumain. Results showed that

AndoSan™ and EIW are very potent inhibitors of the autoactivation of prolegumain (approximately 80 % inhibition at concentrations above 1 mg/mL), of which EIW seems to have somewhat higher potency than unfractionated AndoSan™ powder. In addition, pure AndoSan™ powder was incubated with active legumain, which resulted in 40 % enzyme inhibition at concentrations above 0.5 mg/mL.

EIW was chosen for further purification, and was separated into two fractions by SEC; EIW-A and EIW-B, of which EIW-A exhibited the greatest reduction of legumain activity in RAW264.7 cells. However, the inhibitory activity of EIW-A was not better than that of crude EIW, indicating synergistic or combined effects of the compounds in EIW.
In addition to the characterisation analyses performed with the crude extracts, EIW-A and EIW-B were also analysed by LC-MS and GC-MS. The former was performed with both extracts to attempt to identify any constituents, whereas the latter was done to determine the binding pattern of the polysaccharides in EIW-A.
LC-MS did not result in identification of any compounds, but indicates highly polar components and the presence of a sodium salt in both fractions.
EIW-A proved to be the fraction with most carbohydrate (10 %), which would contain potential β-glucans and other high Mw polysaccharides. In contrast, EIW-B contains only 0.4 % carbohydrate.
The binding pattern analysis of carbohydrates in EIW-A implied only traces of 1,3 bound glucose monomers, which is another indicator of low β-glucan content in AndoSan™.

In conclusion, further purification of AndoSan™ compounds is necessary for identification of specific bioactive agents. In regard to legumain activity it is apparent that EIW is the most interesting choice for further studies, but synergistic and/or combined effects of constituents can complicate the work of identifying a single active component.

Sammendrag

AndoSan™ er et kommersielt produkt som for øyeblikket er gjenstand for forskning for bruk mot multippelt myelom, tarmkreft og Crohn's sykdom. Produktet er et fermentert vandig ekstrakt av tre ulike medisinske sopper, hvor *Agaricus blazei* Murill (AbM) er hovedbestanddelen. Mange vitenskapelige artikler beskriver de biologiske effektene av AbM og mange påstår også at disse effektene er forårsaket av β-glukaner og proteoglykaner, men en avklaring i nøyaktig hvilke komponenter som gir de observerte effektene er enda ikke fastslått. En kompliserende faktor oppstår når forskningsresultater stammer fra ulike fragmenter av soppen, hvilket kan resultere i svært ulike ekstrakter av samme soppart. Dette gjør det vanskeligere å bruke publiserte data til å fastslå hva AndoSan™ kan bestå av. Målene med denne oppgaven har derfor vært å fraksjonere dette produktet, å undersøke biologisk aktivitet i et cellesystem bestående av en makrofagcellelinje (RAW264.7) samt å innlede prosessen med å identifisere komponenter i AndoSan™. Det biologiske testsystemet bruker legumain, en cysteine protease, til å måle aktivitet. Redusert legumainaktivitet anses som fordelaktig, ettersom uttrykk av legumain er økt i flere typer kreftsvulster.

AndoSan™ ble delt opp i fem fraksjoner på bakgrunn av ulik løselighet i ulike løsemidler. Disse ble grovt karakterisert med ^{1}H NMR, SDS-PAGE, UV absorbans, tynnsjiktskromatografi (TLC) og karbohydratanalyser. Sistnevnte analyser påviste et karbohydratinnhold i AndoSan™ på kun 2 %, noe som strider imot det som har blitt funnet for rent, pulverisert soppmateriale (består av 90 % karbohydrat). Dette antyder også at de biologiske effektene av AndoSan™ ikke kan være forårsaket av β-glukaner alene.

^{1}H NMR ble brukt for å si noe om hvilke grupper av komponenter som var tilstede i ekstraktene, og spektrene antydet ulike grupper av komponenter i ulike ekstrakter. De ekstraktene som ble ekstrahert med liknende kjemikalier lignet hverandre (de to alkoholekstraktene samt de to vannekstraktene ga veldig like spektra). TLC av vannekstraktene antydet svært polare bestanddeler og SDS-PAGE påviste protein i det etanoluløselige vannekstraktet.

AndoSan™ ekstraktene ble også screenet for inhibering av legumainaktivitet i RAW264.7 celler, hvorav det etanoluløselige vannekstraktet (EIW) viste høyest inhiberende aktivitet. Rent AndoSan™ pulver ble deretter inkubert med aktivt legumain og autoaktivert prolegumain for å kunne si noe om denne hemmingen i aktivitet skyldes direkte hemming av aktivt enzym, eller om

den skyldes hemming av autoaktiveringen av prolegumain ved pH 4. EIW ble også inkubert med autoaktivert enzym for å undersøke om det ga mer hemming enn ubehandlet AndoSan^{TM}. Resultatene viser at AndoSan^{TM} og EIW er svært potente inhibitorer av autoaktivering av prolegumain, hvorav EIW viser bedre inhibering ved konsentrasjoner 0.25 og 0.5 mg/mL. Ved konsentrasjoner på 1 mg/mL og oppover gir begge ekstraktene like god hemming av autoaktivering (ca. 80 %). Resultater viser også at AndoSan^{TM} hemmer aktivt enzym, men ikke i like stor grad som det hemmer autoaktivering (ca. 40 % hemming ved konsentrasjoner over 0.5 mg/mL).

Det etanoluløselige vannekstraktet ble, på bakgrunn bedre hemming av legumainaktivitet i RAW264.7 celler, valgt for videre opprensing og karakterisering. Ekstraktet ble separert i to fraksjoner vha. SEC; EIW-A og EIW-B, hvorav EIW-A viste best inhibering av legumain i celleforsøk. EIW-A viste ikke bedre hemming enn EIW, hvilket antyder synergi og/eller kombinerte effekter av komponentene i EIW.
I tillegg til karakteriseringsanalysene utført på råekstraktene, ble EIW-A og EIW-B også analysert med LC-MS og GC-MS. Førstnevnte ble utført på både EIW-A og EIW-B for å forsøke å identifisere komponenter i disse, mens sistnevnte ble utført for å bestemme bindingsmønsteret til polysakkaridene i EIW-A. LC-MS førte ikke til identifikasjon av spesifikke komponenter, men antyder polare bestanddeler samt påviste en lavmolekylær natriumkomponent (antakelig et natriumsalt) i både EIW-A og EIW-B. EIW-A er den fraksjonen som viste seg å inneholde mest karbohydrat (10 %), og vil også inneholde eventuelle β-glukaner. I motsetning til EIW-A inneholder EIW-B kun 0.4 % karbohydrat. Analyse av bindingsmønsteret til polysakkaridene i EIW-A antydet at ekstraktet inneholder kun små mengder 1,3 bundet glukose, hvilket indikerer lavt innhold av β-glukaner.

Det konkluderes med at videre opprensing av AndoSan^{TM} fraksjoner er nødvendig for å identifisere enkeltkomponenter som har en spesifikk biologisk effekt, og at eventuelle β-glukaner alene ikke kan forklare de biologiske effektene av AndoSan^{TM}. Med hensyn til legumainaktivitet er det tydelig at EIW er mest interessant, men synergi mellom de ulike komponentene i produktet kan vanskeliggjøre identifikasjonen av en enkelt forbindelse som gir fordelaktig effekt.

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1 Preface

1.1 Introduction

The purpose of this thesis has been to identify and investigate the biologically active chemical compounds in the commercial AndoSan™ product; an aqueous preparation of fermented medicinal mushrooms commonly used as health food in Japan and Brazil.
AndoSan™ is produced in Japan and developed and distributed by ImmunoPharma AS, Oslo, Norway.
The product consists of three different mushrooms; 82.4 % *Agaricus blazei* Murill (AbM, *Himmematsutake* in Japan, *Cogumelo do Sol* in Brazil), 14.7 % *Hericium erinaceum* and 2.9 % *Grifola frondosa* (Johnson et al. 2009), all of which belong to the same phylum; *Basidiomycota.*
It is currently under investigation for use in treatment of multiple myeloma, colorectal cancer and inflammatory bowel disease (ImmunoPharma). AbM has been shown to be the main active ingredient in AndoSan™, with respect to biological activity (Forland et al. 2010).
AndoSan™ was selected among other AbM products as the one of most interest for further research at Oslo University Hospital, due to higher potency of this product compared to other AbM extracts, as was indicated in a pneumococcal sepsis model by Hetland et al. Possible synergistic effects of the three mushrooms in AndoSan™ were proposed as an explanation for this observation (Hetland et al. 2011).
Furthermore, previous research articles using this particular product in their experimental setups', state that 100 g of AbM mixed powder contains 5.8 g moisture, 2.6 g protein, 0.3 g fat, 89.4 g carbohydrates (of which β-glucans constitute 2.8 g) and 1.9 g ash (Ellertsen & Hetland 2009; Forland et al. 2011; Johnson et al. 2009).

AndoSan™ is a commercial product and thus its manufacturing is a business secret. However, ImmunoPharma has revealed that the product consists mainly of mycelia combined with some fruiting bodies, and that the mushrooms are initially grown in peat soil before they are transferred into growth chambers under highly specific conditions.

Numerous studies have been performed to investigate the health benefits of AbM, including its potential in the treatment of different cancers (Jiang & Sliva 2010; Su et al. 2011; Takaku et al. 2001; Yu et al. 2009), allergy (Ellertsen & Hetland 2009), inflammatory bowel disease (Crohn's disease) (Forland et al. 2011), infection (Bernardshaw et al. 2005b; Sorimachi et al. 2001b) and diabetes (Oh et al. 2010).

In human phase I studies, AndoSan™ was found to be free of adverse effects (Forland et al. 2011; Johnson et al. 2009).

1.2 *Agaricus blazei* Murill

The medicinal mushroom *Agaricus blazei* Murill originates from the village Piedate, Sao Paolo in Brazil. It was identified as a natural remedy by a Japanese scientist named Takatoshi Furumoto in 1960, who observed a lower disease rate among the inhabitants of this village compared to the general population (Firenzuoli et al. 2008). He brought the mushroom back to Japan, where it was subject to research and cultivated for use in natural medicine.

Figure 1.1 The fruiting bodies of *Agaricus blazei* Murill. The mushroom belongs to the phylum *Basidiomycota*, and is related to the common food mushroom *Agaricus bisporous*, or champignon. Photo © NutriCon

Previous research on this mushroom has revealed several possible sources for bioactive compounds. However, the highly diverse growth conditions and the use of different parts of the mushroom produce dissimilar preparations of the same mushroom. The results obtained from

other sources of AbM preparations can thus only be used as an indication of which chemical compounds may be responsible for the effects seen with AndoSan^{TM}, and not as a statement. This can also help to explain why some AbM extracts prove to be beneficial for health, whereas others have only very limited or no effect at all.

1.2.1 Health effects and possible stimulants

Several scientific studies on AbM have revealed it to contain components that can act as immunostimulants (Bernardshaw et al. 2005a; Ellertsen et al. 2006; Forland et al. 2010; Hetland et al. 2011), and thereby help to prevent allergy, asthma, Crohn's disease, a number of cancers and infection (Bernardshaw et al. 2005b; Ellertsen & Hetland 2009; Grinde et al. 2006; Hetland et al. 2008; Takimoto et al. 2008).

Research on AbM has proposed several biological response modulators, therein β-glucans (Ohno et al. 2001), proteoglycans (Itoh et al. 1994), agaritine (Endo et al. 2010), steroids (Su et al. 2011), sterols (Takaku et al. 2001) and isoflavonoids (Oh et al. 2010).

β-glucans

β-glucans are polysaccharides of D-glucose monomers linked by β-glycosidic bonds. These bonds are normally in the β(1→3) and β(1→6) conformations, where the former usually makes up the backbone of the polymer and the latter is involved in the attachment of branching side chains. These polymers are most frequently found as structural components in the cell wall of many fungi, but other sources also include bacteria and seaweed (Novak & Vetvicka 2009; Soltanian et al. 2009).

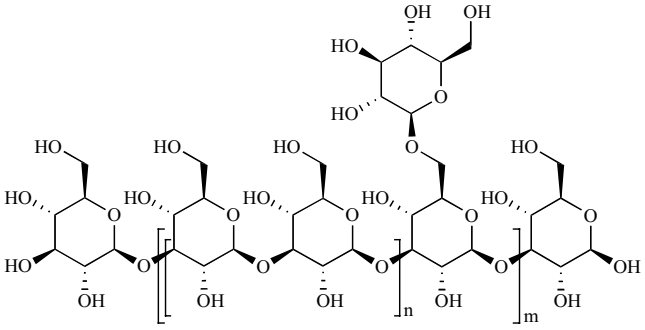

Figure 1.2 The general structure of β-glucans (Novak & Vetvicka 2009).

Since these β-glucans are not found in animals, they are considered to be classic pathogen associate molecular patterns (PAMPs), which can induce immune responses in humans (Brown & Gordon 2003). The ability of fungal β-glucans to induce immune responses is widely utilised for medicinal purposes in Japan; e.g. Lentinan, a β-glucan isolated from *Lentinus edodes*, which has antitumour properties against stomach cancer (Taguchi 1987). Also, the mushroom *Grifola frondosa* contains a β-1,3-D-glucan with β-1,6-glucopyranosyl side chains called Grifolan (Soltanian et al. 2009), which has shown antitumour properties against sarcoma in mice (Ohno et al. 1986).

The yield of β-glucans from mushrooms varies considerably during their different maturation stages, and so the time of harvest and also the isolation method are of great importance. These data are often not included in scientific papers describing β-glucans isolated from AbM, which proposes a problem when trying to use these articles to predict the composition of β-glucans in AndoSan™ (Firenzuoli et al. 2008).

Proteoglycans and glycoproteins

Proteoglycans are proteins that have a high degree of glycosylation (i.e. carbohydrate chains attached to it) and glycoproteins are proteins with a lesser degree of glycosylation. Both have been found in AbM (Fujimiya et al. 1998; Kawagishi et al. 1989).

One fairly known component of AbM is a glycoprotein named FIII-2-b, which has been shown to have antitumour properties against sarcomas in mice (Itoh et al. 1994; Kawagishi et al. 1989; Kawagishi et al. 1990). This component has been characterized as a β-1,6-D-glucan-protein complex, which consists of 43.3 % protein and 50.2 % carbohydrate. This component is isolated from the water insoluble residue of AbM fruiting bodies (Kawagishi et al. 1989).

Agaritine

Agaritine is a phenyl hydrazine conjugated with glutamic acid, and its IUPAC name is 2-[4-(Hydroxymethyl)phenyl]-glutamohydrazide ($C_{12}H_{17}O_4N_3$) (Endo et al. 2010). It is a compound of low molecular weight (267.32 Da).

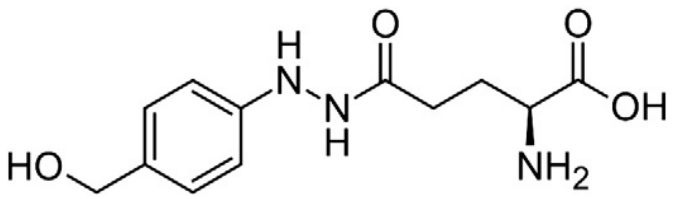

Figure 1.3 The chemical structure of agaritine (Akiyama et al. 2011)

Agaritine has recently been shown to reduce viability of leukemic cells (U937, human monocyte leukaemia cell line) *in vitro* by inducing apoptosis (Akiyama et al. 2011).

There have been some contradictory reports on the compound regarding carcinogenesis, some state that agaritine is mutagenic and a carcinogen (Walton et al. 1997), others state that agaritine has no adverse effects (Roupas et al. 2010; Shephard & Schlatter 1998).
Endo et al. suggested that this might be due to discrepancies in the quality and purity of agaritine that has been used in these experiments. They found no adverse effects of their compound using the *umu* test (a genotoxicity test). In the same paper, they also stated that the Japan National Institute of Health Sciences has concluded that agaritine does not exhibit any genotoxic properties (Endo et al. 2010). The compound has however, been found to be sensitive to oxidative and fermentative degradation (Roupas et al. 2010).

Steroids and sterols

Steroids that have lost their A-ring have been isolated from cultured mycelia of AbM (Hirotani et al. 2002a; Hirotani et al. 2002b). These compounds have been named Blazeispirols A-G, of which blazeispirol A has shown to exhibit antihepatoma activity by inducing apoptosis in hep 3B cells (human liver cancer cells) (Su et al. 2011).

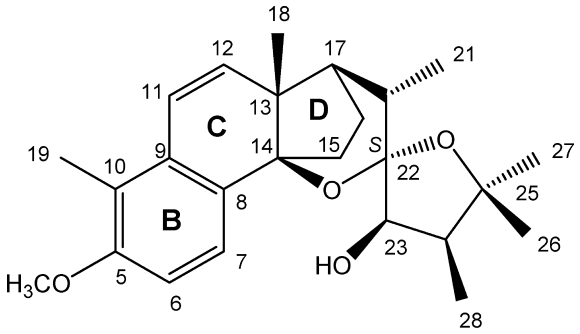

Figure 1.4 The chemical structure of blazeispirol A (Su et al. 2008).

The content of blazeispirols in the fermentation products of cultured AbM is dependent upon the amount of soybean or black soybean in the culture medium (Su et al. 2008). It is unknown whether the AbM in AndoSan™ has been cultivated with soybean, and thus the presence of blazeispirols is uncertain.

Another steroid derived compound that have been found in AbM is ergosterol, the fungal variant of human cholesterol. Sterols are steroid alcohols, which have an alcohol group attached to the A-ring in the steroid structure. Ergosterol is such a sterol and a precursor to ergocalciferol (Vitamin D2). It has been reported that ergosterol (or its metabolites) isolated from *Agaricus blazei* inhibits angiogenesis in solid tumours (Takaku et al. 2001).

Isoflavonoids

A recent review article regarding AndoSan™ states that AbM contains isoflavonoids (Hetland et al. 2011; Oh et al. 2010). Isoflavonoids have been found to be hypoglycaemic (reduce the glucose level in blood) in diabetic rats. However, the paper describing these findings also say that such isoflavonoids (identified as genistein, genistin, daidzein and daidzin) may be derived from the soybean flakes in the culture media with which AbM had been cultivated (Oh et al. 2010). As mentioned above, it is unknown whether the AbM in AndoSan™ has been cultivated with soybean, thus the presence of isoflavonoids in AndoSan™ is unclear.

Other biologically active compounds that have been found in AbM include sodium pyroglutamate (Kimura et al. 2004) and antioxidants (Izawa & Inoue 2004).

Sodium pyroglutamate is the sodium salt of pyroglutamic acid and has a mass of 151 Da. The compound was shown to exhibit antitumour and antimetastatic effects in tumour-bearing mice.

Results indicated that the observed effect was due to its ability to inhibit the reduction of immune responses caused by tumour growth and tumour-induced angiogenesis (Kimura et al. 2004).

1.2.2 Effects on the immune system

AbM has been shown to promote secretion of IL-8, TNF-α and nitric oxide by macrophages derived from rat bone marrow (Sorimachi et al. 2001a), and to promote synthesis of the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α by human monocytes *in vitro* (Bernardshaw et al. 2005a).

The *in vivo* effects however, were demonstrated to be somewhat opposite of those of the *in vitro* effects; daily oral intake of AndoSan™ by healthy human adults resulted in a decreased level of different pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, IL-17 and IL-2) (Johnson et al. 2009). These results corresponded well with the effects observed in mice inoculated with pneumococcal (Bernardshaw et al. 2005b) or faecal bacteria (Bernardshaw et al. 2006); their survival rate increased when given AndoSan™ orally. This indicated anti-inflammatory effects of AndoSan™, which have also been observed in human patients suffering from inflammatory bowel disease (Forland et al. 2011).

The anti-inflammatory effect of AndoSan™ may also reduce the risk of developing cancer, due to the contribution of chronic infection in the development of 1 in 4 cancer cases (Hussain & Harris 2007).

AbM extracts have also shown to rectify the shifted cytokine response in individuals suffering from immunoglobulin E (IgE)-mediated allergy. This condition leads to increased expression of cytokines from T-helper 2 (Th2) cells relative to cytokines from T-helper 1 (Th1) cells, which results in increased production of IgE antibodies. This skewed balance has been shown to be ameliorated by AbM extracts (Takimoto et al. 2008), indicating that AndoSan™ may be a possible remedy against IgE-mediated allergy (Ellertsen & Hetland 2009).

Of the compounds described in section 1.2.1, the β-glucans are the most studied immunomodulators. Harmless β-glucans (e.g. from AbM) are recognized by the innate immune system due to their presence in other pathogenic fungal species, such as *Candida albicans*. The cell receptors involved in the recognition of β-glucans include dectin-1, scavenger receptors,

Toll-like receptor 2 (TLR2) and Complement receptor 3 (CR3), and are generically called pattern-recognition receptors (PRR) (Chen & Seviour 2007; Hetland et al. 2011). These receptors are present on antigen presenting cells (such as dendritic cells and monocytes/macrophages) and natural killer (NK) cells, which in an activated state further stimulate the adaptive immunity by secreting cytokines and chemokines (Ahn et al. 2004; Forland et al. 2010; Kasai et al. 2004). This immune enhancing capacity of fungal β-glucans is of great advantage, in that harmless species can act as immunostimulants and thus help to increase the body's own defence against disease. This is of great interest due to the many adverse effects seen with medicine widely used in the treatment of cancer.

Another factor that affects the great ability of β-glucans to induce immune responses is their longevity in mammalian digestive systems; vertebrates lack an enzyme to degrade β-glucans, and relie on regular oxidative metabolism to degrade them (Brown & Gordon 2003; Nono et al. 1991).

1.3 Legumain

Legumain is an asparaginyl endopeptidase; a lysosomal protease with a cysteine in its active site and thus belongs to the cysteine proteases. Its activity is specific for the hydrolysis of asparaginyl bonds in Z-Ala-Ala-Asn-7-(4-methyl)coumarylamide (where Z is benzyloxycarbonyl) and benzoyl-Asn-*p*-nitroanilide (Chen et al. 1998). In the lysosomes, the proenzyme (56 kDa) is autoactivated at pH 4 by conformational change and sequential cleavage of the C-terminal and N-terminal propeptides, resulting in fully active enzyme (46 kDa) (Berven et al. 2012; Li et al. 2003).

Evidence suggest that legumain has a role in the processing of peptides as antigens presented by the MHC class II molecules on professional antigen presenting cells (such as macrophages and dendritic cells) (Manoury et al. 1998), and as an activator of other proteases; progelatinase A and cathepsin B (Berven et al. 2012; Liu et al. 2003). Under normal conditions, its expression is highest in the kidney, but also detectable in the liver and spleen.

Recent findings reveal an overexpression of legumain in several solid tumours and tumour associated endothelial cells. Tumours that have been analysed and assessed positive for such

overexpression include breast-, colon-, lung-, prostate- and ovarian carcinomas, tumours of the central nervous system, and to some extent lymphoma and melanoma (Gawenda et al. 2007; Liu et al. 2003; Wang et al. 2012).

When present in tumours, legumain facilitates and increases the rate of tissue invasion and metastasis, as may be explained by its activation of progelatinase A and cathepsin B. In order to convert progelatinase A and cathepsin B into their respective active form, an asparaginyl bond needs to be cleaved (Chen et al. 1998; Chen et al. 2001). The active form of these proteases facilitates degradation of the extracellular matrix (Liu et al. 2003).

1.3.1 Legumain activity in RAW264.7 cells

On the basis of these tumour-facilitating properties of legumain, its reduced activity in biological test systems proposes a good indication of possible cancer prevention by different substances, e.g. AndoSan^{TM}. This is what has been implemented into the model using the RAW264.7 macrophage cell line. These cells normally express legumain (unpublished data, Berven et. al.), and when incubated with different extracts of AndoSan^{TM}, it may be possible to differentiate between the fractions if they exhibit different levels of legumain inhibition. This can facilitate further purification and identification of specific chemical compounds responsible for inhibition of legumain.

1.4 Aims of thesis

The aims of this thesis have been:

- To separate the chemical components of AndoSan^{TM} on the basis of different physical and chemical properties
- To use an established system of a macrophage cell line and its enzyme activity to identify more biologically active fractions
- To initiate the process of identifying the biologically active components of AndoSan^{TM}

2 Materials and methods

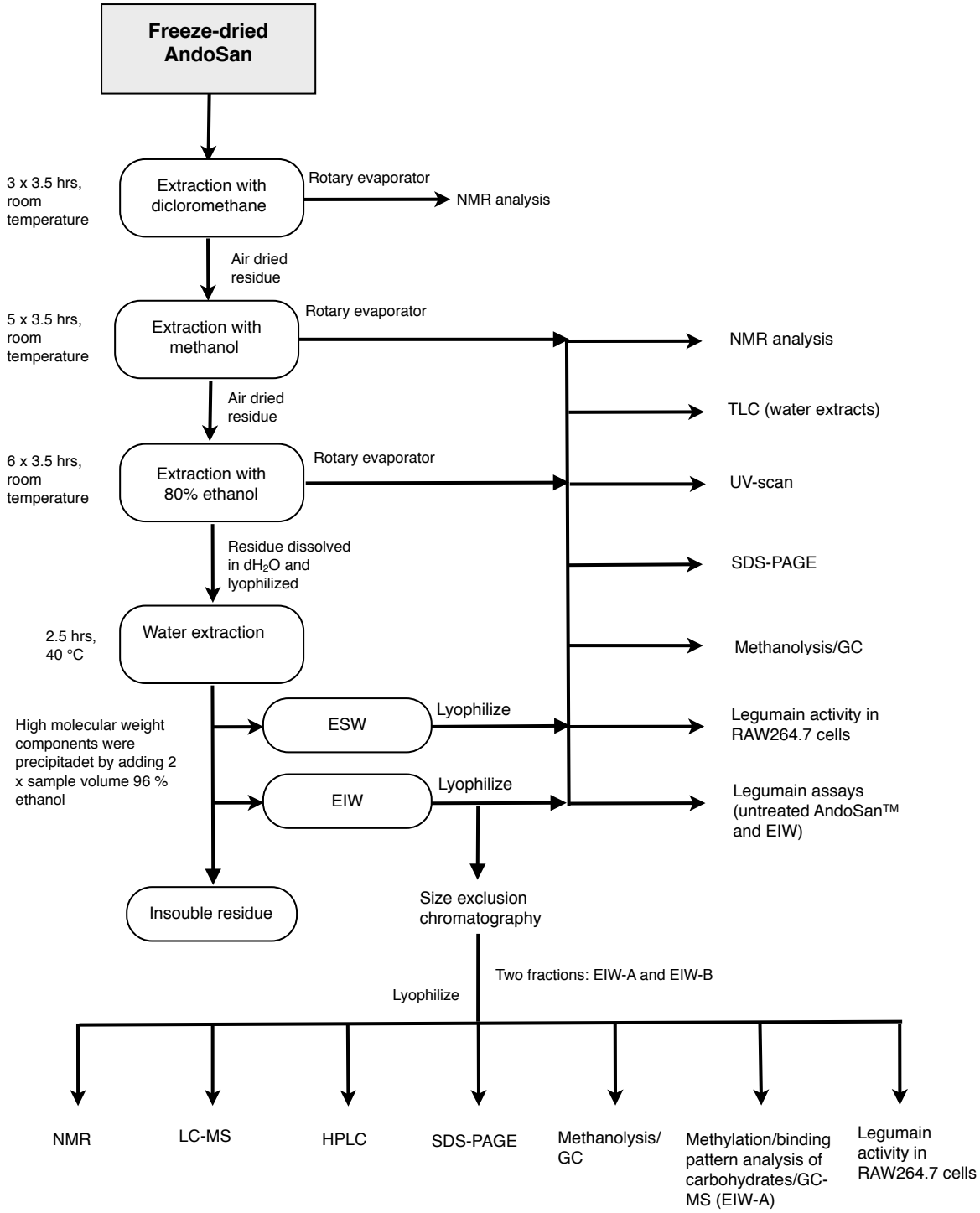

Figure 2.1 Flowchart of the experimental setup of this thesis. Detailed descriptions of each of the procedures are presented below. Chemicals and abbreviations are listed in appendix 1 and 2, respectively.

2.1 Sample preparation

ImmunoPharma AS (Oslo, Norway) kindly provided 14.3 L of AndoSan™. The product was kept in dark glass bottles and stored at 4°C, before it was lyophilized with a Hero Drywinner 6-85 apparatus. The 14.3 L yielded 64.35 grams of dry sample, which corresponds to a dry weight of 4.5 mg/mL. The freeze-dried material was stored in a freezer (-18°C) when not in use.

2.2 Extractions of chemical components

The different components of AndoSan™ were extracted from the crude sample material for further investigation, including biological activity search, purification processes and characterisation analyses.

The purpose of the dichloromethane (DCM) extraction was to isolate any lipid-like substances from the sample material, such as terpenes, fats, steroids and ergosterols. The methanol extraction was performed in order to isolate polar low molecular weight components from the material, whereas the ethanol extraction was performed to extract less polar low molecular weight components from the freeze-dried AndoSan™. The purpose of the water extraction was to isolate proteins, peptides and higher molecular weight carbohydrates.

Procedure

Dichloromethane extraction

Freeze-dried material (30.11 g) was treated with 200 mL of DCM, the beaker was sealed with aluminium foil and stirred on a magnetic stirrer (RAT basic, IKA® labortechnik) at room temperature for 3 hours ± 15 minutes, followed by filtration through paper filters (Schleicher & Schüll). This procedure was repeated until the extract was fairly colourless (and thus most of the compounds soluble in DCM were collected); in this case it took three extractions, in addition to have been left overnight once under the same conditions. The DCM extract was run on a rotary evaporator (IKA® RV 10 Basic Rotavapor with vacuum controller) in order to remove the DCM/solvent and obtain a dry extract sample.

Methanol extraction

Following DCM-extraction, the remaining material was air dried in order to prepare for methanol extraction. The dry sample was treated with 200 mL methanol; the beaker was resealed with aluminium foil and stirred on the magnetic stirrer at room temperature for 3 hours ± 15 minutes, followed by filtration with paper filters. Due to the material being more soluble in methanol than in DCM, it required more iterations of the extraction step in order to collect most of the methanol-soluble fraction. It was therefore repeated five times. In addition, it was left overnight under the same conditions twice. The methanol extract (MeOH extract) was run on the rotary evaporator.

Ethanol extraction

The remaining material was air dried in order to prepare for ethanol extraction. The dry sample was treated with 200 mL of 80 % ethanol; the beaker was resealed with aluminium foil and stirred on the magnetic stirrer in room temperature for 3 hours ± 15 minutes, followed by filtration through paper filters. The material was, just as with the methanol extraction, more soluble in ethanol relative to DCM, and six repetitions of the extraction step were required. In addition, it was left overnight under the same conditions twice. The ethanol extract (EtOH extract) was run on the rotary evaporator.

The remaining sample material was dried free of ethanol with the rotary evaporator, dissolved in 400 mL of dH_2O and lyophilized (Christ Alpha I-4 LD plus), in order to obtain thoroughly dried material ready for further extractions.

Water extraction

The material was then added 500 mL of dH_2O and left on a water bath at 40°C for 2 hours and 30 minutes. The sample was filtered through a 1.6 µm microfiber filter (GF/A, Whatman) with a vacuum pump. In order to precipitate high molecular weight carbohydrates, the filtrate was divided into two bottles and added a 2:1 volume ratio of 96 % ethanol. The bottles were left to settle in a refrigerator (4°C) over night.

The precipitate and supernatant were designated EIW (ethanol insoluble water extract) and ESW (ethanol soluble water extract), respectively, and were separated by centrifuging the samples at 1750 G for 30 minutes. ESW was collected and EIW was washed twice with ethanol.

ESW and EIW were dried free of ethanol with the rotary evaporator. Both were then dissolved in 200 mL of dH_2O and freeze-dried in order to obtain thoroughly dry samples for further analyses. The final insoluble residue was collected on the microfiber filters used in the filtration of the water extract, and left to dry in an incubator at 50°C over night.
The dry extract samples were kept in a freezer (-18°C) when not in use.

2.3 Fractionation of components by chromatography

Two types of chromatography were used for separation of the compounds in AndoSan™: preparative size exclusion chromatography (SEC) and size exclusion high-performance liquid chromatography (SEC-HPLC).
SEC was used to further fractionate the crude fractions of AndoSan™; HPLC was used in order to indicate the size of components in more refined fractions.

2.3.1 SEC

Size exclusion chromatography, also called gel filtration chromatography, was used to separate molecules in EIW by size.

In size exclusion, there is no attraction between the stationary phase (the gel) and the solute (the sample). Instead, the sample goes through pores in the gel, which are of a specific size; any molecules larger than this pore size will be excluded and stream past the gel pores. Smaller molecules within the threshold of the pore size will have partial to complete access to the pores, and therefore have to pass through a larger volume. Thus, the smaller analytes will take longer time to elute than the larger ones (Harris 2007), and the fractions can be separated with a fraction collector.

The column material used for this purpose was Sephacryl S100 HR, which separates globular proteins of sizes between 1 and 100 kDa (peptides and small proteins).

Procedure

Analysis conditions

Column	XK 26/100 column (2.6 cm x 100 cm), Pharmacia
Column volume	400 mL
Pump	P-50, Pharmacia
Mobile phase	dH_2O, filtered through 45 μm filters, gassed with helium
Injector	Valve 1V-7 2MPa injector, Pharmacia
Flow rate	0.5 mL/min
Fraction size	3.5 mL
Fraction collector	Pharmacia LKB Superfrac, Pharmacia
Detector	RID-6A Refractive Index Detector, Shimadzu
Software	Chromeleon version 7.0

The mounted column facility was packed with Sephacryl S-100 HR in accordance with the protocol provided by the manufacturer. 5 mL of sample (dry powder dissolved in dH_2O, filtered through 45 μm) were applied to the column, with concentrations ranging from 3 mg/mL to 4 mg/mL. The elution was performed with a total volume of 630 mL. The fractions that appeared to belong to the same peak on the chromatogram were collected and mixed, before they were lyophilized for further analysis. This resulted in two fractions; EIW-A and EIW-B.
The column material was stored in 20 % ethanol after use.

2.3.2 SEC-HPLC

Size exclusion high performance liquid chromatography (SEC-HPLC) was used to estimate the molecular size of components in the fractions obtained from SEC of EIW; EIW-A and EIW-B.

HPLC utilizes high pressure to force components through closed columns packed with very fine particles, resulting in high-resolution separations (Harris 2007). The column used is another type of gel filtration column, containing gel particles (7 μm, hydroxylated polymethacrylate) that are ideal for separation of water-soluble linear polymers with molecular weights up to 50 kDa (Tosoh).

The HPLC equipment included a reservoir for the mobile phase, a pump, an auto injector, a guard column, a gel filtration column and a detector. The samples were dissolved in mobile phase and pumped through both the guard column and the gel filtration column at a constant pressure. This resulted in separation of components by sizes, which were detected by a refractive index (RI) detector. The RI detector was equilibrated with solvent (can thus only be used with isocratic elution) and detected all solutes. The signals from the detector were interpreted by computational analysis and presented as chromatograms.

Procedure

Analysis conditions

Apparatus	Hitachi LaChrom Elite
Detector	RI Detector L-2490
Software	EZ. Chrom Elite
Column	TSK Gel® G3000PW_{XL} HPLC column (7.8 mm x 30 cm), Tosoh
Guard column	TSK Gel® PW_{XL} Guard column (6 mm x 4 cm), Tosoh
Mobile phase	0.05 M Na_2SO_4 filtered through 45 µm filter, gassed with helium
Flow rate	0.5 mL/min
Injection volume	95 µL
Elution	Isocratic elution for 30 minutes per sample
Standards	β-glucan from oat of known molecular weights (35.6 kDa and 70.6 kDa, Megazyme)

The column was prior to analysis washed with dH_2O and mobile phase ($NaSO_4$) sequentially, until the detector had stabilized. Each sample (1 mg) and both standards were dissolved in 1 mL 0.05 M $NaSO_4$, filtered through a 45 µm filter and transferred to HPLC sample tubes, which were placed in the autosampler. After analysis, the column was washed with dH_2O, and stored in 20 % ethanol.

2.4 Characterisation of extracts

Several methods were utilised for the characterisation of extracted compounds, including proton nuclear magnetic resonance (^{1}H NMR), thin layer chromatography (TLC), liquid chromatography-mass spectrometry (LC-MS), UV-absorption measurements, methanolysis and identification of monosaccharides by gas chromatography (GC), methylation/binding pattern analysis of carbohydrates by GC-MS and sodium dodecyl sulphate protein gel electrophoresis (SDS-PAGE),

2.4.1 ^{1}H NMR

The ^{1}H NMR spectrum was obtained from each of the extracts in order to do rough compositional analyses.

Nuclear magnetic resonance spectroscopy utilizes the magnetic properties of some nuclei; those having nuclear spin will, in the presence of a strong magnetic field, behave like bar magnets. In ^{1}H NMR these nuclei are hydrogens/protons, which have magnetic spin of ½. In the presence of a magnetic field, these can take up only two orientations, a low-energy orientation aligned with the applied field and a high-energy orientation opposed to the applied field. The number of nuclei in the high-energy state and the number in the low-energy state will change when a radio-frequency signal is applied. If the radio frequency matches the frequency with which the nuclear magnets precess with the applied magnetic field, more nuclei are promoted from the low-energy state to the high energy state. This resonance frequency is dependent upon the applied magnetic field and the nature of the nucleus in question (Williams & Fleming 2008). Protons bound to different compounds behave differently in NMR spectroscopy due to surrounding electrons, and thus ^{1}H NMR can be used to identify groups of compounds.

There has been no determination of exact structures. In order to determine the structures of the compounds, it is necessary to combine the obtained ^{1}H NMR spectrum with its respective ^{13}C NMR spectrum.

Procedure

Samples of 18.7 mg, 25.7 mg and 24.4 mg were taken of the DCM-, MeOH- and EtOH extracts, respectively, which were dissolved in deuterated methanol (CD_3OD). Samples of 50 mg were taken of ESW and EIW, which were dissolved in deuterated water (D_2O). Samples of 5 mg were taken of EIW-A and EIW-B, which were also dissolved in D_2O.
The samples were analysed on a Bruker DPX 300 MHz NMR instrument equipped with a BACS-60 automatic sampler. Trimethylsilyl (TMS) was used as reference in the samples dissolved in CD_3OD (TMS is not soluble in D_2O), and the software used for processing the spectra was MestReNova.

2.4.2 TLC

Revers phase thin layer chromatography (TLC) was performed on both ESW and EIW in order to try to separate and indicate the chemical properties of the compounds.

TLC is a chromatographic method where the stationary phase is attached to a sheet of aluminium foil, glass or plastic. The samples are blotted to this sheet, which is placed vertically in a beaker filled with mobile phase. Capillary forces make the mobile phase move up the stationary phase, and depending upon the properties of the stationary and mobile phases, the components in the samples can be separated based on chemical properties (Cheng et al. 2011). Revers phase TLC has a non-polar stationary phase, thus a more polar mobile phase will move more rapidly up the stationary phase.

Procedure

Two reverse phase TLC plates (TLC Silica Gel 60 RP-18 F_{254}s, Merck) were prepared and both ESW and EIW (the samples from the ^1H NMR experiment, dissolved in D_2O) were added with capillary tubes. The plates were put in respective beakers, one containing methanol:dH_2O (1:2) as mobile phase and one containing methanol:dH_2O (1:9) with 1 drop trifluoroacetic acid as mobile phase, and left for about 20 minutes. After treatment involved exposure to short-wave UV (254 nm), long-wave UV (366 nm) and 1% $Ce(SO_4)_2$ in 10 % aqueous H_2SO_4. The latter is a detection reagent for organic compounds.

2.4.3 LC-MS

Liquid chromatography-mass spectrometry (LC-MS) of EIW-A and EIW-B was performed by Leon Reubsaet (Department of Pharmaceutical Chemistry Bioanalytics at the University of Oslo).

The components in both fractions were separated based on polarity in LC. The column used separated components based on hydrophobic interactions. In addition, the gradient elution involved an initial polar mobile phase that became gradually less polar during elution. Thus, highly polar substances would be eluted first, less polar substances would elute later.

The mass spectrometer determines the mass of the compounds by bombarding them with electrons in vacuum (giving them electric charge), and then separates them on the mass-to-charge ratio (m/z). This produces characteristic mass spectra, which can be used in the identification of compounds.

Procedure (summary)

Analysis conditions

Injection volume:	5 µL
Mobile phase 1:	10 mM ammonium acetate with 1 % formic acid
Mobile phase 2:	Methanol
Column:	Nucleodur C_{18} (Machery-Nagel)
Software:	Thermo Xcalibur

EIW-A and EIW-B were dissolved in dH_2O, both at a concentration of 1 mg/ml and analysed by liquid chromatography combined with ion trap mass spectrometry.

2.4.4 UV scan of extracts

The extracts were scanned for absorption of ultraviolet light at wavelengths between 200 and 900 nm, in order to assess whether the samples contained any polyphenols and/or aromatic compounds.

Polyphenols and aromatic compounds give an absorption maximum between 250 and 300 nm.

Procedure

The samples were dissolved in dH_2O and transferred to a quartz cuvette. The cuvette was placed in a two beam UV apparatus (Biochrom Libra S32 PC), with one blank sample containing only dH_2O in the reference cell. The apparatus was set to scan the sample for absorption between 200 nm and 900 nm.

2.4.5 Methanolysis

The purpose of the methanolysis analysis was to determine the carbohydrate content in the samples, and the monosaccharide composition of these carbohydrates.
The method was developed by R.E. Chambers and J.R. Clamp in 1971, and is based on the theory that methanolysis is equally efficient at cleaving glycosidic linkages as the more typical hydrolysis with aqueous acids or cation-exchange resins, in addition to cause less destruction of carbohydrate (Chambers & Clamp 1971).

The method involves cleavage of the glycosidic bonds in the polysaccharides with methanolic hydrochloric acid. This reaction is carried out in anhydrous methanol, which yields methyl glycosides. These are not very volatile or thermally stable, and thus need to be derivatised in order to prepare them for analysis by gas chromatography. For this purpose trimethylsilyl (TMS) is used, which makes the polar groups non polar and thereby increases volatility and thermal stability.

Procedure

Sample preparation

Samples of 3-4 mg of the crude extracts and unfractionated freeze-dried AndoSan^{TM} and 1 mg of EIW-A and EIW-B were collected in methanolysis tubes (acid-washed). To thoroughly dry the samples, the tubes were placed in a desiccator containing phosphorous pentoxide (P_2O_5) and incubated over night at 40°C (the ethanol extract were dried for 4 days). This was followed by adding 100 µL of internal standard (1.02 µg/µL D-mannitol in anhydrous methanol), and the mixtures were left on the bench for a few minutes to vaporize some of the methanol.

Sample methanolysis

The samples were treated with 1 mL anhydrous methanol and 100 µL anhydrous 3M hydrochloric acid (HCl) and incubated at 80°C for 24 hours. Following incubation, they were dried free of methanol and HCl using nitrogen gas (Reacti-Vap III, Thermo Scientific). The samples were then treated with 200 µL anhydrous methanol and dried with nitrogen gas. This cleaning step was repeated twice. The sample tubes were placed in the desiccator for at least one hour before derivatisation.

TMS derivatisation

The dried samples were treated with 100 µL TMS and the tubes were sealed and vortexed for 30 seconds. The samples were left standing in room temperature for 30 minutes before centrifuging for a few minutes and transferring the supernatant to GC sample tubes.

2.4.6 Identification of monosaccharides by GC

Gas chromatography (GC) was used to identify the monosaccharides in the samples. The resulting peak areas were summed to provide a calculation of carbohydrate content.

In GC the mobile phase is a gas and the components are separated based on different affinity to the stationary phase. The components with greater affinity to the stationary phase will take longer time to elute than components with less affinity. This affinity to the stationary phase is dependent upon the components' structure and chemical properties (Harris 2007).

In order to identify monosaccharides in the samples by gas chromatography, standard curves were created beforehand. Such standard curves have been calculated for arabinose, ribose, rhamnose, xylose, mannose, galactose, glucose, glururonic acid and galacturonic acid, and were calculated by measuring the retention time of each monosaccharide with respect to the internal standard (D-mannitol).

Procedure

Analysis conditions

Carrier gas:	Helium
Column:	Restek capillary column (30m, 0.32 mm inner diameter, 0.25 µm film)
Injector temperature:	250°C
Detector temperature:	300°C
Injection mode:	Split (ratio: 1/10)
Injection volume:	1 µL
Flow mode:	Constant pressure (0.70 bar)
Temperature program:	140°C (initial)
	140°C-170°C at rate: 1°C/min
	170°C-250°C at rate: 6°C/min
Software:	Chromeleon version 6.8

Samples were prepared by methanolysis (described in section 2.4.5) and analysed by capillary gas chromatography (Thermo Scientific, Focus GC), with a Flame Ionization Detector (FID) and a split injector.

2.4.7 Determination of binding pattern in polysaccharides by methylation

The binding pattern of the polysaccharides in EIW-A was determined by methylation analysis.

This method was developed by Ciucanu and Kerek (Ciucanu & Kerek 1984) and modified by McConville et al (McConville et al. 1990), and involves the following steps: methylation, hydrolysis, reduction, acetylation and GC-MS.

The GC-MS detects the resulting partially methylated alditol-acetates, which are separated in the GC column and their respective masses are determined in the mass spectrometer. The mass spectrometer determines the mass of the compounds by bombarding them with electrons in vacuum, which results in fragmentation and ionisation of the alditol-acetates. This produces

characteristic mass spectra of each peak from GC, of which binding patterns can be deduced (Harris 2007).

Methylation

The initial step was methylation; free hydroxyl-groups on the polysaccharides were ionised with a base and were then methylated by a methylation reagent. The end result was the conversion of free OH-groups into O-CH_3-groups.

Procedure

A sample of EIW-A (3.4 mg) was collected in an acid washed methylation tube. It was added 500 µL dimethyl sulfoxide (DMSO), followed by shaking at 200 rpm for 20 minutes (Vibrax-VXR, IKA Labortecnik). A slurry of NaOH in DMSO (500 µL, approximately 120 mg/mL) was added with a SMI glass pipette. The tube was flushed with nitrogen gas, and shaken for 40 minutes. The sample was treated with 100 µL methyl iodide (CH_3I) and shaken at 200 rpm for 10 minutes. The latter step was repeated, prior to adding another 200 µL of methyl iodide and shaking for 20 minutes. The sample was then added 10 mL sodium thiosulfate (100 mg/mL) and 2 mL chloroform, mixed thoroughly (40 sec. on a whirlimixer) and centrifuged at 1000 g for 3 minutes. The aqueous phase was removed from the sample, and the remaining chloroform phase was washed with 4 x 5 mL dH_2O. The chloroform phase was then transferred to another methylation tube, and dried with nitrogen gas.

Hydrolysis

The methylated polysaccharides were hydrolysed to monosaccharides with trifluoroacetic acid (TFA).

Procedure

The sample was treated with 500 µL TFA, followed by flushing with nitrogen gas. It was then set to hydrolyse in a heating cabinet at 100°C for 2 hours, before it was cooled down and dried with nitrogen gas.

Reduction

Partially methylated monosaccharides were reduced to alditols by treatment with sodium borodeuteride ($NaBD_4$) in an alkaline environment. Sodium borodeuteride introduced a deuterium atom in the anomeric carbon, and thus made it possible to differentiate between C1 from C6 in the monosaccharide. This also resulted in more volatile, open ring structures of the monosaccharides.

Procedure

The sample was added 500 µL 2M NH_3, followed by 500 µL 1M $NaBD_4$. It was then thoroughly mixed and incubated in the heating cabinet at 60°C for 60 minutes. The superfluous reductant was eliminated by carefully adding 3 x 50 µL glacial acetic acid. The sample was dried on a SpeedVac (SPD121P, Thermo Scientific). Repeated removal of boric acid was carried out by first adding 2 x 2.5 mL 5% acetic acid in methanol, then 2 x 2.5 mL methanol (the sample was dried on SpeedVac between every step).

Acetylation

The partially methylated alditols were reduced to alditol-acetates with acetic anhydride. The resulting products were more lipophilic and contained an acetyl-group, which made them easier to separate by GC.

Procedure

The sample was added 200 µL 1-methyl imidazole, followed by 2 mL acetic anhydride. It was mixed well, and left standing for 10 minutes. To eliminate the surplus of acetic anhydride, 10 mL of dH_2O was added. The sample was mixed well and left standing for 10 minutes. Partially methylated monosaccharides were extracted from the sample with 2 x 1 mL dichloromethane (done by adding DCM, mixing well and centrifuging at 1000 x g for 3 minutes). The organic phase was transferred to a new methylation tube and washed with 2 x 5 mL dH_2O, followed by transferring the organic phase to a supelco. The sample was dried with nitrogen gas.
Just prior to GC-MS analysis, the sample was dissolved in 50 µL dichloromethane.
Hoai N.T. Aas performed the GC-MS analysis, and the spectrum analysis was performed in cooperation with Anne Berit C. Samuelsen.

2.4.8 SDS-PAGE

The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed to investigate whether there were any proteins present in the different extracts.

SDS-PAGE is based in the electrophoretic separation of proteins by size. SDS is a detergent that denatures the proteins' secondary and tertiary structure and covers them with negative charge. Thus, the proteins have charge proportional to their size and when applied to a gel electrophoresis, the larger proteins will migrate slower through the gel than the smaller ones (Jensen 2012). In order to provide an indicator of protein size, a standard containing proteins of known sizes is applied to the same gel as the samples.

Procedure

Two protein gels were run with the different AndoSan^{TM} extracts, using two different protocols. The first protocol used was developed by BioRad, with which the crude extracts were analysed.

The BioRad gel casting form was mounted, and a running gel with 12.5 % acrylamide (containing acrylamide, dH_2O, Tris, SDS, ammonium persulfate and TEMED) was cast according to protocol. dH_2O was applied on top of the gel and it was left to solidify for 45 minutes. The stacking gel with 4 % acrylamide was then cast and left to solidify for 50 minutes. The setup of the gels in the gel chambers followed the procedure set by BioRad.
The samples were prepared by mixing 50 µL sample (50 mg dry sample/mL dH_2O) with 50 µL of 2X sample buffer (0.125 M Tris-Cl, 0.14 M SDS, 20 % w/v glycerol, 2 % w/v DTT, 0.03 M bromophenol blue, dH_2O) followed by boiling the samples for 5 minutes. They were cooled down to room temperature, and 10 and 20 µL of each samples were applied to respective wells. The protein standard (Amersham LMW Calibration Kit, 17-0446-01, GE Healthcare) was also applied. The electrophoresis was run in 1X running buffer (0.25 M Tris, 1.92 M glycine, 34.7 mM SDS, dH_2O) at 200 V for 45 minutes. Following electrophoresis, the gel was added warm fixing solution (20 vol% methanol at 50 °C) for 10 minutes, and then warm colouring solution (1 g Coomassie R-250, 40 vol% methanol, 10 vol% acetic acid, dH_2O) for 20 minutes. The gel was washed with decolouring solution (10 vol% methanol, 10 vol% acetic acid, dH_2O) in order to remove non-specific colouring over night and then photographed.

The second protocol utilised, Amersham ECL Gel Box, was developed by GE Healthcare, and used to analyse EIW, EIW-A and EIW-B.

The gel was bought pre-cast, containing a 12 % acrylamide running gel and a 4 % stacking gel. It was mounted in the gel box and running buffer was made and added in accordance with protocol, before the samples (prepared in the same manner as above) were applied. The same standard as described above was also applied to both the first and last lane. The electrophoresis was run at 200 V for 50 minutes. The after treatment of the gel was the same as described above.

2.5 Incubation of RAW264.7 with AndoSan™ extracts

Legumain is an asparaginyl endopeptidase, and is overexpressed in some solid tumours where it is involved in tumour invasion and metastasis (Liu et al. 2003). The test system described here uses the RAW264.7 macrophage cell line, which under natural conditions produce some legumain. The aim was to look for changes in this legumain activity as a consequence of incubation with the different AndoSan™ fractions, and thus identify fractions exhibiting higher inhibitory activity.
The cell experiments were performed in a LAF-bench with a vertical laminar airstream in order to prevent microbial contamination of the cell cultures.

Procedure

Cell culture

RAW264.7 cells, a monocyte/macrophage cell line from *Mus musculus* (acquired from American Type Culture Collection), were grown in medium containing 500 mL DMEM (Dulbecco's Modified Eagle Medium, with 4 mM L-glutamine and 4.5 g/l glucose), 10 mL sodium bicarbonate, 50 mL foetal bovine serum and 5 mL penicillin (1000 U/mL)/streptomycin (10 mg/mL).

Splitting of cells

When the density of the cells in the growth flasks increased to an upper limit, they were split. The culture medium was removed and 10 mL of fresh medium were added. The cells were released

from the container wall with a cell scrape. 1 mL of the cell culture were then extracted and added to a new growth flask containing 12 mL of fresh medium. The passage number (i.e. the number of splits) was kept below 40.

Cell count

The cells were counted in order to determine the concentration of cells in the culture flasks. This was performed by an automatic cell counter (Countess Counter, Life Technologies Co.). A suspension of cells (15 µL) were added the same amount of trypane blue before it was transferred to the count chamber. The results were given with the number of living cells.

Casting of wells

Following the cell count, the cell suspension was diluted in culture medium to reach desired concentration of 500 000 cells/mL. 1 mL of this cell suspension together with additional 1 mL culture medium was cast in 6-well plates. The plates were incubated at 37°C and 5 % CO_2 for 24 hours, resulting in a final number of cells per well of 1 million.

Stimulation with AndoSan™ extracts

Following incubation, the medium was removed from the cell wells and 1 mL of serum-free medium (medium without FBS) was added in order to wash the cells free of serum. Following removal of washing-medium, 1 mL of the AndoSan™ extract preparations (dry powder dissolved in serum-free medium) at different concentrations was added to respective cell wells. The plates were then re-incubated for 48 hours at the same conditions as described above.

Extraction of cell lysates

The AndoSan™ extract solutions were removed from the wells, and 1 mL of phosphate buffered saline (PBS) was added in order to wash the cells free of sample. Lysis buffer (100 mM sodium citrate, 2 mM disodium EDTA and 1 % Octyl β-D-glucopyranoside/detergent) was then added to the wells, and cell scrapes were used to help release the cells from the wells. The amount of lysis buffer were adapted to the differing concentration of protein in the samples (the concentration had to be within the standard curve of the protein measurement, see section 2.5.1), and ranged from 200 µL to 1000 µL per well.

In order to ensure proper lysis of the cells, they were freeze-thawed; they were put in a freezer at -70°C for about 10 minutes and transferred directly to a heat block at 30°C. After repeating this twice, the lysates were centrifuged at 10 000 x g for 10 minutes at 4°C and the supernatant was transferred to new eppendorf tubes. The samples were kept in a freezer (-20 °C) prior to protein and legumain analysis.

2.6 Legumain assays and western blot

The legumain assay procedures were performed in order to indicate if incubation with AndoSan™ had any inhibitory or stimulatory effect on legumain expression in RAW264.7 cells, and if so, how the enzyme activity is affected by AndoSan™. The former was done by measuring legumain activity in cell lysates (their procurement is described in section 2.4), the latter was done by assessing whether AndoSan™ extracts inhibited the autoactivation of the enzyme at pH 4 or the activity of active enzyme.

The method by which legumain is measured was developed by the original work of Chen et al. and Johansen et al. (Chen et al. 1997; Johansen et al. 1999). An assay buffer containing the reduction reagent DTT (Cleland's reagent), which reduces the SH-groups of cysteine proteases and activates them, is added to the samples. A substrate (Z-Ala-Ala-Asn-NHMec, where NHMec is 4-methyl-7-coumarylamide) containing a fluorescent group (NHMec) is added to the mix. Active legumain can hydrolyse the peptide bond at the C-terminus of Asn in this substrate, and cause the release of NHMec (emits light at 460 nm). A fluorescence microplate reader can measure the change in this fluorescence over change in time, and thereby calculate legumain activity in each sample.

2.6.1 Legumain activity in cell lysates

The legumain activity in cell lysates was calculated by measuring the legumain activity (dF/dS) and dividing this by the amount of protein in the samples. This calculation was necessary in order to normalise the results with respect to a differing number of cells in each cell well.

Procedure

Measurement of total protein

The measurement of the total amount of protein in the samples was done by adding BioRad Protein assay reagent (contains Coomassie Brilliant Blue), which turns blue when bound to protein (absorbs light at 595 nm). A standard curve was constructed by measuring albumin of concentrations 0, 50, 100, 150, 200, 250 and 300 µg/mL, which were used by computer software to calculate the amount of protein in the samples.

Standards and samples (20 µL) were mixed with 200 µL of diluted (1:5) BioRad Protein Assay reagent in a clear 96 well plate (Corning Inc.). The reaction was left to settle for 5 minutes, before absorbance was detected by a microplate reader (Wallac Victor 1420 counter, Perkin Elmer Life and Analytical Sciences), and protein content was calculated by the Workout software.

Measurement of legumain

The measurement of active legumain was carried out in a similar fashion. Each sample, in addition to a control with 0.9 % NaCl, were pipetted (10 µL) to respective wells in a black 96 wells plate (Corning Inc.). The plate was inserted into the microplate reader, which was programmed to inject 100 µL 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). After 10 minutes (the time given for activation of legumain by DTT), 50 µL enzyme substrate was automatically injected and the fluorescence measurements started. These measurements were taken by measuring fluorescence in the wells 10 times, with 3 minutes lag between each measurement. The results were given by the Workout software, as a calculation of change in fluorescence divided by change in time (dF/dS).

2.6.2 Assay with active legumain

Active legumain (provided by Harald T. Johansen, isolated from ox kidney) was incubated with different concentrations of lyophilized AndoSan™ in the same type of plates used for fluorescence detection in cell lysates described above. The activity was measured in the same fashion as described above.

Due to dark colour in some of the samples with higher concentrations of AndoSan™, a control experiment was necessary to rule out quenching ("engulfing" of fluorescent signal by the colour in the samples). This was carried out by incubating the same samples with a fluorescent substrate (NHMec) giving a constant fluorescence when unaffected by quenching.

Procedure

The active enzyme was added (20 µL) to a black 96 wells plate. Each sample (concentrations ranged from 1 µg/mL to 1000 µg/mL) was added (17 µL) to respective wells, and the plate was inserted into the microplate reader. The latter was programmed to inject 83 µL legumain assay buffer, and wait 30 minutes before injecting 50 µL substrate and measuring the activity in the same manner as described in section 2.6.1.

The control experiment was carried out in an identical manner, but instead of incubating active enzyme and AndoSan™ with an enzyme substrate, a fluorescent group giving out constant fluorescence (the same intensity as the sample with the strongest fluorescent signal in the enzyme inhibition experiment) was incubated with the AndoSan™ samples.
This procedure was performed by Harald T. Johansen, and the results were given in decrease in fluorescence as a consequence of quenching.

2.6.3 Assay with prolegumain

The assay with prolegumain was performed in order to assess whether AndoSan™ and EIW inhibit the autoactivation of prolegumain at pH 4.
This was done by bufferswitching the cell medium containing prolegumain, using a PD-10 column packed with Sephadex™ G-25 medium (GE Healthcare, 17-0851-01) and sodium acetate buffer pH 4 (200 mM sodium acetate, 4 mM disodium EDTA). The prolegumain (200 ng/mL) was obtained from serum-free media of HEK (human embryonic kidney) 293 cells, designated M38L cells, which have been transfected with cDNA for human legumain (Berven et al. 2012)

The buffer switched prolegumain (undergoing autoactivation) was collected manually and incubated with different concentrations of AndoSan™ and EIW for 17 hours, before measuring legumain activity and performing western blot.

Procedure

The PD-10 column was washed with 25 mL buffer at pH 4 prior to applying 2.5 mL prolegumain to the column. When the prolegumain was contained in the column, another 3.5 mL buffer at pH 4 were added, and buffer switched prolegumain was collected. Prolegumain (180 µL) was added to 20 µL of each sample and a control containing 0.9 % NaCl and incubated at 37°C for 17 hours, before measuring legumain activity in the same manner as described in section 2.6.1.
In addition, 26 µL of each sample were collected for immunoblotting.

2.6.4 Western blot of prolegumain samples

Western blot was performed with the samples incubated with prolegumain in order to determine whether AndoSan™ and EIW inhibited the activation of prolegumain at pH 4. Any prolegumain present in the sample would result in the detection of a band at 56 kDa, whereas the active legumain would give a band at 46 kDa.

Western blotting involves the separation of proteins by size with SDS gel electrophoresis, followed by their transfer to a membrane using electric voltage and detection using antibodies (Jensen 2012). The detection of proteins involves the use of two antibodies; one primary antibody that is specific for the target protein (in this case legumain, AF2199, R&D Systems) and one secondary antibody (rabbit anti-goat IgG HRP-conjugate, P 0160, DAKO, Denmark) that bind to the primary antibody and that has a peroxidase attached to it. This peroxidase reacts with SuperSignal West Dura Extended Duration Substrate, which results in the release of luminescence. This luminescence is detected by a light sensitive film and captured by a scanner.

Procedure

The samples were prepared by transferring 26 µL to respective eppendorf tubes, followed by mixing them with 4 µL DTT and 10 µL NuPage® LDS sample buffer. The tubes were then put on a heat block at 70°C for 10 minutes.

A pre-cast NuPage® Bis-Tris 4-12 % protein gel (Life Technologies) was placed in the electrophoresis chamber (Novex Mini Cell, Invitrogen), and the chamber was filled with

NuPage® MOPS SDS running buffer and 500 µL NuPage® antioxidant (to neutralise the reduction agent, DTT). Each of the samples, in addition to two control samples containing NaCl instead of AndoSan™ or EIW (one right after buffer switch, t_0, and one after the 17 hours incubation, t_{17}), were applied (10 µL) to respective wells, before running the electrophoresis at 200 V and 110 mA for 1 hour. Following electrophoresis, the gel was placed in a "sandwich" between filter paper and membrane (3 filter papers, membrane, gel, 3 filter papers), all pre-moistened in blotting buffer (25 mM Tris, 192 mM glycine, 20 % methanol).

The proteins were then transferred from the gel to the membrane with a TE 70 ECL Semi-Dry Transfer Unit (Amersham Biosciences) at 30 V and 32 mA in blotting buffer for 1 hour.
To avoid non-specific binding of antibody to the membrane, it was covered with milk proteins by washing with 5 % fat-free dry milk in 1 x T-TBS buffer (0.05 mM Tris, 0.9 % NaCl, 0.05 % Tween 20) for 1 hour. Legumain primary antibody was diluted 1:1000 in 5 % dry milk in 1 x T-TBS and added to the membrane, which was left over night at 4°C. The membrane was then washed thoroughly with 1 x T-TBS buffer; 3 x 10 minutes. Secondary antibody was diluted 1:5000 in 2.5 % fat-free dry milk in T-TBS and added to the washed membrane, which was left for 1 hour. A final washing with 1 x T-TBS was performed (3 x 10 minutes) before scanning the membrane.
Induction of signals was performed using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific), in accordance with the protocol given by the manufacturer and detected by ChemiDoc™ XRS+ molecular imager with Image Lab™ software (BioRad).

2.7 Statistics

Statistical analysis of the effect of AndoSan™ extracts on legumain activity was utilised in order to state whether a reduction in activity had any statistical significance, and to investigate whether any extracts had significantly higher inhibitory effect than others. For this purpose Student's t-test was used.

The legumain activity divided by protein content for each sample was compared to the activity in the respective control sample, or to the activity in another sample from the same experiment. The tests were performed with 95 % significance, i.e. $\alpha = 0.05$. Thus, inhibitory activities of the extracts were considered significant if the corresponding p-values were less than 0.05 ($p < 0.05$).

Procedure

The hypotheses tested were formulated as follows:

H_o: There is no significant reduction in legumain activity $\mu = 0$

H_1: There is a significant reduction in legumain activity $\mu < 0$

The null hypothesis was rejected if the p-value was less than 0.05.

For the calculation of each p-value, the software R (downloaded from http://www.r-project.org) was used. The UMB library was loaded, making the R Commander available and the data for each pair was entered as new data set. Two samples t-test was chosen from the statistics menu, followed by choosing the sample column as first variable and the control column as the second. The null hypotheses were set as described above and tested with a confidence level of 95 %.

3 Results

3.1 Extractions of chemical components

AndoSan™ was initially lyophilized with a yield of 4.5 mg/mL (method 2.1), and its components were extracted with different solvents; dichloromethane, methanol, ethanol and water (method 2.2). The water extract was separated into two fractions, one ethanol soluble water extract (ESW) and one ethanol insoluble water extract (EIW). Table 3.1 shows the respective yield of each extraction.

Table 3.1 The yield in percent and grams of extracted material from the initial sample amount (30.11 g freeze-dried AndoSan™)

	Extracted material	**Yield %**
Dichloromethane extract	0.1039 g	0.4
Methanol extract	3.7034 g	12.3
Ethanol extract	5.4598 g	18.1
Ethanol insoluble water extract	3.5828 g	11.9
Ethanol soluble water extract	7.0125 g	23.3
Insoluble residue	2.3552 g	7.8

The largest fraction is ESW with a yield of 23.3 %, followed by the ethanol extract (18.1 %), methanol extract (12.3 %), EIW (11.9 %) and the DCM extract (0.4 %). The insoluble residue constitutes 7.8 %, and was not used in any experiments due to most experiments involving samples dissolved in water or aqueous solutions. Furthermore, the low yield of the DCM extract prevented further experimental focus on this extract. The combined yields only constitute 73.5 % of the initial sample material, and the loss of sample material was 26.5 %.

3.2 Characterisation of crude extracts

Several characterisation analyses were utilised in order to do compositional estimates of the extracts, including ^{1}H NMR, thin layer chromatography (water extracts only), UV-absorption, carbohydrate analysis by methanolysis and SDS-PAGE.

3.2.1 ^{1}H NMR

The extracts were analysed by ^{1}H NMR according to method 2.4.1. The results are given as spectra for each extract with corresponding compositional analyses.

Dichloromethane extract

The DCM extract was dissolved in deuterated methanol and analysed by ^{1}H NMR. The resulting spectrum is presented in figure 3.1.

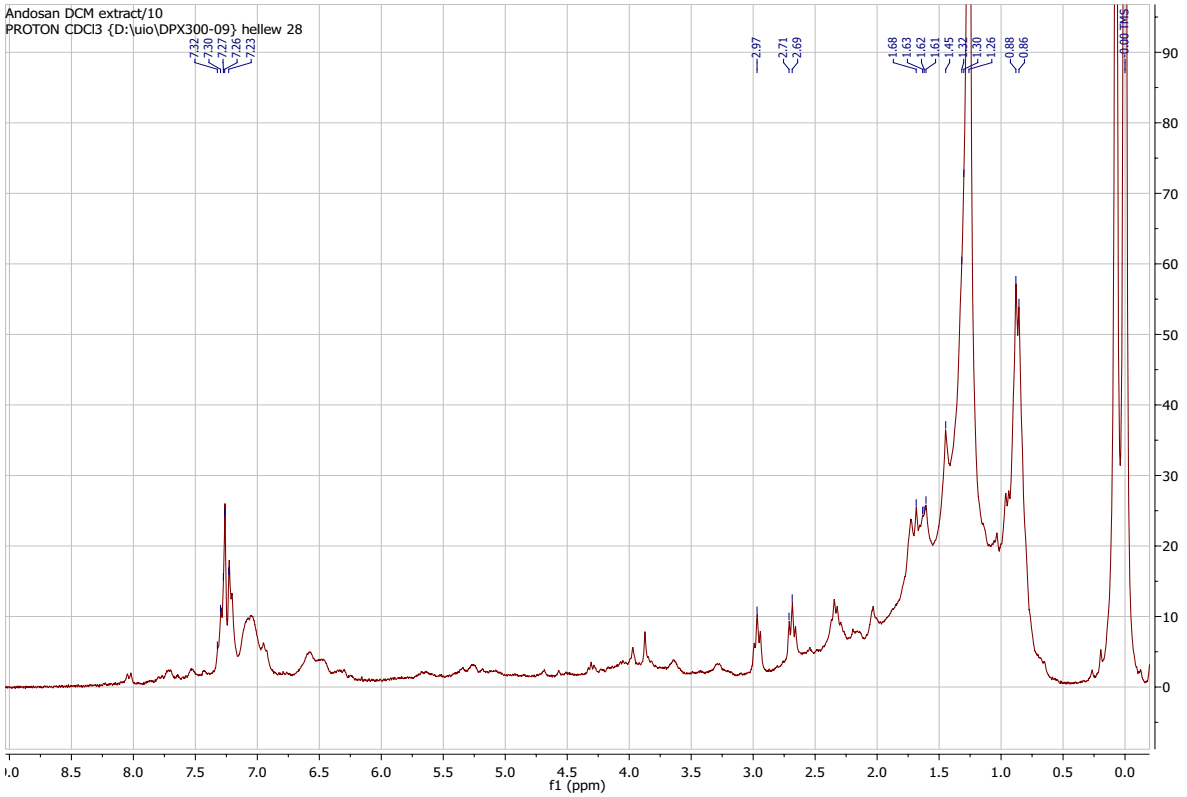

Figure 3.1 The ^{1}H NMR spectrum of the DCM extract

The ^{1}H NMR spectrum of the DCM extract (figure 3.1) shows relatively many peaks due to this being a crude extract. The peaks at < 2 ppm are identified as different fat molecules; the ones at 0.86/0.88 are CH_3- groups, and the one at 1.26 is -CH_2- groups. The many signals in this area can also be other aliphatic structures. There are some peaks in the area for aromatic compounds (between 7.0 and 7.5 ppm), which could be caused by compounds such as phenols and alkenes.

Methanol extract

The methanol extract was dissolved in deuterated methanol and analysed by ^{1}H NMR. The resulting spectrum is presented in figure 3.2.

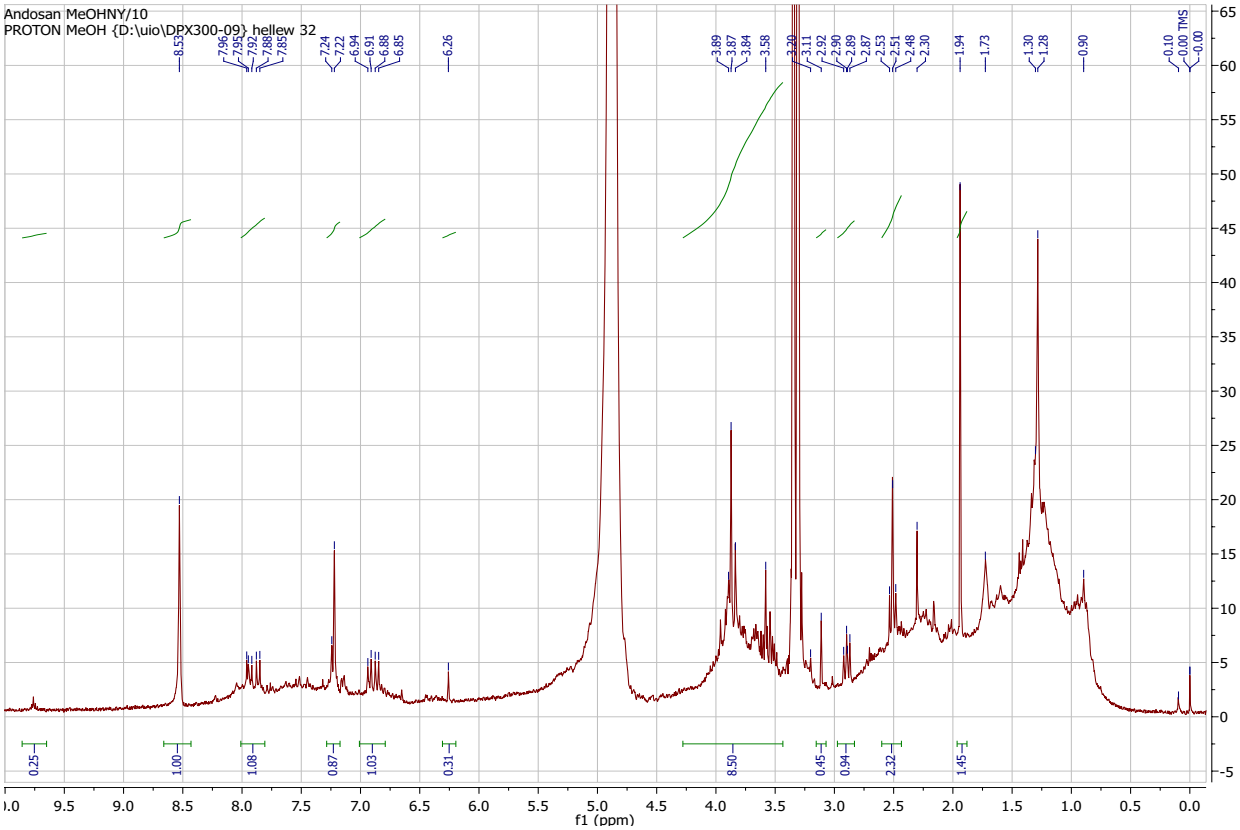

Figure 3.2 The ^{1}H NMR spectrum of the MeOH extract

The methanol extract is also a crude extract, and thus results in many peaks on the ^{1}H NMR spectrum (figure 3.2). The peaks between 3.5 and 4.2 ppm are low molecular carbohydrates, whereas the large peak at approximately 4.9 ppm is H_2O and the one at 3.3 ppm is methanol (both solvents). The extract also contains some aliphatic components, as can be seen by the peaks in the area below 2 ppm, in addition to some aromatic components detected in the area between 7.0 and 8.5 ppm. The latter may be phenols or alkenes.

Ethanol extract

The ethanol extract was dissolved in deuterated methanol and analysed by ^{1}H NMR. The resulting spectrum is presented in figure 3.3.

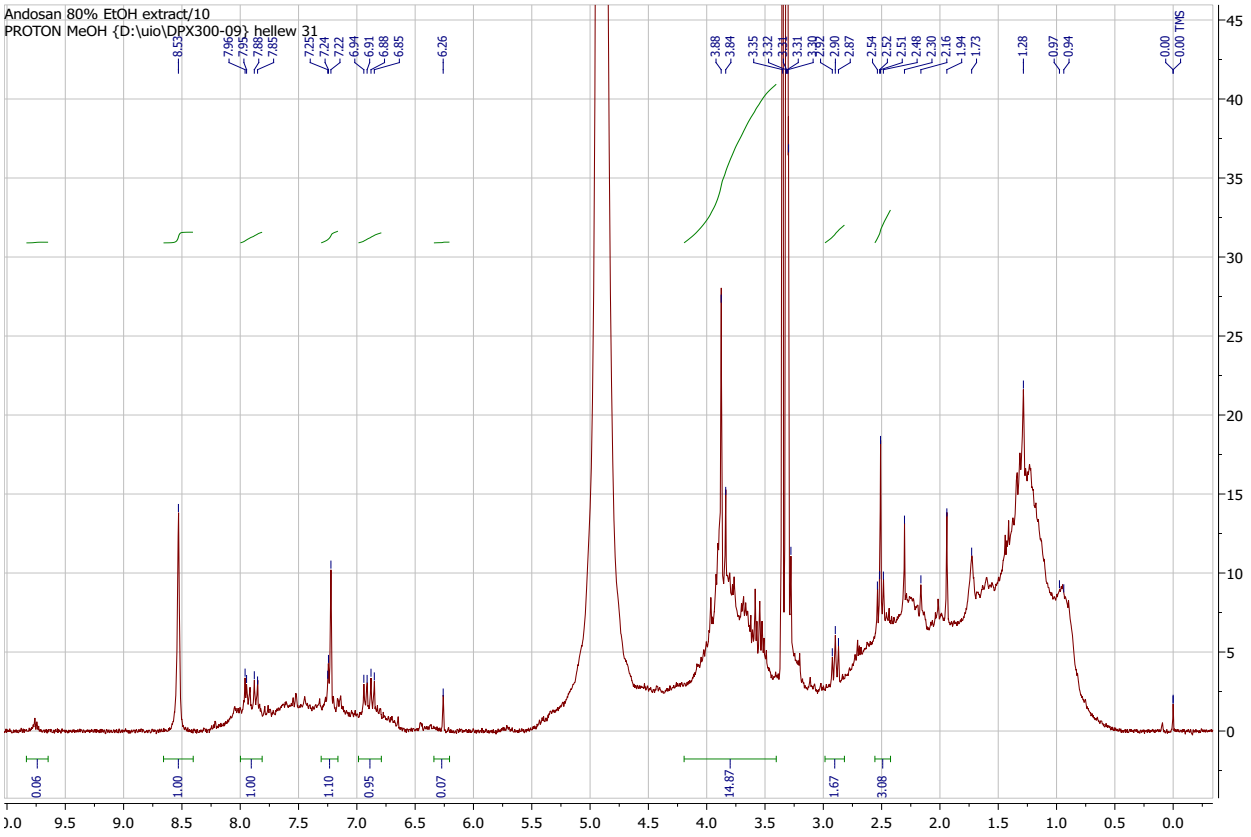

Figure 3.3 The ^{1}H NMR spectrum of the 80 % ethanol extract

The ^{1}H NMR spectrum of the ethanol extract (figure 3.3) is very similar to the spectrum from the methanol extract (figure 3.2), with many coinciding peaks; the large peak at approximately 4.9 ppm is H_2O and the one at 3.3 ppm is methanol (both solvents).

The low molecular carbohydrates are shown in the peak area between 3.5 and 4.2 ppm, and the extract also shows peaks in the aliphatic area (< 2 ppm) and aromatic area (7.0 – 8.5 ppm).

Ethanol soluble water extract

ESW was dissolved in deuterated water and analysed by ^{1}H NMR. The resulting spectrum is presented in figure 3.4.

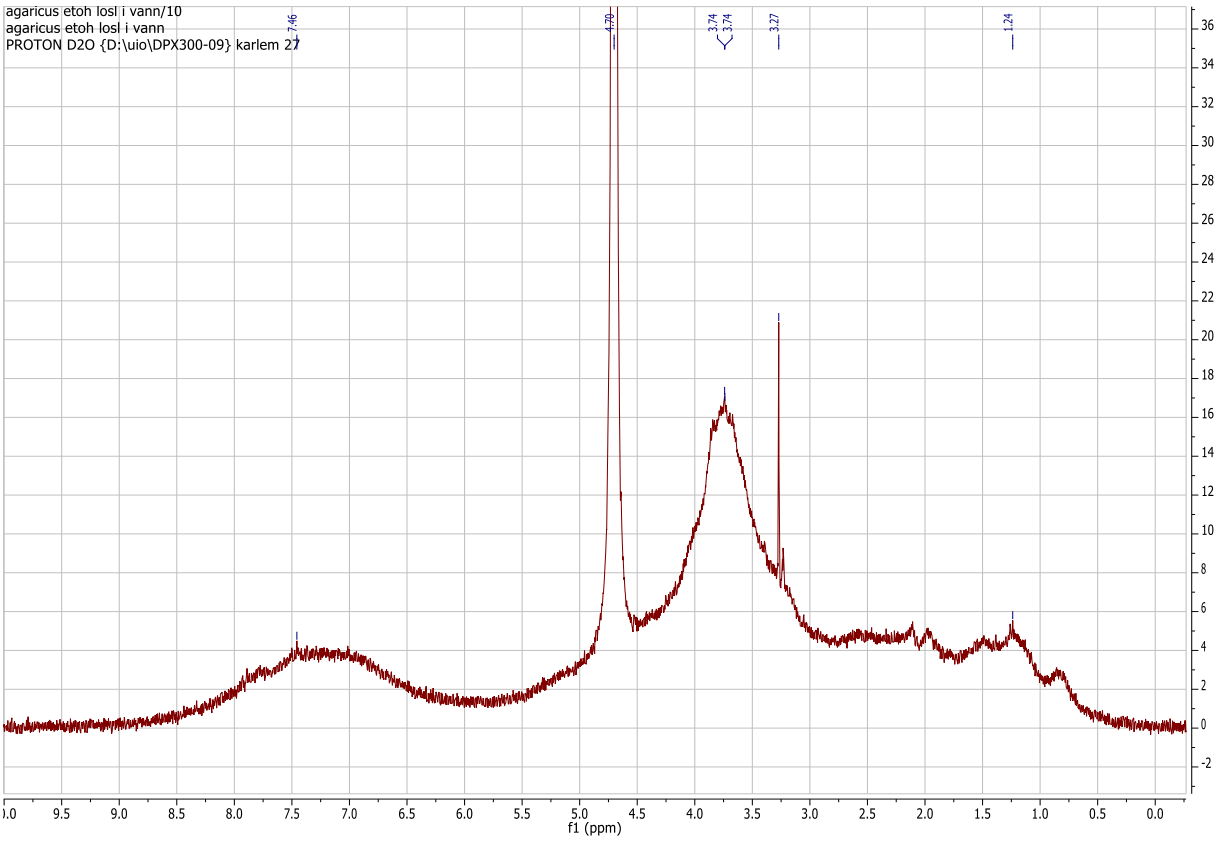

Figure 3.4 The ^{1}H NMR spectrum of ESW

The ^{1}H NMR spectrum of ESW (figure 3.4) does not contain any sharp peaks other than H_2O (at approximately 4.9 ppm) and methanol (3.2 ppm), but indicates that the main component is carbohydrates, as can be seen with peaks between 3.5 and 4.2 ppm.

The extract also contains some aliphatic structures (small peaks at < 2 ppm) and the broad peak in the area 6.5 to 8 ppm indicates the presence of some phenolic or aromatic compounds or alkenes.

Ethanol insoluble water extract

EIW was dissolved in deuterated water and analysed by ^{1}H NMR. The resulting spectrum is presented in figure 3.5.

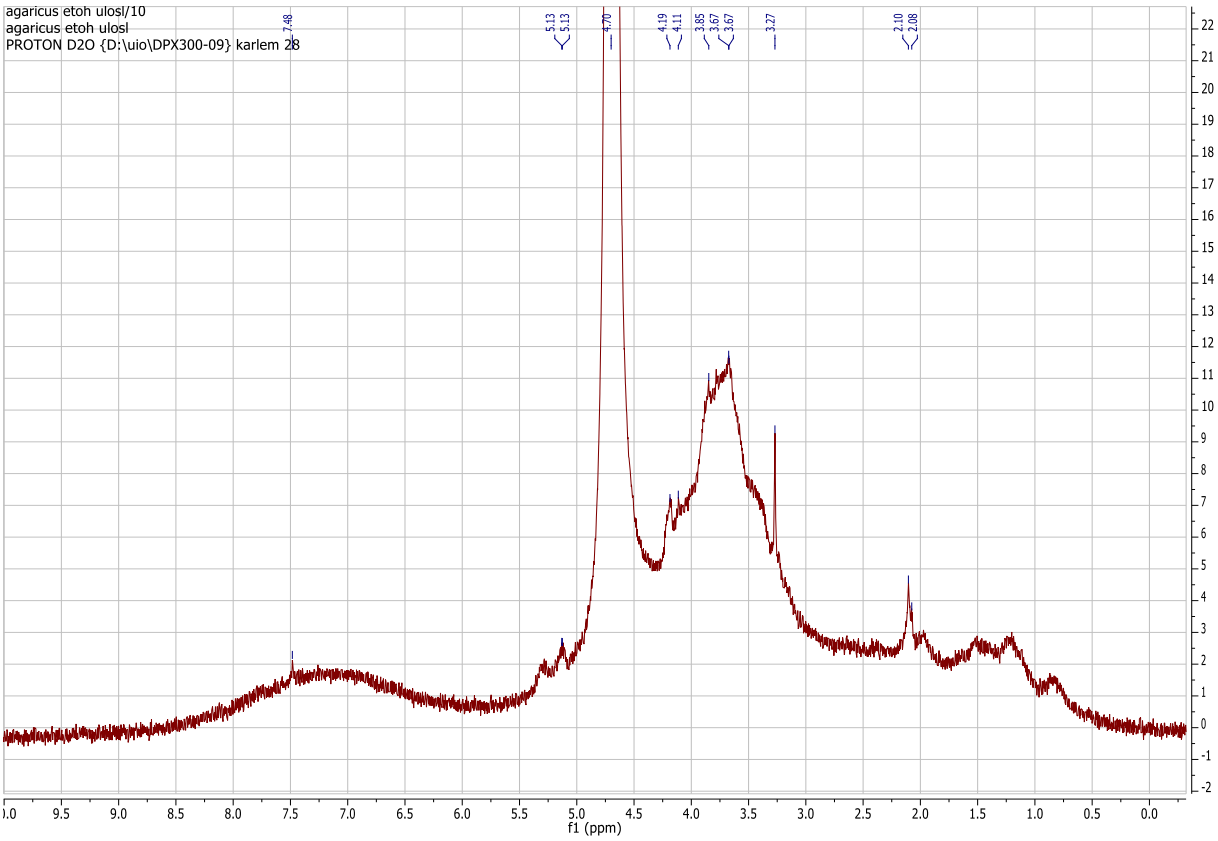

Figure 3.5 The ^{1}H NMR spectrum of EIW

The ^{1}H NMR spectrum of EIW (figure 3.5) resembles that of ESW (figure 3.4), in that carbohydrates are seemingly the main component (peaks between 3.5 and 4.2 ppm) and that it contains some aliphatic structures (peaks < 2 ppm) and aromatic/phenolic structures or alkenes (peak 6.5 – 8 ppm). It differs in that this extract gives two small peaks just prior to the dH_2O peak (at approximately 4.9 ppm), which are protons attached to anomeric carbons (C-1) in carbohydrates. These anomeric protons at about 5.1 and 5.25 ppm are in the α configuration. In order to determine the presence of anomeric protons in the β configuration, the analysis needs to be carried out at a higher temperature. This will make the large H_2O peak at 4.9 ppm shift, and reveal possible β-anomeric signals in this area.

3.2.2 TLC

ESW and EIW were analysed by reverse phase thin layer chromatography (TLC, method 2.4.2). Two plates were run with respective mobile phases, one more polar then the other. Reverse phase TLC utilizes a non-polar stationary phase, on which a more polar mobile phase will move more rapidly than a less polar mobile phase. The two mobile phases used were methanol in dH_2O (1:2) and methanol in dH_2O (1:9) with a drop of trifluoroacetic acid (TFA). The latter were thus more polar then the former. The plates were detected in short-wave UV (254 nm), long-wave UV (366 nm) and with 1 % $Ce(SO_4)_2$ in 10 % aqueous H_2SO_4 ("Ceric", a detection reagent for different organic compounds). The resulting plates are presented in figure 3.6.

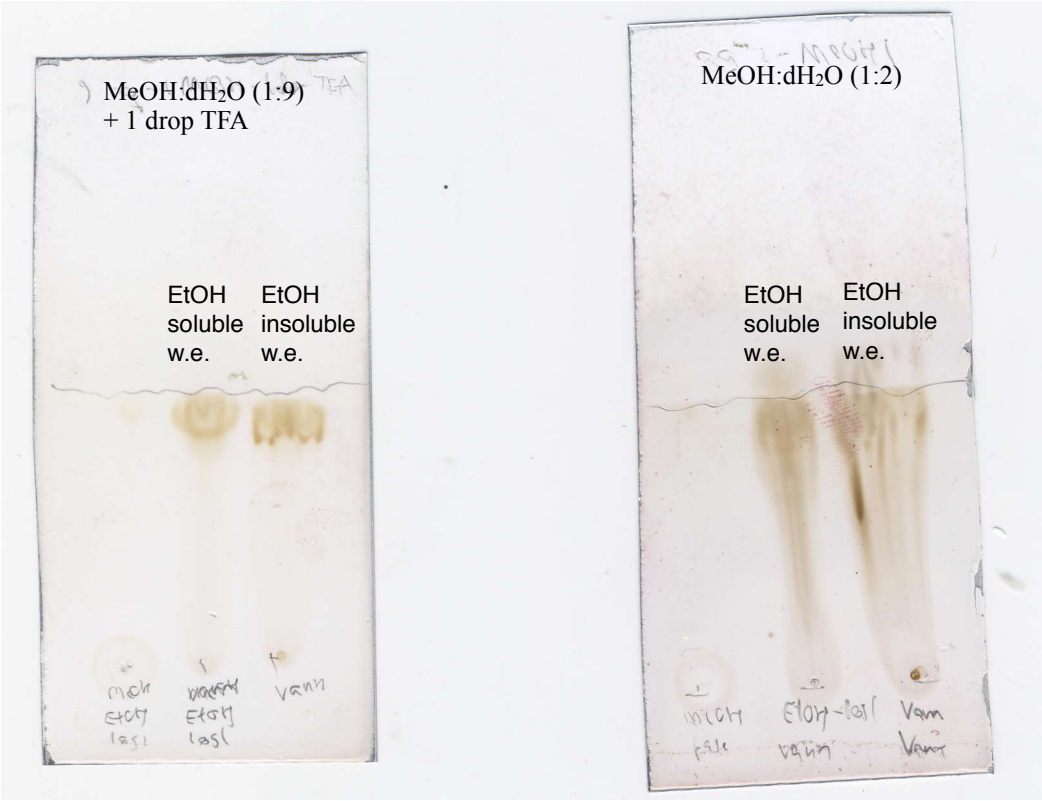

Figure 3.6 The TLC plates of the ESW and EIW

Components were detected only in front with the more polar mobile phase, i.e. the retardation factor (the fraction of an analyte in the mobile phase) is close to 1. This indicates that the components in the samples are highly polar. The Ceric reagent did not detect any organic compounds, probably due to all components being in front.

3.2.3 UV-scan of crude extracts

The crude fractions (except the DCM extract) were scanned for absorption of UV light in the region between 200 nm and 900 nm (method 2.4.4). Absorption between 250 nm and 300 nm would indicate the presence of aromatic compounds/phenols.

Neither of the two water extracts showed any absorption of UV between 250 and 300 nm, but the two alcohol extracts gave absorption curves that indicated presence of small amounts of aromatic components. Their absorption spectra are given in figures 3.7 and 3.8.

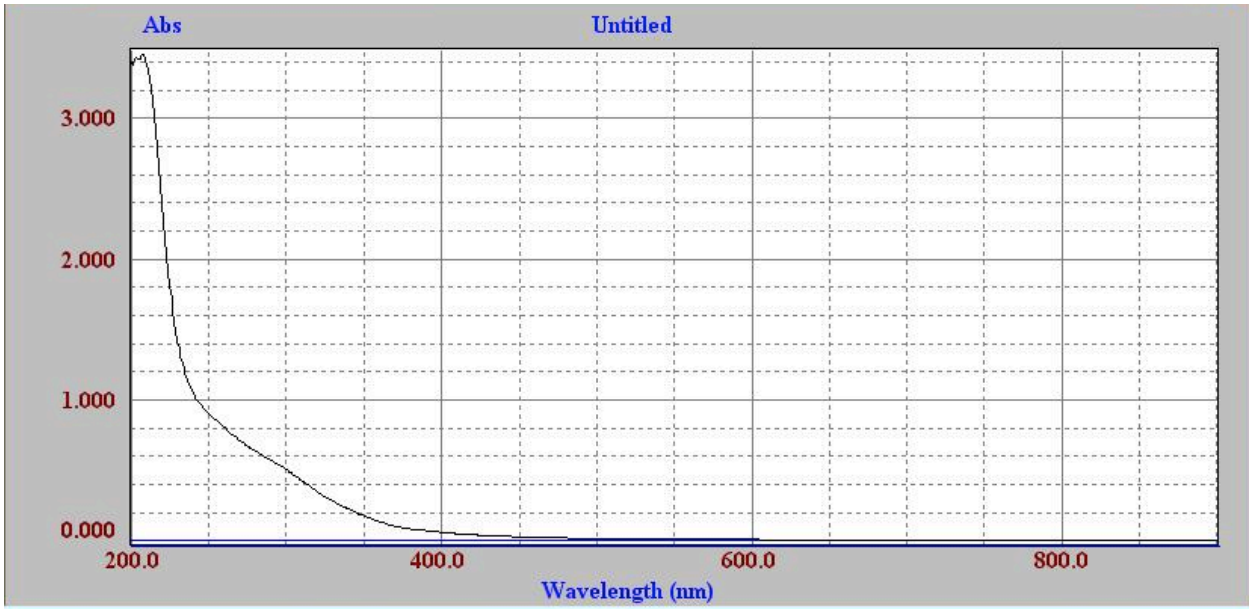

Figure 3.7 The UV wave scan of the MeOH extract

The UV wave scan of the MeOH extract shows a slight “shoulder” in the absorption graph at approximately 300 nm, indicating the presence of aromatic structures.
There is absorption in the region below 250 nm, but this region is of no use for identification purposes.

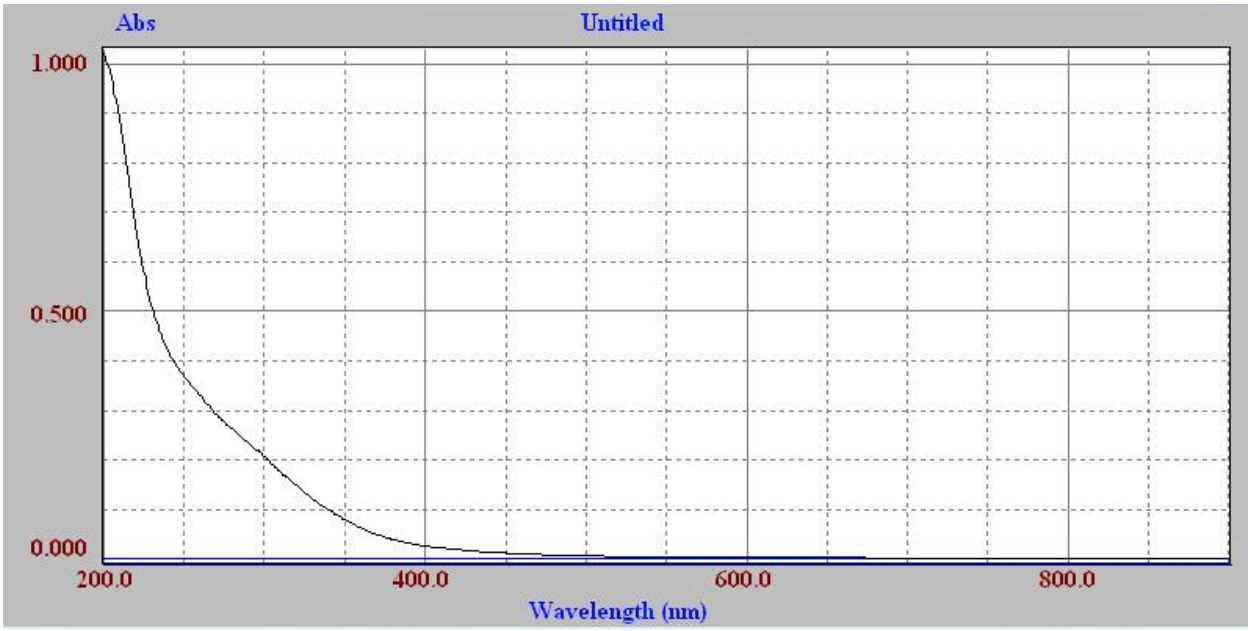

Figure 3.8 The UV wave scan of the EtOH extract

The EtOH extract also shows a slight “shoulder” in the absorption graph at approximately 300 nm, indicating the presence of aromatic compounds.

3.2.4 Carbohydrate analyses

The crude extracts were analysed for monosaccharide composition by methanolysis (method 2.4.5) and gas chromatography (method 2.4.6).
Mannitol was used as internal standard, and monosaccharides were identified based on standard curves constructed beforehand from retention times of known monosaccharides relative to the internal standard.

The content of each monosaccharide in each sample was estimated by calculating their respective peak area, and all the peak areas were summed in order to provide a calculation of total carbohydrate content in the samples.

Table 3.2 shows the calculation of carbohydrate content in the different extracts.

Table 3.2 The carbohydrate content in the different crude extracts of AndoSan™

	Percent carbohydrate in sample
Unfractionated AndoSan™	*2.0*
Methanol extract	5.7
Ethanol extract	1.6
Ethanol soluble water extract	1.7
Ethanol insoluble water extract	6.3

It is evident that AndoSan™ contains very little carbohydrate; the unfractionated lyophilized AndoSan™ contains only 2 % carbohydrate, which corresponds to 0.09 mg carbohydrate per mL of AndoSan™.

The extracts show varying carbohydrate content; the EtOH extract and EIW contains only 1.6 % and 1.7 % carbohydrate, respectively, whereas the MeOH extract and ESW contains 5.7 % and 6.3 % carbohydrate.

The MeOH and EtOH extracts contain monosaccharides and disaccharides, in correlation with their extraction methods, and the water extracts contain larger, more complex carbohydrates (polysaccharides, e.g. β-glucans).

The monosaccharide compositions of the polysaccharides in the different extracts are presented in table 3.3.

Table 3.3 The percentage distribution of monosaccharides in the carbohydrates detected in the extracts.

	Unfractionated AndoSan™	**MeOH extract**	**EtOH extract**	**ESW**	**EIW**
Arabinose	19.4	11.1	31.4	19.4	10.6
Galactose	10.0	16.6	2.4	8.5	17.3
Galacturonic acid	8.3	13.2	0.3	Trace	11.6
Glucose	23.0	21.8	31.3	17.9	22.7
Glucuronic acid	Trace	2.1	8.3	0.6	3.0
Mannose	6.8	9.8	1.1	7.4	9.5
Rhamnose	6.8	6.6	6.1	8.0	6.2
Ribose	n.d.*	n.d.*	Trace	Trace	n.d.*
Xylose	25.6	18.7	19.0	38.2	19.1

*n.d. = not detectable

The polysaccharides in unfractionated AndoSan™ contain a mixture of several monosaccharide units, where the three most abundant ones are xylose (26 %), glucose (23 %) and arabinose (19 %). The product was also found to contain some galactose (10 %), galacturonic acid (8 %), mannose (7 %) and rhamnose (7 %).

The different extracts show varying composition. The MeOH extract contains mostly glucose (22 %), xylose (19 %) and galactose (17 %), but also galacturonic acid (13 %), arabinose (11 %) and mannose (10 %), and small amounts of rhamnose and glucuronic acid.
The EtOH extract contains mostly arabinose (31 %) and glucose (31 %), but also xylose (19 %), glucuronic acid (8 %) and rhamnose (6 %), and small amounts of galactose, galacturonic acid and mannose.

ESW contains mostly xylose (38 %), but also glucose and arabinose (18 % and 19 %) and small amounts of rhamnose, mannose, galactose and glucuronic acid.
EIW contains mostly glucose (23 %), xylose (19 %) and galactose (17 %), but also arabinose (11 %), galacturonic acid (12 %) mannose (10 %) and small amounts of rhamnose and glucuronic acid.
Only trace amounts of ribose were found in the EtOH extract and in ESW.

3.2.5 SDS-PAGE

SDS-PAGE was performed with the crude extracts for detection of proteins present in the samples (method 2.4.8). SDS-PAGE separates proteins by sizes, which were detected using the Coomassie.

The resulting gel is presented in figure 3.9.

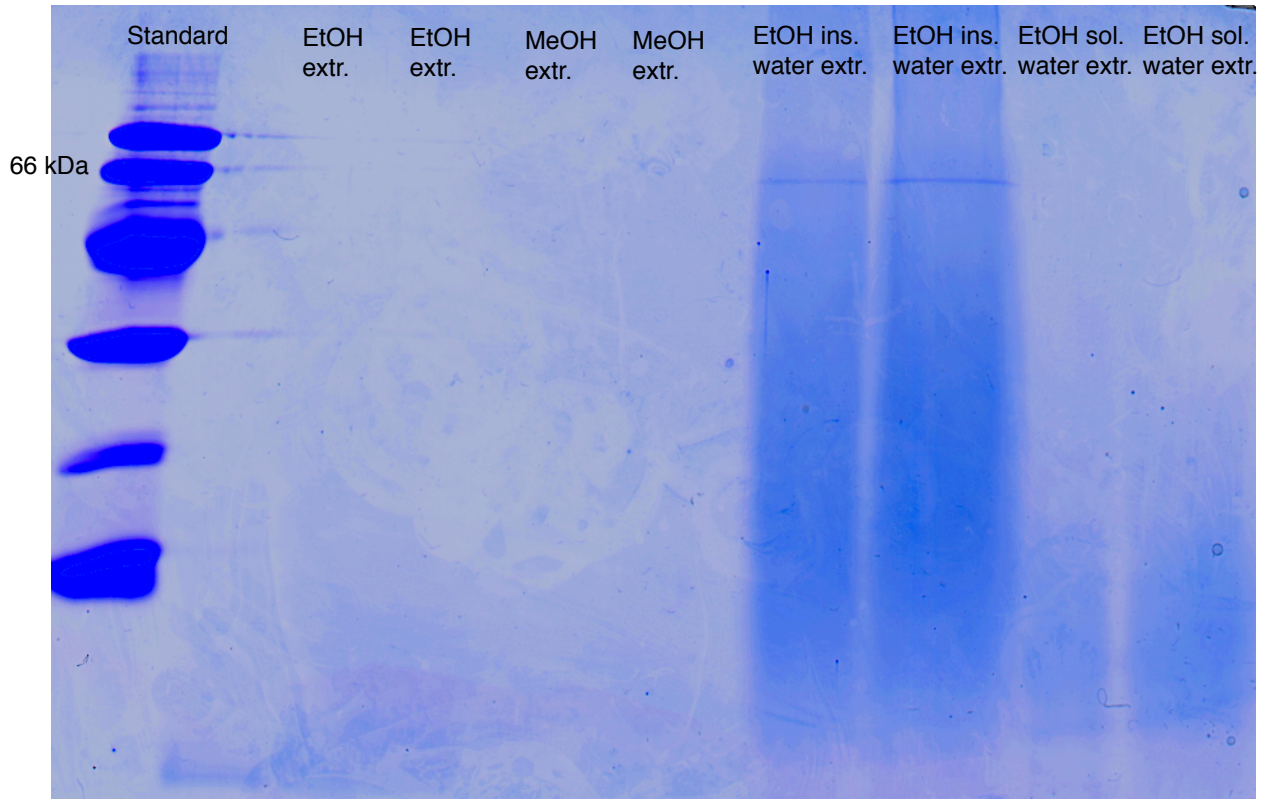

Figure 3.9 Protein detection by SDS-PAGE analysis of the crude extracts.

The only fraction was shown to contain any protein by this method is EIW (figure 3.9). In both lanes there are protein bands coloured with Coomassie approximately juxtaposed to the 66 kDa ladder.

There was no detection of protein in the other lanes.

3.3 Legumain assays with AndoSan™ crude fractions

The legumain assays were performed in order to investigate the inhibitory properties of AndoSan™ on legumain activity in RAW264.7 cells, in addition to how this inhibitory effect is mediated. The latter was done by incubation of AndoSan™ with both active enzyme and proenzyme, respectively.

3.3.1 Inhibition of legumain activity in RAW264.7 cells

The RAW264.7 cells were incubated with unfractionated AndoSan™ and the crude extracts for 48 hours before they were lysed (method 2.5).

Both protein content and enzyme kinetics were measured in the lysates (method 2.6.1), and legumain activity for each sample was calculated by dividing the resulting change in fluorescent over change in time (dF/dS) by the protein content. This calculation was necessary in order to normalise the measurements with respect to differing number of cells in the wells, and thus make them comparable.

The resulting activities are presented as percentages of the enzyme activity in the control cells (incubated with serum-free medium instead of AndoSan™ fractions), and the inhibition of legumain activity was considered statistically significant if the corresponding p-value was less than 0.05, as calculated by Student's t-test (method 2.7).

The results are presented in figure 3.10.

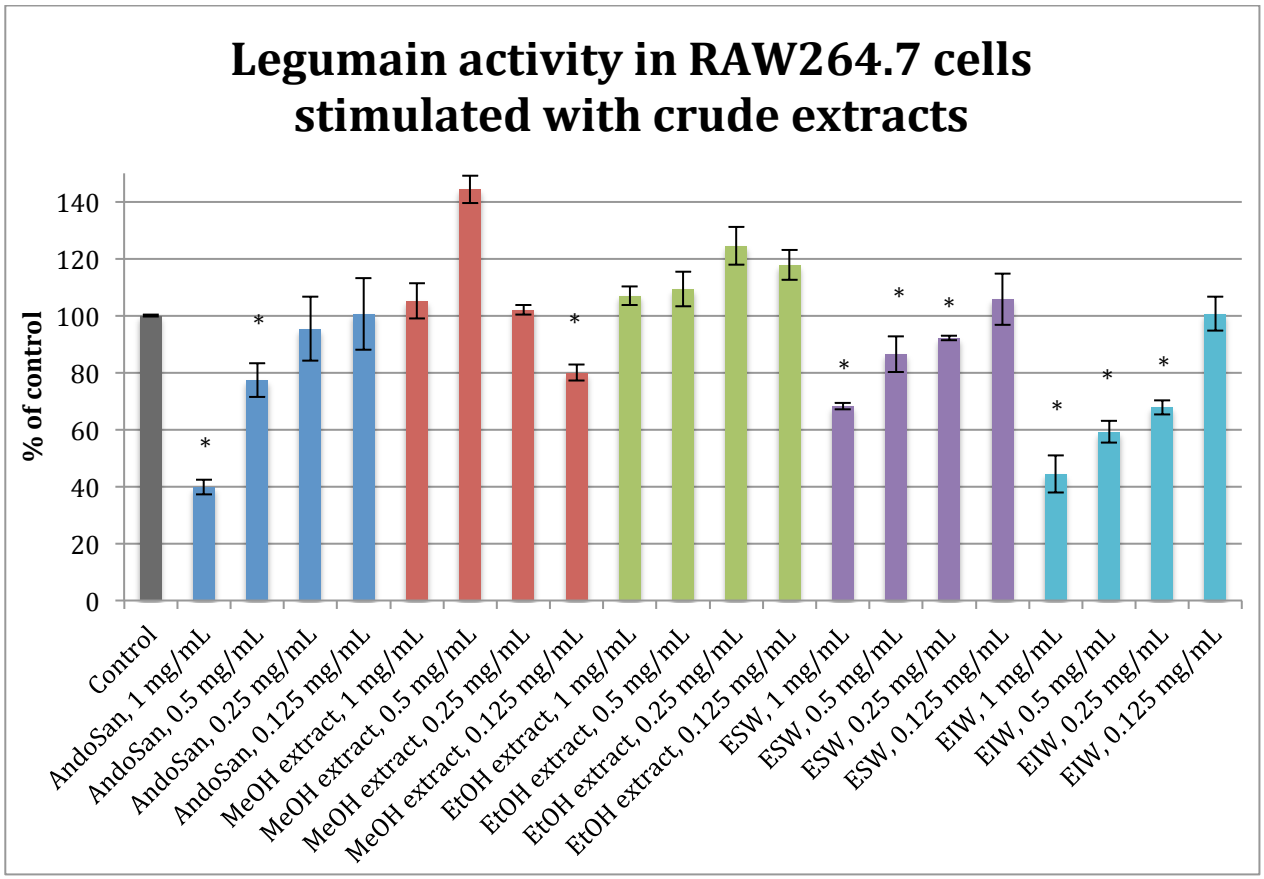

Figure 3.10 The legumain activity in RAW264.7 cells incubated with different extracts of AndoSan™ at different concentrations. The results are calculated as percent activity with respect to control. The results are representative for two independent cell experiments. Bars denoted with an asterisk are significantly lower than the control, $p < 0.05$.

Incubation of RAW264.7 cells with crude extracts of AndoSan™ resulted in a statistical significant inhibition of activity by unfractionated AndoSan™ and the two water extracts at concentrations 1 mg/mL and 0.5 mg/mL. ESW and EIW also exhibit significant inhibition at concentrations of 0.25 mg/mL.
Unfractionated AndoSan™ and both the water extracts show a clear dose-response in legumain activity (figure 3.10).

The extract exhibiting the most potent inhibition of legumain activity is EIW, which is also demonstrated by performing a t-test of EIW against ESW ($p < 0.05$ at concentrations 1, 0.5 and 0.25 mg/mL). For this reason, EIW was chosen for fractionation by size exclusion chromatography.

The MeOH and EtOH extracts show no significant inhibition of activity, except the MeOH extract at 0.125 mg/mL, and were therefore not considered for further separation and purification.

3.3.2 Inhibition of active legumain

Active legumain was incubated with different concentrations of unfractionated AndoSan™ for 30 minutes before measuring enzyme activity (method 2.6.2). A control experiment incubating the same AndoSan™ samples with a fluorescent group was performed by Harald T. Johansen, in order to measure quenching of fluorescent signal by the colour in the AndoSan™ samples. The results from the enzyme activity were given as dF/dS, whereas the results from the control experiment were given in fluorescence units. For this reason, both the enzyme activity and the quenching activity were recalculated as activity in percent of the control sample (0.9 % NaCl instead of AndoSan™).

The enzyme activity was corrected for quenching by subtracting the percentage inhibition caused by quenching from the inhibition caused by AndoSan™, which is equivalent to the formula:

$$\text{I:} \qquad Y = i_{ASx} - i_{Qx}$$

Where i_{ASx} is the inhibition of legumain activity by AndoSan™ at concentration x, and i_{Qx} is the inhibition of fluorescent signal caused by quenching in AndoSan™ at concentration x.

The calculations are presented in figure 3.11.

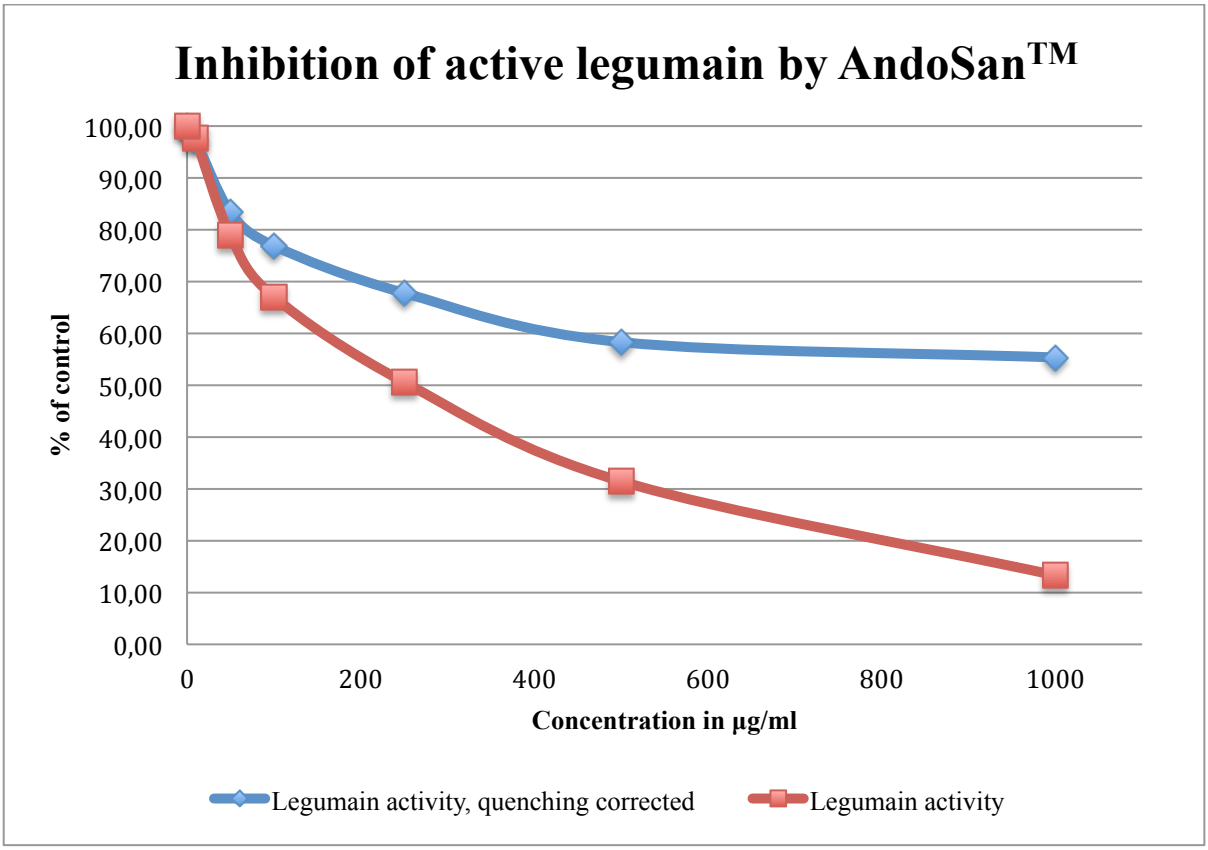

Figure 3.11 The inhibition of active legumain by different concentrations of AndoSan™, measured in percent of the activity in the control sample (0.9 % NaCl, enzyme and substrate). The legumain activity corrected for quenching was calculated by using equation I.

Despite quenching having a significant impact on the fluorescence measurements (~ 40 % quenching with a concentration of 1000 µg/ml), it is clear that AndoSan™ inhibits active legumain even at very low concentrations (16 % inhibition at a concentration of 50 µg/mL). At concentrations between 500 µg/mL and 1000 µg/mL, the activity of active legumain is reduced by approximately 40 %, where it seems to flatten despite increasing concentration of inhibitor/AndoSan™.

3.3.3 Inhibition of autoactivation of prolegumain and corresponding western blot

Different concentrations of AndoSanTM and EIW were incubated with autoactivated prolegumain, as performed by bufferswitching using a PD-10 column and buffer pH 4 (method 2.6.3). After 17 hours incubation, the samples were analysed for enzyme kinetics and by immunoblotting.

The reduction of legumain activity as a consequence of incubation with AndoSan™ and EIW is presented in figure 3.12.

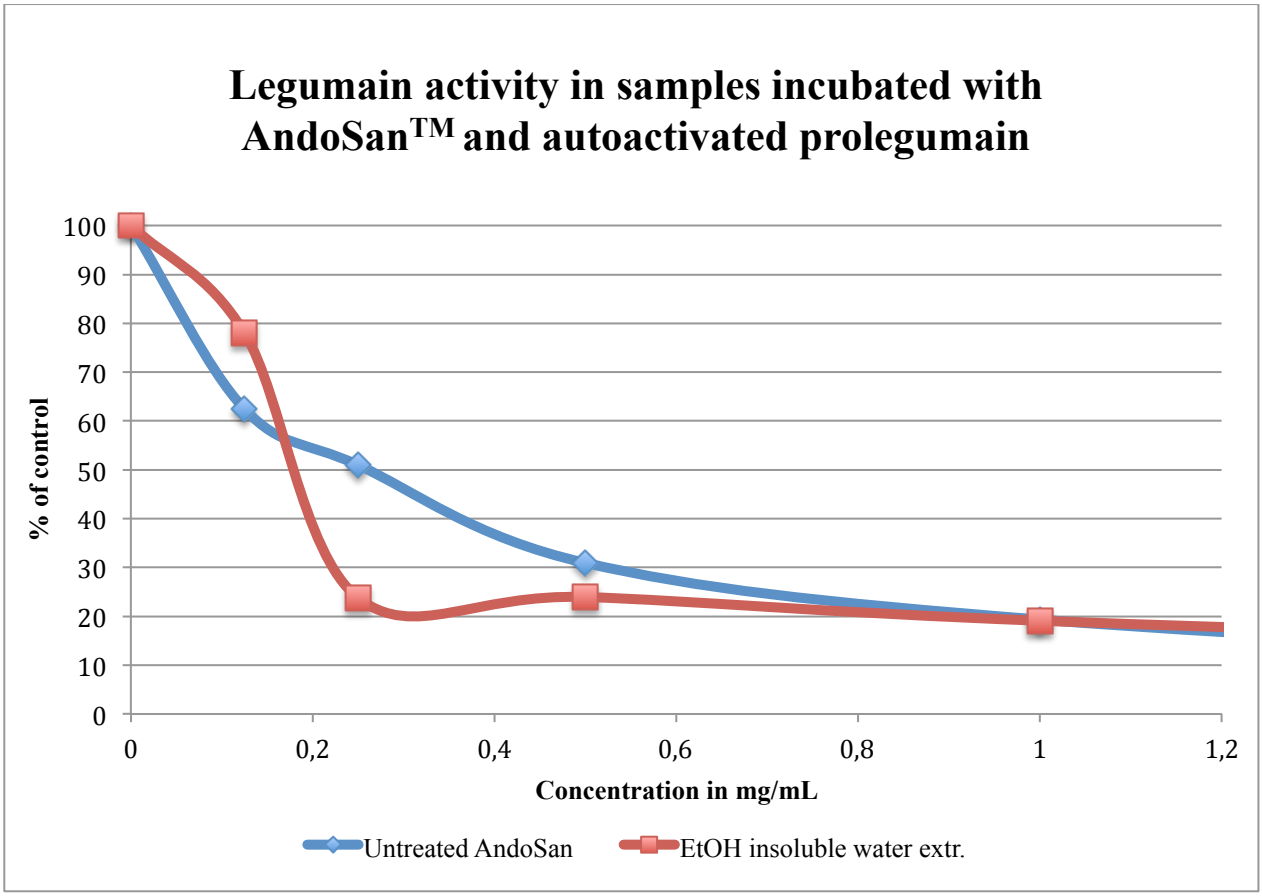

Figure 3.12 The reduced legumain activity in samples incubated with autoactivated prolegumain (pH 4) and different concentrations of AndoSan™ (blue) or EIW (red), measured in percent of control (n = 2).

The results from the incubation with prolegumain indicates that autoactivation of prolegumain at pH 4 is strongly inhibited by both AndoSan™ and EIW (figure 3.12).

The inhibition is better with EIW than with unfractionated AndoSan™ at concentrations of 0.25 mg/mL and 0.5 mg/mL, before they both flatten at approximately 20 % activity (and thus 80 % inhibition) at concentrations above 1 mg/mL. At a concentration of 0.125 mg/mL unfractionated AndoSan™ shows better inhibition than EIW.

Samples were measured with concentrations up to 2 mg/mL, but these also stabilise at an activity of about 20 % and are therefore not shown.

The samples were also analysed by western blot to verify that the inhibition of activity was due to inhibition of autoactivation and not inhibition of active enzyme. This was done by determination of the presence of prolegumain and active legumain in the samples. The former was detected with a 56 kDa band, the latter was detected with a 46 kDa band. The resulting blot is presented in figure 3.13.

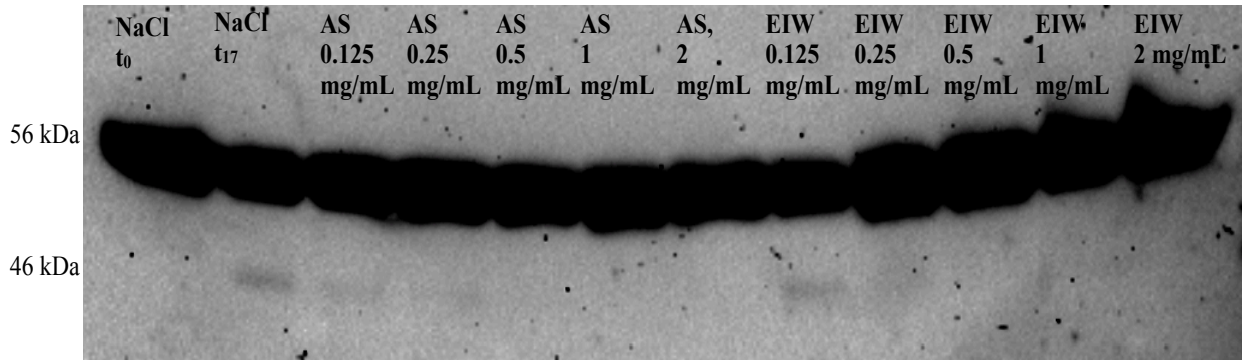

Figure 3.13 The western blot of samples incubated with unfractionated AndoSan™ or EIW and autoactivated prolegumain at pH 4. AS is unfractionated AndoSan™. NaCl t_0 is a negative control with only prolegumain (56 kDa), whereas NaCl t_{17} is a positive control for active legumain (46 kDa) after 17 hours incubation.

The western blot of samples incubated with AndoSan™ and autoactivated prolegumain resulted in very strong 56 kDa bands and only weak 46 kDa bands (figure 3.13). This called for prolonged exposure during signal induction, which resulted in a relatively dark blot.

However, the results indicate that activation of prolegumain is inhibited by both unfractionated AndoSan™ and EIW, as can be seen by the weaker active bands in these lanes compared to the control sample (NaCl, t_{17}). The inhibition seems apparent even at low concentrations.

3.4 Separation of components in EIW

Preparative size exclusion chromatography (SEC) and size exclusion high performance liquid chromatography (SEC-HPLC) were used to separate components in EIW by molecular size.

3.4.1 SEC

The components in EIW were separated with a Sephacryl S-100 HR column (method 2.3.1), which resulted in two fractions; EIW-A and EIW-B. The resulting chromatogram is presented in figure 3.14.

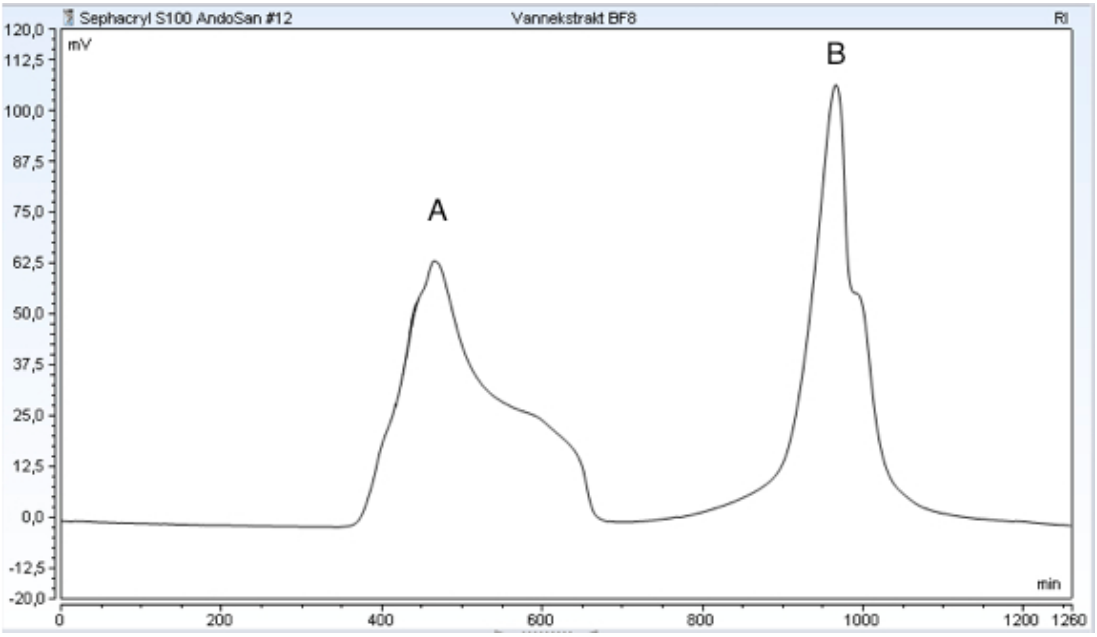

Figure 3.14 The separation of the ethanol insoluble water extract on the Sephacryl S-100 column resulted in two fractions; EIW-A and EIW-B.

Peak A (figure 3.14) was eluted before peak B, indicating higher molecular weight compounds present in A than in B.

The fractions that seemingly belonged to the same peak were mixed and lyophilized for further analyses. The two fractions were designated EIW-A and EIW-B

3.4.2 SEC-HPLC of EIW-A and EIW-B

EIW-A and EIW-B were analysed by SEC-HPLC (method 2.3.2). They were compared with the retention times of two β-glucan standards (35,6 kDa and 70.6 kDa).

The standard with the highest Mw is larger than the area of optimal separation set by the column (dextrans ≤ 50 kDa), and would therefore elute in the void (the components too large to enter the pores in the gel column). The resulting chromatogram is presented in figure 3.15.

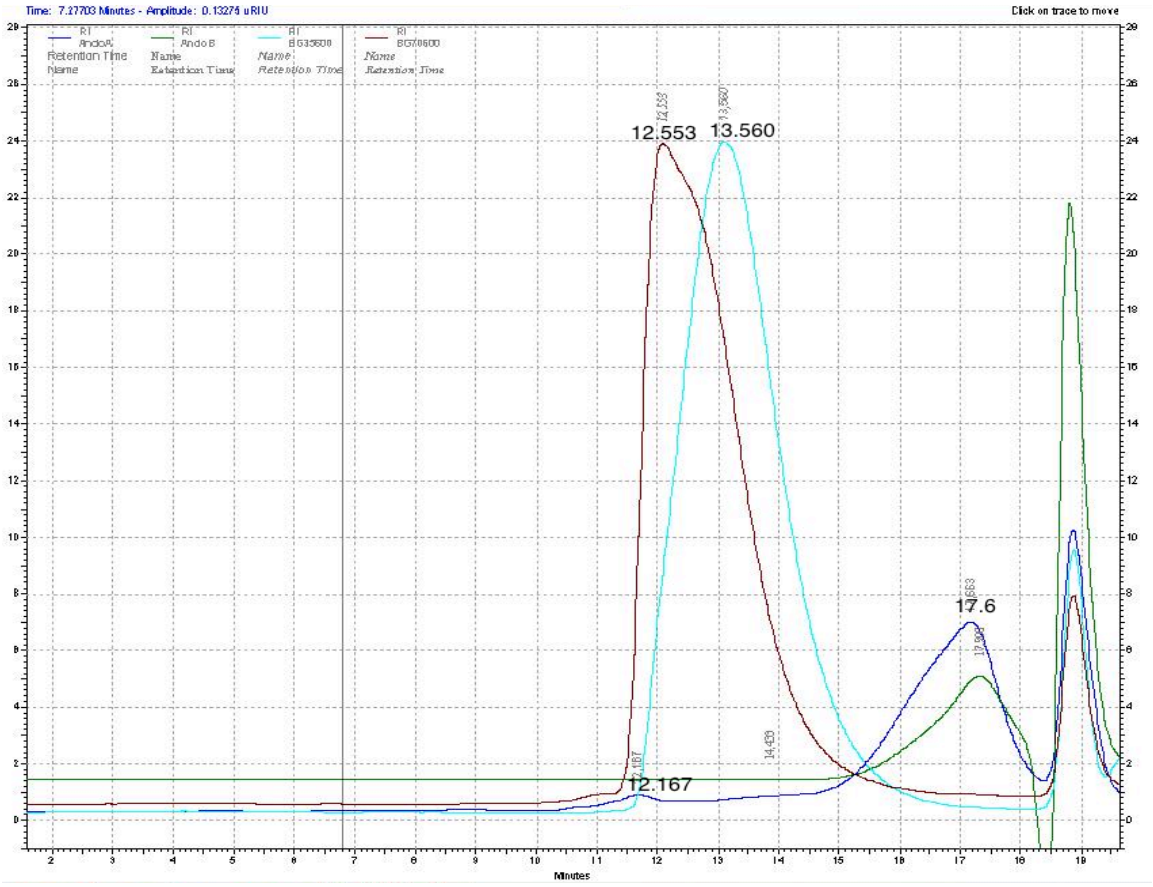

Figure 3.15 The chromatogram from the HPLC analysis of EIW-A and EIW-B, together with two β-glucan standards. The samples are graphed in different colour; the burgundy is the standard with Mw 70.6 kDa, the cyan is the standard with Mw 35.6 kDa, the dark blue is EIW-A and the green is EIW-B.

The components in EIW-B were not separated by the column and were eluted in a single peak; in the low molecular weight region (figure 3.15, retention time 17.6 minutes). The chromatogram of EIW-A looks like that of EIW-B with the largest peak in the low molecular weight region, but with a small additional peak at 12.167 min.

The standard peaks are large compared to the sample, and they are partly overlapping due to a certain polydispersity of the standards. 35.6 kDa and 70.6 kDa are the peak molecular weights of the respective standards, but they also contain a range of molecular sizes.
In addition, the components in the standard with a Mw of 70.6 kDa were not separated by this column, which only separates compounds below 50 kDa (dextrans). This indicates that the 70.6 kDa standard was eluted in the void volume of the column. The small peak of EIW-A seems to elute close to this standard, and could therefore be close to or larger than 50 kDa.

3.5 Characterisation of EIW-A and EIW-B

Several characterisation analyses were utilised to do compositional estimates of the different extracts, including ^{1}H NMR, LC-MS, carbohydrate analysis by methanolysis and methylation (EIW-A) and SDS-PAGE.

3.5.1 ^{1}H NMR

Both EIW-A and EIW-B were dissolved in D_2O and analysed by ^{1}H NMR (method 2.4.1). The resulting spectra are given in figures 3.16 and 3.17.

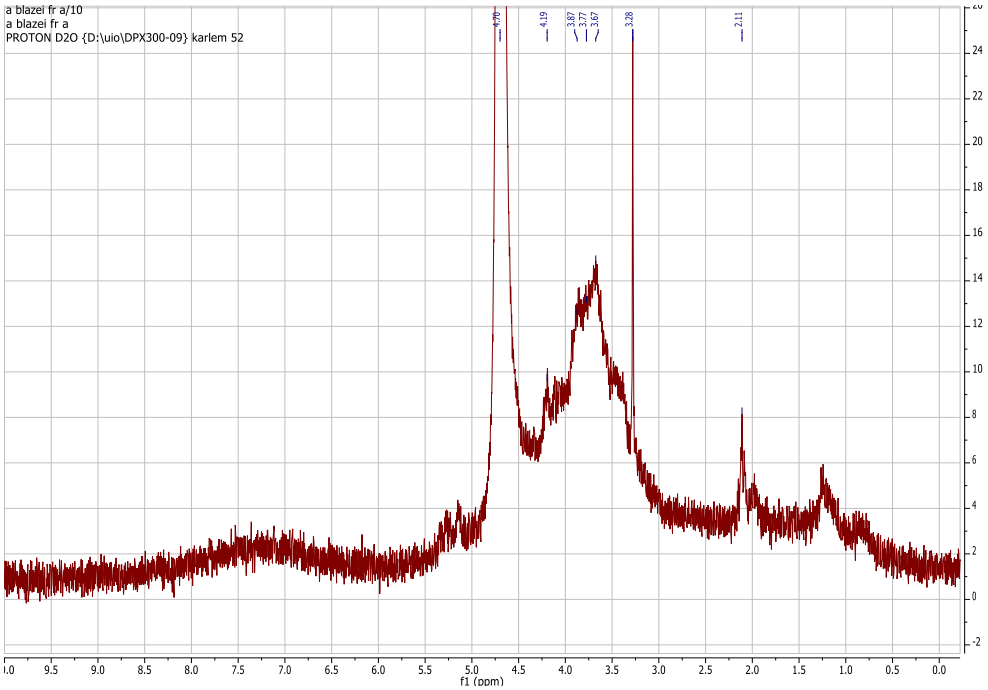

Figure 3.16 The ^{1}H NMR spectrum of EIW-A

The ^{1}H NMR spectrum of EIW-A (figure 3.16) is very similar to that of EIW (figure 3.5). The main component seems to be carbohydrates (peaks between 3.5 and 4.2 ppm), but it also contains some aliphatic structures (< 2 ppm) and perhaps traces of aromatic compounds, phenols or alkenes (peaks between 7 and 8.5 pm). The large peak at 4.9 ppm is H_2O.

EIW-A also resembles EIW in that it contains the two small peaks just prior to the water peak. These are protons bound to anomeric carbons (C-1) in carbohydrates. These signals around 5.2 ppm are in the α-anomeric configuration. The presence of β-anomeric signals (e.g. from β-glucans) are not revealed due to the signal from water that appears at 4.9 ppm. This can be eliminated with higher temperatures during analysis, which makes the water signal shift.

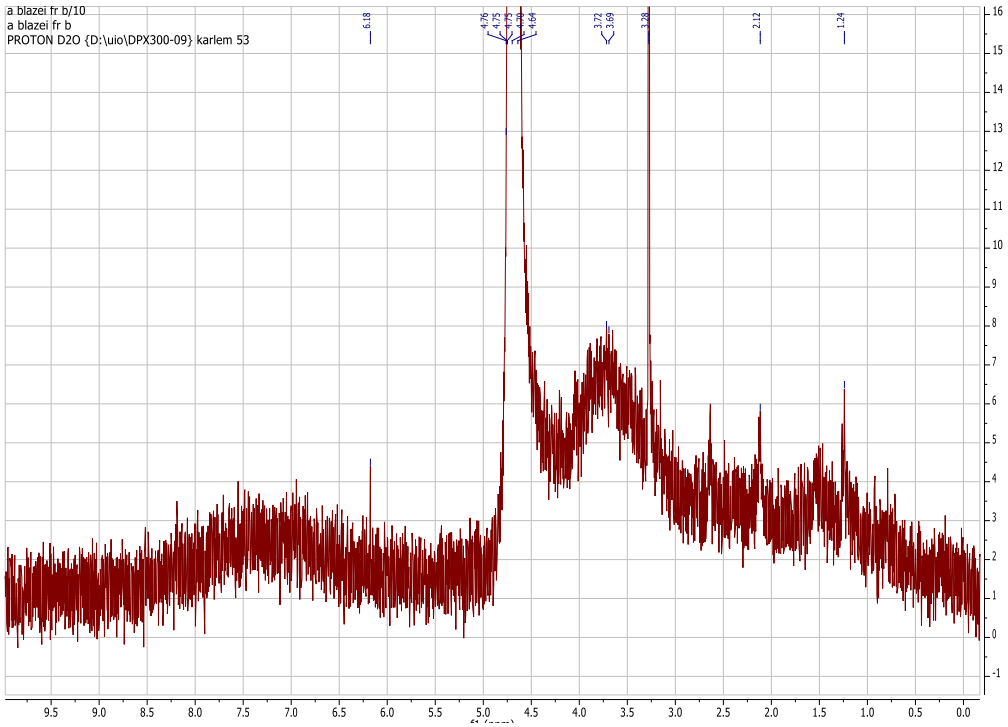

Figure 3.17 The ^{1}H NMR spectrum of EIW-B

The ^{1}H NMR spectrum of EIW-B shows broad peaks in the carbohydrate area (between 3.5 and 4.2 ppm), in addition to some peaks in the aliphatic area (small peaks < 2 ppm) and the aromatic area, which can be caused by phenols or alkenes (small peaks between 7.0 and 8.5). The large peak at approximately 4.9 ppm is H_2O.

3.5.2 LC-MS

EIW-A and EIW-B were analysed by LC-MS (method 2.4.3), which initially separated the components based on polarity, followed by a mass analysis of resulting peaks in the chromatogram.

The liquid chromatography method utilised uses a gradient eluent, with an initial polar eluent becoming gradually less polar. In addition, the column separated components based on hydrophobic interactions.
The resulting LC-chromatogram of EIW-A is presented in figure 3.18.

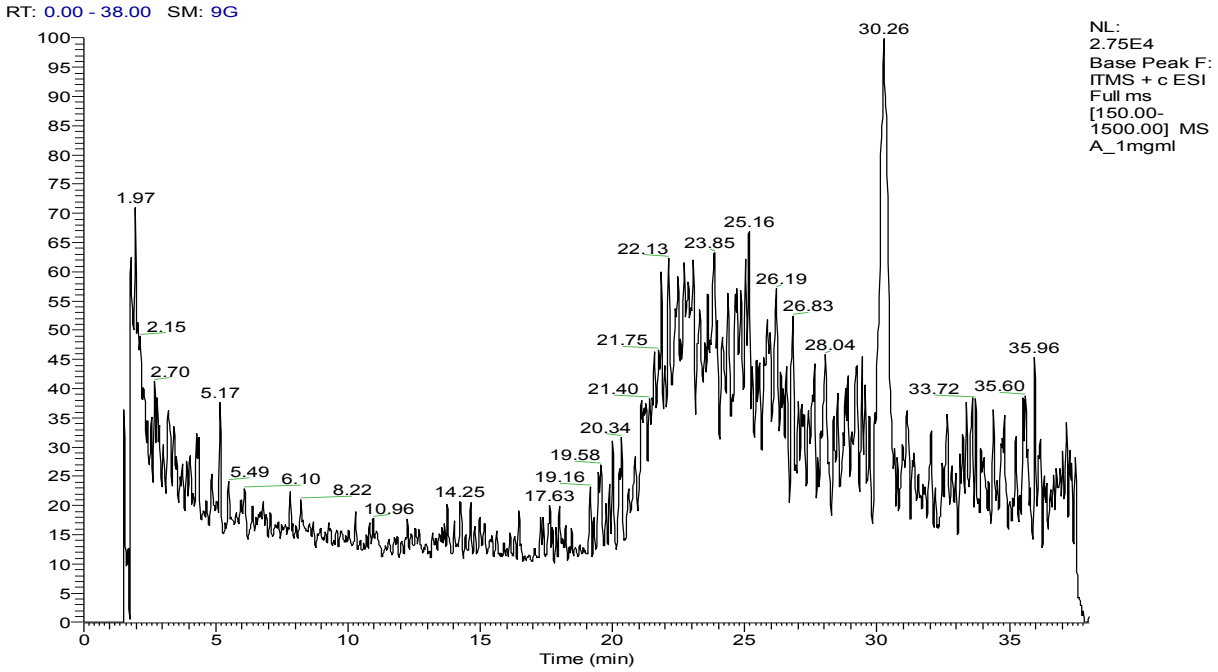

Figure 3.18 The LC chromatogram of EIW-A

The noise in the spectrum was caused by solvent (H_2O). The highly polar compounds, which are very soluble in the polar eluent, were eluted instantly and are not detected. The less polar constituents were eluted later, which resulted in one peak at 30.26 minutes.
The detection of only one peak indicates highly polar components in EIW-A.

The large peak that eluted after 30.26 minutes was analysed by ion trap mass spectrometry, and its spectrum is presented in figure 3.19.

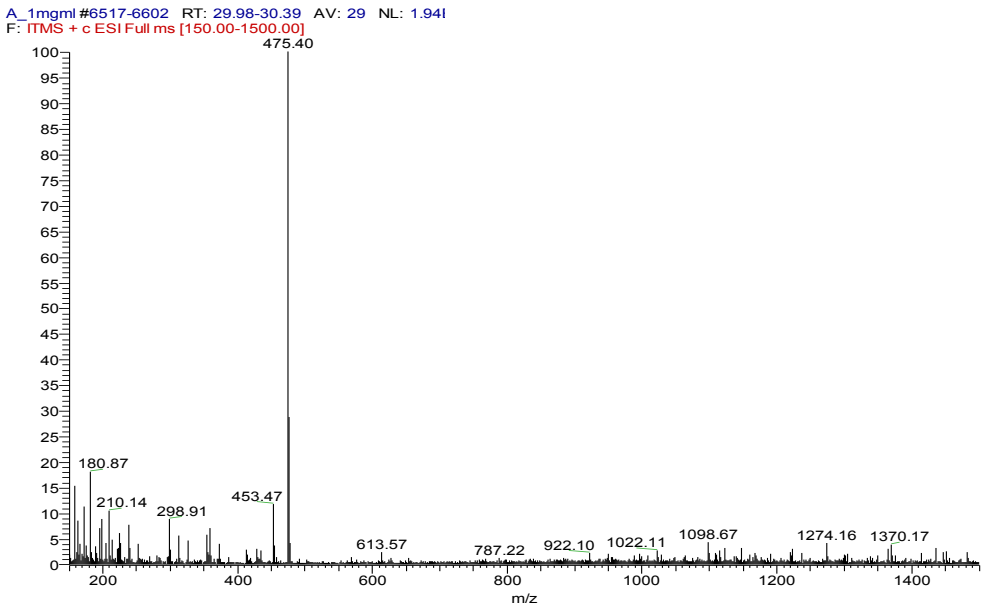

Figure 3.19 The MS spectrum of the peak with retention time 30.26 from the LC-run of EIW-A (fig. 3.18)

The masses in Dalton (Da) of the respective compounds are presented with a numerical value on the top of each peak in figure 3.16. The major peak has a mass of 475.40 Da, and its neighbouring peak has a mass 453.47 Da. The difference in mass between these two is the mass of sodium, thus the compound in the peak that elutes after 30.26 minutes may be a sodium salt.

It is not possible to predict a specific structure form the detected masses alone, additional analyses are necessary to identify specific compounds (e.g. elemental analysis).

The LC chromatogram of EIW-B is presented in figure 3.20.

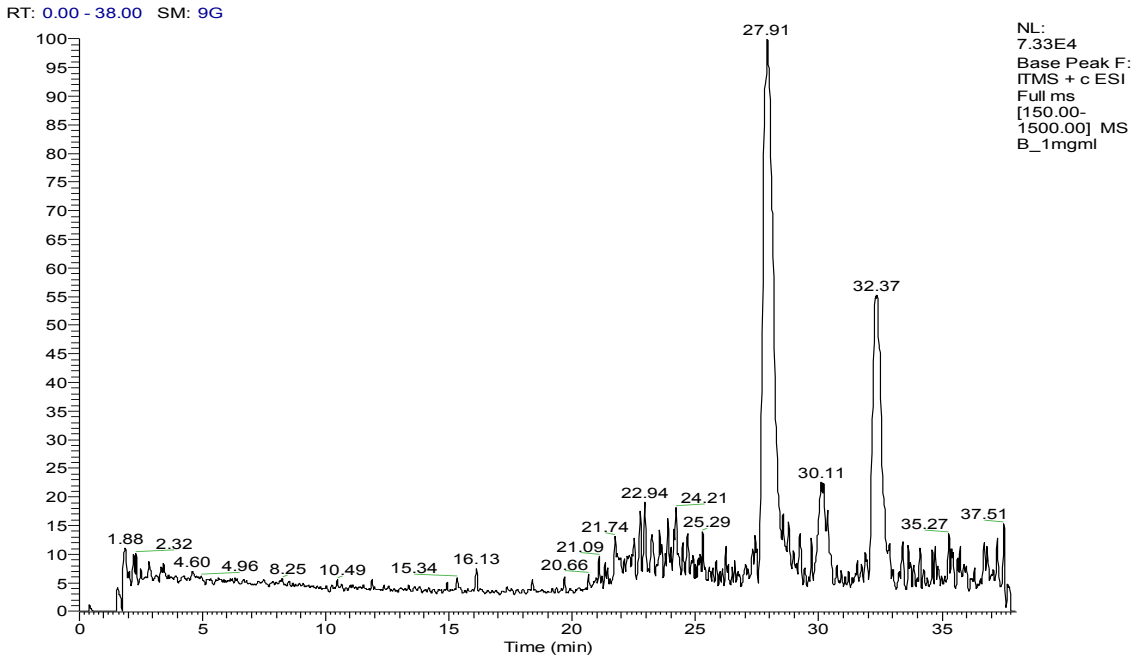

Figure 3.20 The LC chromatogram of EIW-B

The two peaks that elute after 27.91 and 32.37 minutes, respectively, were analysed by ion trap MS. The mass spectrum of the first peak is presented in figure 3.21.

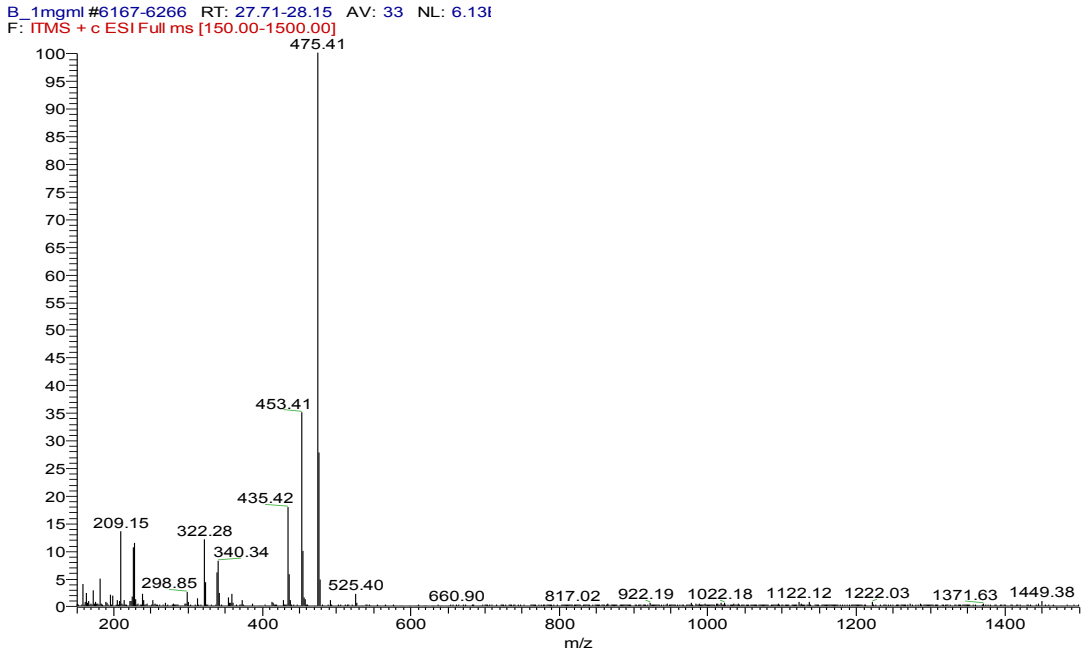

Figure 3.21 The MS spectrum of the peak with retention time 27.91 minutes from the LC run of EIW-B (fig. 3.20)

The masses in Da of the compounds in the first peak of EIW-B are presented with numerical values on the top of each peak in figure 3.21. The largest peak has a mass of 471.41 Da, and its neighbouring peak has a mass of 453.41 Da. The difference between these is the mass of sodium, indicating the presence of a sodium salt. This has the same mass as the compound found in EIW-A (figure 3.16), which points to a compound that is present in both EIW-A and EIW-B.

The mass spectrum of the compounds in peak two of EIW-B (eluted after 32.37 minutes) is presented in figure 3.22.

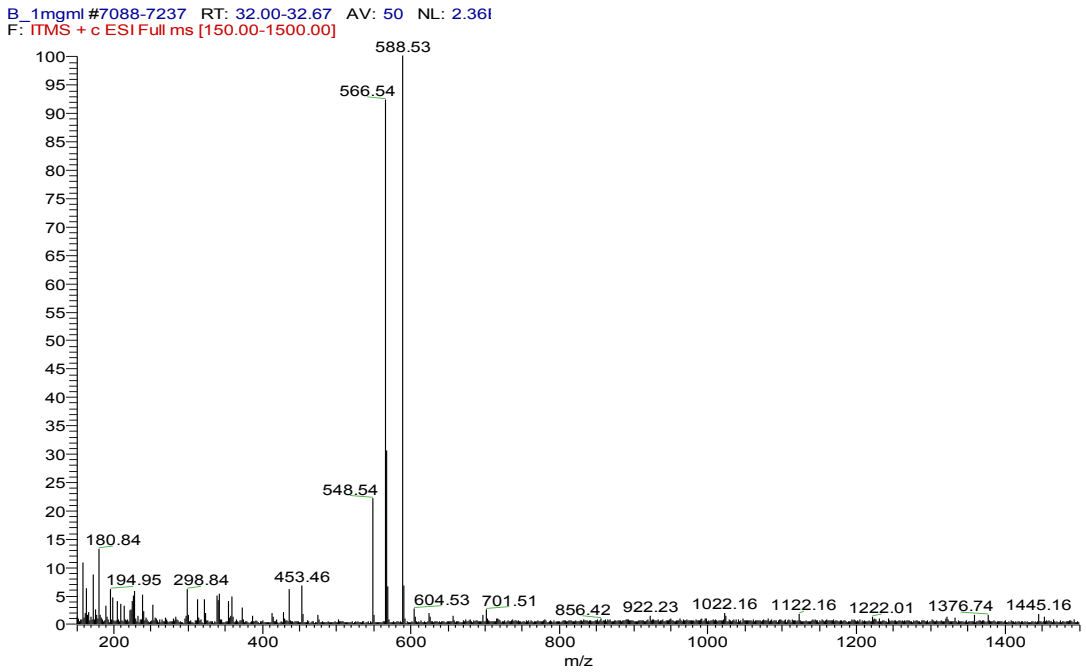

Figure 3.22 The MS spectrum of the peak with retention time 32.37 minutes from the LC run of EIW-B (fig. 3.20)

The masses in Da of the compounds in peak two are presented with numerical values on top of each peak in figure 3.22. As with EIW-A, it is not possible to deduce structures from the detected masses alone. Further analyses are required.

3.5.3 Carbohydrate analyses

EIW-A and EIW-B were analysed by methanolysis to determine the carbohydrate content and monosaccharide composition of their respective polysaccharides (method 2.4.5 and 2.4.6). EIW-A was also analysed by methylation and GC-MS in order to determine the binding pattern of the polysaccharides (method 2.4.7).

The carbohydrate contents of the two fractions are presented in table 3.4.

Table 3.4 The carbohydrate content in EIW-A and EIW-B

	Percent carbohydrate in sample
EIW-A	9.7
EIW-B	0.4

EIW-A contains more carbohydrates (9.7 %) than EIW-B (0.4 %). The composition of the polysaccharides in these samples is presented in table 3.5.

Table 3.5 The monosaccharide composition of the polysaccharides in EIW-A and EIW-B

	EIW-A	**EIW-B**
Arabinose	9.2	Trace
Galactose	16.7	Trace
Galacturonic acid	12.6	Trace
Glucose	24.7	6.4
Glucuronic acid	n.d.*	n.d.*
Mannose	11.4	Trace
Rhamnose	5.4	n.d.*
Ribose	n.d.*	n.d.*
Xylose	20.0	93.6

* n.d. = not detectable

The two fractions of the ethanol insoluble water extract, EIW-A and EIW-B have distinct monosaccharide compositions. The latter contains mostly xylose (94 %) and some glucose (6 %), with trace amounts of other monosaccharides (see table 3.5), while the former has a lot more complex monosaccharide composition. EIW-A contains mostly glucose (25 %) and xylose (20 %), but also galactose (17 %), galacturonic acid (13 %), mannose (11 %), arabinose (9 %) and rhamnose (5 %).

EIW-A was also analysed by methylation and GC-MS to determine the binding pattern of the carbohydrates in the sample. The peaks from the GC separation were fragmented and charged by ionisation, followed by mass/charge separation by MS. Monosaccharides partaking in different linkages in the polysaccharides resulted in different fragmentation patterns, and thus the binding pattern of monosaccharides were deduced.

Table 3.6 presents the retention time and the respective binding pattern of each component in the GC-MS spectrum of EIW-A, in addition to a percentage calculation of each monosaccharide in the total amount of carbohydrate.

The calculations of percent in total carbohydrate were done by first summing all the peak areas of the same monosaccharide from the monosaccharide fragment analysis by GC-MS (e.g. all the peaks containing different glucose units). This was followed by calculating the fraction of the different configurations of the monosaccharide using their respective peak area in GC-MS (set all areas of e.g. glucose to 100 %, then calculate the percentage of each configuration of glucose), and then this percentage was multiplied with the percentage of the monosaccharide present in the sample divided by 100 (GC, from table 3.5).

Table 3.6 Binding pattern analysis of polysaccharides in EIW-A

	Ret. time (min)	% in carbohydrates in sample
T Ara*f*	13.03	9.2
T Fuc*p*	15.83	Trace
1,3 Fuc*p*	16.95	Trace
T Glc*p*	17.32	16.6
1,3 Glc*p*	19.33	2.3
1,6 Glc	20.36	3.3
1,3,6 Glc*p*	22.67	2.5
T Gal*p*	17.88	8.3
1,3 Gal*p*	20.16	8.4
T Man*p*	17.54	11.4
T Rha*p*	14.68	1.5
1,2 Rha*p*	16.26	3.4
1,2,3 Rha*p*	18.41	0.5
T Xyl*p*	13.94	11.0
1,3 Xyl*p*	16.38	9.0

p = pyranose, *f* = furanose

Table 3.6 shows that the carbohydrates in EIW-A contain monosaccharides with varying binding patterns. Galacturonic acid in the sample (12.6 %) was not detected. For timing purposes, the required initial carboxyl reduction of galacturonic acid was not performed.

The most abundant fragment is terminal glucose, which constitutes 16.7 % of the total carbohydrate content. Terminal xylose and terminal mannose both constitute about 11 %, and there is also a considerable content of terminal arabinose (9.2 %), 1,3 bound xylose (9 %), terminal galactose (8.3 %) and 1,3 bound galactose (8.4 %).
There were also detected small amounts of 1,3 bound glucose, 1,6 bound glucose, 1,3,6 bound glucose, terminal rhamnose, 1,2 bound rhamnose and 1,2,3 bound rhamnose.
The trace amounts of fucose were not detected by methanolysis, probably due to low concentration.

3.5.4 SDS-PAGE

EIW, EIW-A and EIW-B were analysed by SDS-PAGE to determine whether there were any proteins in the extracts. The scan of the gel is presented in figure 3.23.

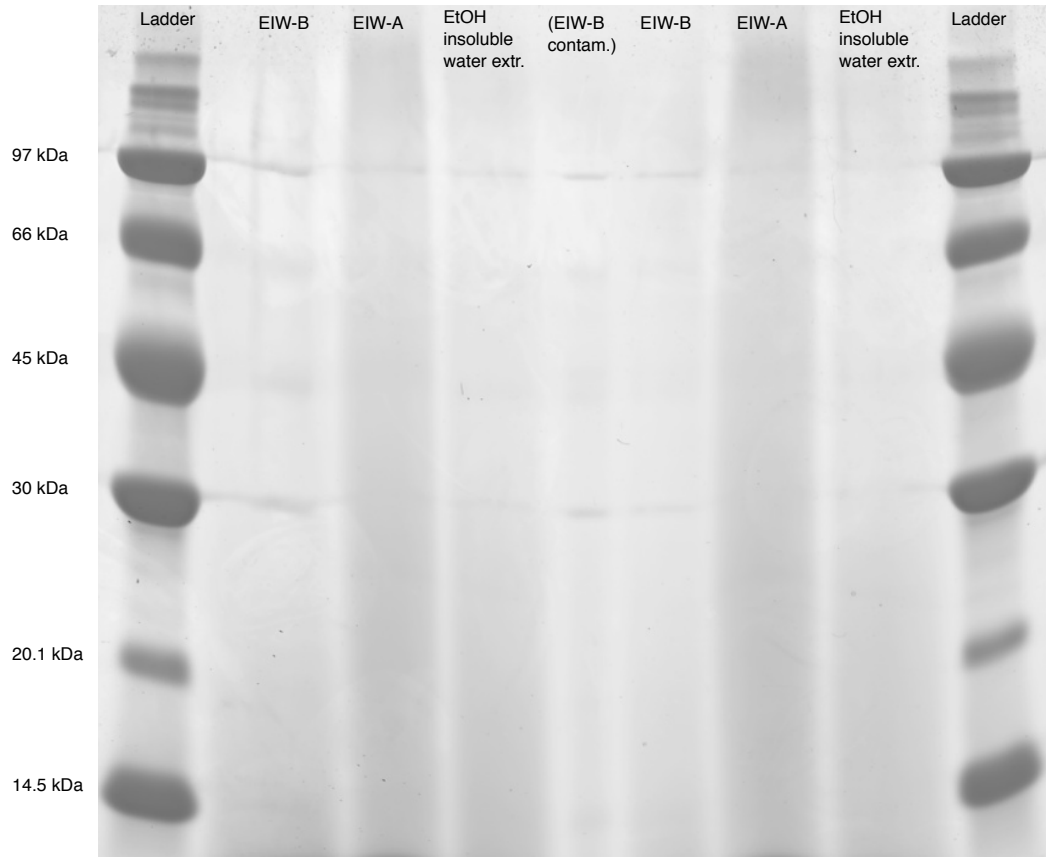

Figure 3.23 SDS-PAGE analysis of EIW, EIW-A and EIW-B.

The protein gel with EIW, EIW-A and EIW-B shows weak bands in all lanes. EIW-B has two bands, one approximately juxtaposed to the 97 kDa marker and one to the 30 kDa marker. EIW-A and EIW have one band approximately juxtaposed to the 97 kDa marker.

The lack of detection of the 30 kDa protein in EIW was probably due to low a concentration of this protein in the extract. Coomassie blue is not particularly sensitive for protein detection. If the gel was stained with silver, which is the most sensitive colorimetric detection of protein, there would most likely be a corresponding 30 kDa band in EIW.

3.6 Inhibition of legumain activity in RAW264.7 cells by EIW-A and EIW-B

The RAW264.7 cells were incubated with EIW and its two fractions, EIW-A and EIW-B, in order to investigate their ability to inhibit legumain activity in these cells.

The results are calculated as percent of control, and are presented in figure 3.24.

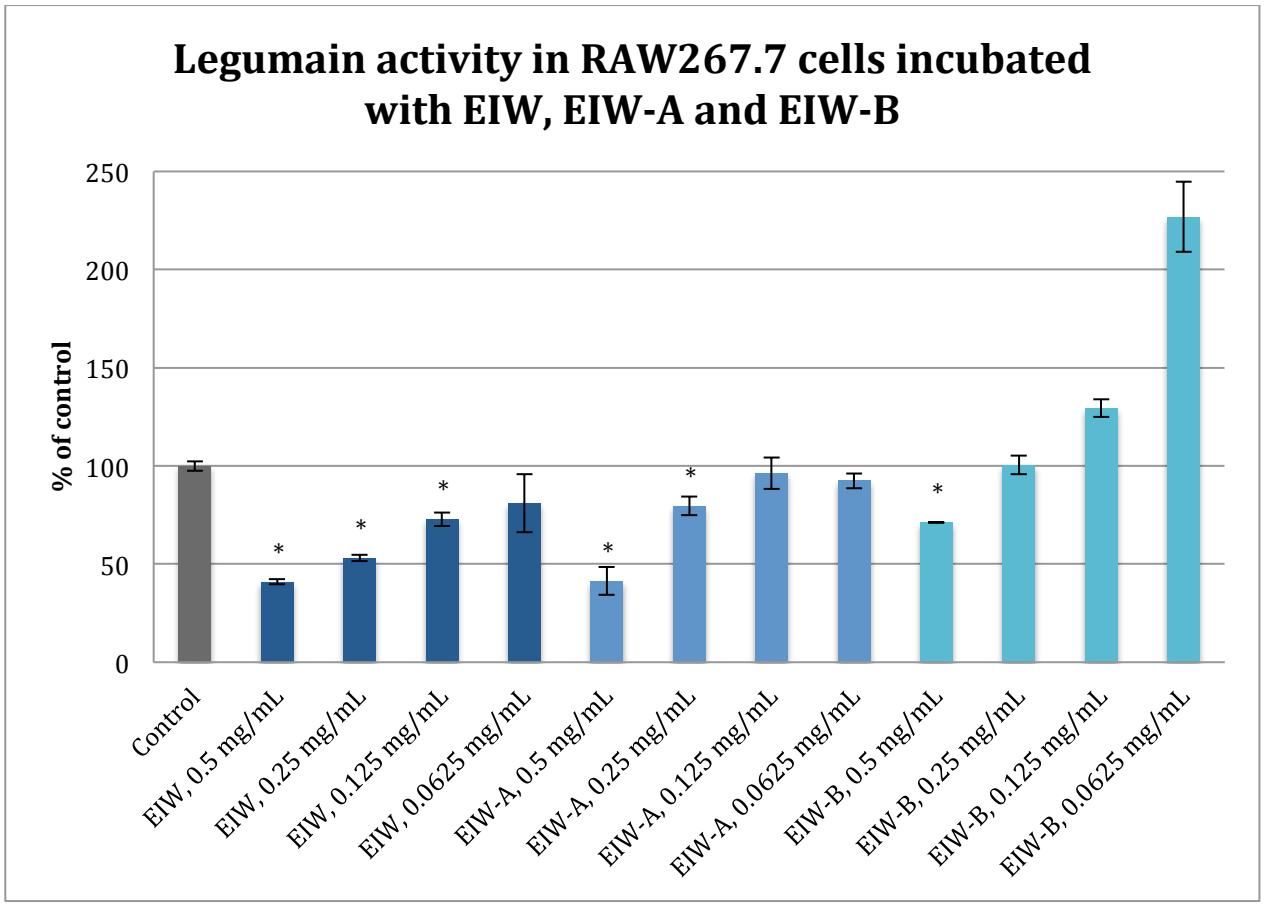

Figure 3.24 The legumain activity in RAW264.7 cells incubated with EIW and its associated fractions EIW-A and EIW-B, calculated as percent activity with respect to control. The results are representative for two independent cell experiments. Bars denoted with an asterisk are significantly lower than the control, $p < 0.05$.

EIW shows a significant inhibition of legumain activity at concentrations of 0.5, 0.25 and 0.125 mg/mL, which is in compliance with the results from the cells incubated with raw extracts of AndoSan^{TM} (section 3.3.1).

EIW-A and EIW-B both show significant inhibition of legumain activity at a concentration of 0.5 mg/ml. EIW-A also shows a significant decrease in legumain activity at a concentration of 0.25 mg/ml.

EIW-A exhibits significantly higher inhibitory activity than EIW-B at concentrations of 0.5 and 0.25 mg/ml, and they both show a dose-dependent response in activity. EIW-A does not show significantly better inhibition than EIW.

4 Discussion

AndoSan™ is a medicinal mushroom extract currently being subject to research for treatment of multiple myeloma, inflammatory bowel disease and colorectal cancer (ImmunoPharma). Its constituents are unknown, and the work with this thesis focused on extracting and identifying fractions exhibiting higher biological activity than others. The extracts were both characterised and tested in a biological test system using the RAW264.7 macrophage cell line. The bioassays focused on the expression of legumain, a cysteine protease that is overexpressed in several solid tumours (Gawenda et al. 2007; Liu et al. 2003; Wang et al. 2012).

4.1 Extractions of AndoSan™

The extractions performed with AndoSan™ resulted in varying yields of crude extracts (table 3.1). The yield of the methanol and ethanol extracts constitutes 30 % (12.3 % and 18.1 %, respectively). These contain lower molecular weight components, with the more polar components in the methanol extract. Any agaritine (described in section 1.2.1) in AndoSan™ would most likely be eluted in the alcohol extracts, due to its low molecular weight and polar groups.

The largest fractions are the two water fractions, which have a combined yield of approximately 35 %. Of these two, the ethanol soluble water extract (ESW) is largest (yield 23.3 %) and contains polysaccharides and/or proteins of lower molecular weight. The larger polysaccharides and/or proteins were precipitated by adding two volumes of 96 % ethanol to the water extract, and are thus in the ethanol insoluble water extract (EIW, yield 11.9 %).

The dichloromethane (DCM) extraction resulted in a yield of only 0.4 % or 0.1039 g, and for this reason the number of analyses on the extract was restricted. The only analysis performed on this extract was ^{1}H NMR.

The high loss of sample material (26 %) can be explained by the many filtrations between each extraction, some sample material stuck to the filters and was irretrievable.

4.2 Characterisation of AndoSan™ crude fractions

All extracts were coloured with a dark brownish colour, particularly the ethanol extract and the two water extracts. The compounds that caused this colour remain unknown, but phenols and Maillard products have been suggested as possible colouring agents. The latter are products of chemical reactions between proteins/amino acids and reducing sugars (e.g. xylose and glucose).

Unfractionated lyophilized AndoSan™

The carbohydrate analysis of unfractionated AndoSan™ indicated that the mushroom extract contains very little carbohydrate (only 2 %, table 3.2), which is in stark contrast to what has been reported for pure AbM powder. Reports state that the dry powder of the mushroom consists of approximately 90 % carbohydrate, of which 2.8 % is β-glucans (Ellertsen & Hetland 2009; Forland et al. 2011; Johnson et al. 2009). The β-glucans are polymers of glucose, and the low content of sugars thus indicates that the beneficial biological properties of AndoSan™ cannot be attributed to the β-glucans alone.

The compositional analysis of the polysaccharides in the product revealed that it contains mostly xylose (26 %), glucose (23 %) and arabinose (19 %), with small amounts of galactose (10 %), galacturonic acid (8 %), mannose (7 %) and rhamnose (7 %). This is contradictory to other reports revealing that the polysaccharides in AbM consist of more than 50 % glucose and about 25 % galactose, with small amounts of mannose, fucose, rhamnose, arabinose and xylose. These were reported to be present β-glucans, α-glucans and mannogalactans (Kozarski et al. 2011; Smiderle et al. 2011). The latter are chains of galactose monomers with mannose side chains. One report also states that hot water extracts of AbM fruiting bodies contain 46 g carbohydrates per 100 g sample material (Kozarski et al. 2011), in contrast to what has been reported for AbM dry powder (89 g per 100 g material). Thus, deviations of carbohydrate content and composition in AndoSan™ compared to other reports regarding AbM, might be caused by the method by which AndoSan™ is produced; it is a fermented mushroom extract. This fermentation may lead to the degradation of some carbohydrates, resulting in reduced carbohydrate content and shifted monosaccharide ratios.

Dichloromethane extract

The DCM extract gave a distinct ^{1}H NMR spectrum (figure 3.1), which indicated that the extract contains mostly fats, other aliphatic structures and aromatic components. The ergosterols and blazeispirols (described in section 1.2.1) will, if present in AndoSan™, most likely be extracted with DCM. The analyses on the DCM extract were limited due to small amount of sample.
The low yield indicates that the components in this extract are of minor importance to the combined biological effects of AndoSan™.

Alcohol extracts

The two alcohol extracts were analysed by ^{1}H NMR, UV-absorption, SDS-PAGE and methanolysis/GC analysis of monosaccharide composition.
They showed very similar NMR spectra (figure 3.2 and 3.3), which indicate that the extracts consist of low-molecular carbohydrates, some aliphatic compounds and some aromatic and/or phenolic compounds. The small amounts of aromatic compounds were also detected by UV-absorption, which resulted in small absorption "shoulders" at approximately 300 nm (figures 3.7 and 3.8). Agaritine contains an aromatic ring (figure 1.3) and would give absorption in this region of the UV-spectrum. However, synthetic agaritine has proven to be sensitive to oxidative and fermentative degradation (Roupas et al. 2010), which indicates that the presence of agaritine in these extracts is unlikely. Neither of the extracts contains any proteins, as detected by SDS-PAGE (figure 3.9).

This resemblance between the two alcohol extracts is expected, due to the many shared properties of ethanol and methanol as solvents (they are both alcohols and dissolve low molecular compounds). They differ in their respective polarity, and the components in the methanol extract should in theory be slightly more polar than the components in the ethanol extract. This is accounted for by the different content and composition of carbohydrates in the two extracts (tables 3.2 and 3.3). The methanol extract contains 5.7 % carbohydrates, which have a fairly even distribution of glucose, xylose and galactose (constitute ~58 %) and also a substantial amount of galacturonic acid, arabinose and mannose (constitute ~34 %). In contrast, the 80 % ethanol extract contains only 1.6 % carbohydrates, which consist mostly of arabinose and glucose (constitute ~62 %), a substantial amount of xylose (20 %) and small amounts of galactose,

galacturonic acid, glucuronic acid, mannose and rhamnose. Thus, the methanol and ethanol extracts contain different low molecular carbohydrates.

Water extracts

The crude water extract was separated into two sub-fractions by precipitating the high molecular components with ethanol, which resulted in an ethanol soluble and an ethanol insoluble water extract. They were both analysed by ^{1}H NMR, TLC, UV-scan and SDS-PAGE.
Their respective NMR spectra (figures 3.4 and 3.5) showed similar compositions of the two extracts; they both contain mainly carbohydrates, but also some aliphatic compounds and traces of aromatic compounds. UV-absorption analysis did not indicate presence of aromatic compounds in neither of the extracts.
In the ^{1}H NMR spectrum, EIW shows two additional peaks (not present in ESW) just prior to the H_2O peak, which are hydrogens attached to anomeric carbon (C-1) in carbohydrates. These appear at approximately 5.2 and 5.3 ppm, which indicates that they are in the α-anomeric configuration. The presence of these protons in EIW and not in ESW indicates that EIW contains α-anomeric hydrogens, whereas ESW does not. These may perhaps be derived from the previously reported α-glucans in hot water extracts of AbM fruiting bodies (Kozarski et al. 2011; Smiderle et al. 2011).
In order to detect any protons in the β-anomeric configuration, the large water peak needs to be shifted by increasing the temperature during analysis. Such signals are of interest because AndoSan^{TM} is thought to contain β-glucans, and would appear in the 4.9 ppm region.

TLC analysis indicated highly polar components; all of the compounds in the samples were detected in front with the more polar mobile phase (Rt close to 1). This suggests that the extract contains a considerable amount of inorganic salts, which are highly polar and very soluble in water. They can produce ions in solution, which can interfere with ^{1}H NMR analysis and result in broad peaks. Such broad peaks have been proven for both water extracts, and a possible route for further analyses would therefore be to desalinate the samples by dialysis. Dialysis separates compounds by size, which depends upon the size of the pores in the dialysis membrane. This has not been performed due to the co-occurring loss of some sample material, which might be important for biological activity.

EIW was shown to contain protein by SDS-PAGE (figure 3.9), whereas the opposite was shown for ESW. This is in accordance with the procedure by which EIW was obtained; it is the precipitate of a water extract that had been treated with ethanol, which indicates high molecular components.
The detected protein has a size of approximately 66 kDa, but further analysis, such as MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight mass spectrometry) is necessary to determine the nature of the protein. It may be the protein part of a proteoglycan or a glycoprotein, or it may be an autonomous protein.

The carbohydrate analyses of the water extracts revealed that EIW is the crude extract containing most carbohydrates (6.7 %, table 3.2). These mainly consist of glucose, galactose and xylose (constitute ~60 %), a substantial amount of galacturonic acid, arabinose and mannose (constitute ~30 %) and some rhamnose and glucuronic acid (table 3.3). ESW contains only 1.7 % carbohydrate, which mostly consists of xylose (about 40 %), but also glucose and arabinose (constitute ~40 %) and small amounts of galactose, rhamnose, mannose and glucuronic acid. These findings are contradictory to what was found with ^{1}H NMR, which indicated that the water extracts mainly consist of carbohydrates. However, the peaks in these spectra are very broad, and elimination of ions by desalination should be performed before repeated analyses by ^{1}H NMR.

4.3 Legumain assays with AndoSan™ crude fractions

The AndoSan™ crude fractions were assessed for inhibition of legumain activity in RAW264.7 cells. Reduced legumain activity is a good indication of antitumour properties of AndoSan™, in that this may help to prevent tumour invasion and metastasis.
In addition to incubation of RAW264.7 cells, unfractionated AndoSan™ was also assessed for inhibition of both active enzyme and proenzyme that had been autoactivated by conformational change and sequential cleavage of propeptides at pH 4. The latter was also performed with EIW.

Student's t-test was used examine whether the inhibition had statistical significance with respect to control and/or to unfractionated AndoSan™.

Significant reduction of legumain activity in RAW264.7 cells with respect to control was observed with unfractionated AndoSan™ and the two water extracts at concentrations 1 mg/mL and 0.5 mg/mL (figure 3.10). EIW and ESW also exhibited significant reduction of activity at a concentration of 0.25 mg/mL.
The two alcohol extracts did not show any reduced legumain activity, with the exception of the methanol extract at a concentration of 0.125 mg/mL, and was therefore not chosen for further purification.

EIW was shown to be the extract with the greatest inhibition of legumain, and further research was therefore focused on this extract. In addition to exhibiting better inhibition of legumain activity when compared to ESW, it also showed better inhibition when compared to unfractionated AndoSan™ at concentrations 0.25 mg/mL and 0.125 mg/mL ($p < 0.05$), but not at a concentration of 0.5 mg/mL. This indicates that the compounds responsible for the legumain inhibition have been somewhat purified in EIW. This theory was strengthened when incubating both unfractionated AndoSan™ and EIW with autoactivated prolegumain (figures 3.12 and 3.13). Results showed that the inhibition of autoactivation was better with unfractionated AndoSan™ than EIW at a concentration of 0.125 mg/mL, but at concentrations above 0.25 mg/mL the inhibition was better for EIW than unfractionated AndoSan™. The inhibition by both extracts flattened at an inhibition of approximately 80 % at high concentrations (above 1 mg/mL), which indicates a maximum inhibitory capacity of 80 %.

Unfractionated AndoSan™ was also incubated with active legumain, in order to assess if the product inhibits active enzyme. Results showed that, despite quenching having a significant impact, AndoSan™ exhibits inhibition of active enzyme even at low concentrations (figure 3.11). The inhibition is weaker than that observed with AndoSan™ and autoactivation of prolegumain. The maximum inhibition of active enzyme is approximately 40 % (flattens at concentrations of above 0.5 mg/mL), whereas the maximum inhibition of activity prolegumain samples is 80 %.

Results indicate that the strong inhibition of legumain activity in RAW264.7 cells by some AndoSan™ extracts (i.e. water extracts), is a combination of their ability to inhibit the

autoactivation of prolegumain and the active enzyme, where the former seems to pose the most significant contribution.

4.4 Characterisation of EIW-A and EIW-B

EIW was separated into two fractions by gel filtration chromatography, which were designated EIW-A and EIW-B (figure 3.12). EIW-A was eluted before EIW-B and thus contains components of larger molecular size than EIW-B.
EIW-A and EIW-B were characterised by SEC-HPLC, ^{1}H NMR, LC-MS, carbohydrate analyses with both methanolysis/GC and methylation/GC-MS and SDS-PAGE.
SEC-HPLC analysis of EIW-A and EIW-B resulted in separation of EIW-A into two peaks; one small peak eluted close to the void, indicating components of high molecular size (close to or larger than 50 kDa), and one larger peak in the low Mw area (figure 3.15). There was no separation of the components in EIW-B, which all eluted in a single peak in the low Mw region. These findings are in accordance with the method by which these fractions were obtained; EIW-A consists of larger components than EIW-B, which should also be apparent by HPLC.
The corresponding low Mw peaks in both samples might indicate components that are present in both fractions. This similarity is also indicated by ^{1}H NMR of the two fractions (figures 3.16 and 3.17) and LC-MS (figures 3.18-22).

^{1}H NMR indicates that both extracts mainly consist of carbohydrate and traces of aliphatic and aromatic structures. The height of the peaks in EIW-B is lower than those of the peaks in EIW-A, indicating lower amounts in EIW-B compared to EIW-A. There are also detected protons in the EIW-A spectrum not present in the EIW-B spectrum; the two peaks just prior to the water peak. These are protons in α-anomeric configuration and are also present in the ^{1}H NMR spectrum of EIW (figure 3.5). Due to their presence in EIW-A and not in EIW-B, they are believed to be found in carbohydrates of high Mw.

LC-MS analysis of EIW-A and EIW-B also revealed a compound common to both extracts, most likely a sodium salt (figures 3.19 and 3.21). It may be present as two isomers of the same compound, since they are found in two different fractions. Sodium pyroglutamate (described in

section 1.2.1), which is the sodium salt of pyroglutamic acid, has been shown to exhibit antitumour properties (Kimura et al. 2004). However, sodium pyroglutamate has a molecular weight of 151 Da and the compound found in EIW-A and EIW-B has a molecular weight of 475 Da, thus the compound found in the two fractions cannot be sodium pyroglutamate. Furthermore, the mass of the detected sodium compound is too general to be of any use for identification purposes, as is also the case with the other masses detected by LC-MS.
Agaritine, the phenyl hydrazine mentioned in section 1.2.1, has a Mw of 267.32 Da. This compound was not detected in either EIW-A or EIW-B by LC-MS.

It is worth noting that the LC-MS analysis was run on a revers phase column, where the components are separated based on hydrophobic interactions with the column particles; the less polar, the longer elution time. TLC of EIW indicated highly polar components (figure 3.6), which would elute in the void with this type of column and therefore not be detected and analysed by mass spectrometry. Further purification (e.g. desalination by dialysis) and analyses (e.g. elemental analysis) are therefore necessary to identify specific components.

The carbohydrate analyses of the fractions showed that EIW-A contains 9.7 % carbohydrates (table 3.4), in contrast to EIW-B that contains only 0.4 % carbohydrates.
The compositional analysis of the carbohydrates revealed that EIW-B consists mostly of xylose (94 %) and some glucose (6 %), with trace amounts of other monosaccharides. These carbohydrates are most likely of low Mw, due to their presence in EIW-B.
The carbohydrates in EIW-A have a more complex composition; the two most abundant monosaccharides are glucose and xylose (constitute ~ 45 %), but also include galactose, galacturonic acid, mannose, arabinose and rhamnose. The presence of galactose, galacturonic acid, rhamnose and arabinose points to the presence of pectin. The relatively high content of glucose (25 %) might indicate the presence of glucans

Analysis of EIW-B by SDS-PAGE resulted in the detection of two proteins, one with an approximate Mw of 97 kDa and one with an approximate Mw of 30 kDa (figure 3.23). Perhaps the carbohydrates in EIW-B are parts of a proteoglycan or glycoprotein. The presence of proteoglycans may also help to explain why there is an observed increase in legumain activity in

RAW264.7 cells incubated with EIW-B at concentrations below 0.25 mg/mL (figure 3.24). Several glycosaminoglycans from proteoglycans have shown to increase legumain activity in these cells (Berven et al. 2012). Thus, it is hypothesised that EIW-B contains components that are both advantageous and disadvantageous (i.e. glycosaminoglycans) for legumain activity, and the former prevails at low concentrations.

Protein analysis of EIW-A indicated the presence of a protein with an approximate Mw of 97 kDa, most likely the same protein that was also found in EIW-B. This 97 kDa protein is just within range for separation by the column used to separate EIW-A and EIW-B, which separates globularly proteins between 1 and 100 kDa. The detected bands are stronger for EIW-B than EIW-A, which indicates that most of the protein is found in EIW-B. It is likely that traces of the protein have eluted with the components in EIW-A. This leads to the conclusion that the components in EIW-A were eluted in the void of the column, i.e. the components are too large to interact with the gel pores, which means they have a Mw close to or above 100 kDa. This is in accordance with the SEC-HPLC analysis, which indicated that EIW-A contains high Mw components that were eluted close to or together with the void (>50 kDa).

The carbohydrates in EIW-A were also analysed by methylation/GC-MS to determine their respective binding patterns (table 3.6). Results showed that the content of terminal groups is high, indicating small molecules. This finding may also be a result of incomplete methylation of the polysaccharides, which might be due to the presence of highly polar components in the extract that may interfere with the chemical reactions in the methylation process. In addition, the data for galacturonic acid was unattainable, due to the lack of the carboxyl reduction process, and the concentration of carbohydrates in the sample was low (9.7 %). Normally, this analysis is performed with samples containing about 90 % carbohydrate. The results from the methylation analysis must therefore be considered with precaution, and can only be used as an indication of binding patterns. Thus, the results indicate that the content of branched (1,3,6 bound) and chained (1,3 bound) glucose, which are the building blocks of β-glucans, is very low. This is yet another indicator of low β-glucan content in AndoSan^{TM}. However, the polysaccharides in EIW-A should be subject to further purification and repeated analysis by methylation, in order to be able to determine the presence and quantity of β-glucans and other carbohydrate polymers.

To summarise, EIW-A and EIW-B were shown to have many compositional similarities; proteins with Mw 97 kDa, a sodium compound (perhaps a salt) with Mw 475 Da, highly polar components and similar ^{1}H NMR spectra. But they also exhibit properties that distinguish them from one another; EIW-B has a 30 kDa protein and contains little carbohydrates, whereas EIW-A does not contain the 30 kDa protein and has a much higher carbohydrate content.

4.5 Legumain assays with EIW-A and EIW-B

Both EIW-A and EIW-B showed significant inhibition of legumain in RAW264.7 cells at concentrations of 0.5 mg/mL, and EIW-A also showed significant inhibition at 0.25 mg/mL. Their inhibitory activities were compared to those of EIW, which indicated that neither of the EIW fractions is better at inhibiting legumain activity than the crude extract ($p > 0.05$). These findings points to synergistic or combined effects of the compounds in EIW, which are necessary for optimal inhibition of legumain activity in RAW264.7 cells.

The mechanisms by which components of AndoSan^{TM} manage to inhibit legumain activity in RAW264.7 cells are unresolved. In order to directly inhibit autoactivation of prolegumain, the compounds need to be taken up by the cells, since this process occurs in the lysosomes. In order to inhibit active enzyme, the product may contain a protease inhibitor, which is a protein that interact directly with proteases, or it might contain components that interact with cell surface receptors. Such receptors may be involved in cellular signalling cascades that affect the expression of legumain.

A few protease inhibitors have been isolated from fungi, e.g. clitocypins (MW close to 18 kDa) from *Clitocybe nebularis* and macrocypins (MW close to 25 kDa) from *Macrolepiota procera*, both of which have shown inhibition of legumain (Sabotic et al. 2007; Sabotic et al. 2009). These mushrooms belong to the same order as AbM; the *Agaricales*. Thus, it poses an interesting theory that AbM and AndoSan^{TM} might contain a cysteine protease inhibitor. A possible route for identification of such proteins is to isolate and identify the proteins found in EIW-A and EIW-B. Furthermore, on the basis of the obtained data it is hypothesised that the protein with Mw close to 30 kDa detected in EIW-B is such a protease inhibitor. This may help to explain why neither

EIW-A or EIW-B show better inhibition of legumain activity in RAW264.7 cells than EIW; perhaps the protease inhibitor that acts on active legumain is present in EIW-B, whereas the components(s) inhibiting autoactivation of prolegumain is present in EIW-A. This theory is somewhat supported by the observation that AndoSan™ showed greater inhibition of autoactivation of prolegumain (maximum 80 %) than active enzyme (maximum 40 %), in that EIW-B showed weaker inhibition of legumain than EIW-A.

These theories can be easily tested by obtaining more of EIW-A and EIW-B and incubate both with active enzyme and autoactivated enzyme, respectively. If the results indicate that the theory reflects reality, the protein in EIW-B should be isolated and identified, and the components in EIW-A should be further purified and identified.

4.6 Future work

Future work on AndoSan™ should focus on further purification of fractions and identification of compounds in all extracts, perhaps particularly those in EIW, based on its inhibition of legumain activity in RAW264.7 cells.
The mechanisms by which legumain expression is reduced in these cells should also be investigated. Furthermore, the purified fractions could be tested in a different biological test system, e.g. cultured cancer cells that have been assessed positive for increased legumain activity. The extracts should also be assessed for legumain activity *in vivo*, due to the contradictory findings with pro-inflammatory cytokines *in vitro* versus *in vivo* (described in section 1.2.2).

In addition, the alcohol extracts could be purified and tested with different bioassays to investigate possible health beneficial properties of the low Mw compounds in AndoSan™. Perhaps the extracts contain agaritine, which has been shown to induce apoptosis in leukemic cells (Akiyama et al. 2011).

5 Conclusions

Components in AndoSan™ were separated into five crude fractions based on chemical and physical properties, which were characterised and tested for biological activity in RAW264.7 cells. The expression of legumain in these cells was used to indicate antitumour properties of the fractions.

Results indicated that, with regard to legumain activity, EIW is the most promising extract. It showed a statistical significant decrease in legumain activity *in vitro*, both when compared to control and to unfractionated AndoSan™. In addition, the extract showed 80 % inhibition of active enzyme. On the basis of these properties, EIW was separated into two fractions by SEC; EIW-A and EIW-B, of which EIW-A exhibited better inhibition of legumain. However, the inhibition by EIW-A was not better than for the crude EIW, which indicates synergy or combined effects of components in this extract. Furthermore, the effects on legumain expression *in vivo* need to be determined before any conclusions regarding beneficial properties of AndoSan™ on legumain activity can be drawn.

The alcohol extracts, containing low Mw components, showed no significant inhibition of legumain activity, and was therefore not selected for further purification and characterisation. These should be tested with other bioassays to look for biological effects of the low Mw components of AndoSan™.

The carbohydrate analyses of unfractionated AndoSan™ and AndoSan™ extracts indicated low carbohydrate content in the product. This implies that the biological activity of AndoSan™ cannot solely be attributed to β-glucans, which is indicated by previous research articles regarding AbM.

Only rough compositional analyses were performed with the extracts; there has been no identification of specific bioactive compounds. For this purpose, the extracts need to undergo further separations and purification.

6 Literature

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Appendix 1: Chemicals

- 1-methyl imidazole, Sigma-Aldrich Co., USA
- Acetic acid, Merck KGaA, Germany
- Acetic anhydride, Merck
- Acrylamide-bis, Merck
- Acrylamide/bis-Acrylamide, A3699, Sigma-Aldrich Co.
- Amersham™ LMW Calibration Kit for SDS Electrophoresis (17-0446-01) GE Healthcare, UK
- Ammonia, Merck
- Ammonium peroxodisulfate, Merck
- BioRad Protein Assay, Bio-Rad Laboratories, USA
- Bromophenol Blue, Bio-Rad Laboratories
- Ceric ($Ce(SO_4)_2$ in H_2SO_4), Sigma-Aldrich Co.
- CHAPS, Sigma-Aldrich Co.
- Chloroform, VWR International, Norway
- Citric acid, Merck
- Coomassie Blue R250, LKB AB (Now GE Healthcare)
- Deuterated methanol, Sigma-Aldrich Co., USA
- Deuterated water, Sigma-Aldrich Co., USA
- Dichloromethane, Sigma-Aldrich Co., USA
- Disodium EDTA, Ferak Berlin, Germany
- Disodium phosphate, VWR International, Norway
- DL-Dithiothreitol, Sigma-Aldrich Co.
- D-Mannitol, Sigma-Aldrich Co.
- DMEM 1X, GIBCO®, 42430, Invitrogen, Life Technologies Co., USA
- DMSO, Sigma-Aldrich Co.
- Ethanol, VWR International, Norway
- Fetal bovine serum (FBS), PAA Laboratories, Pasching, Australia
- Glacial acetic acid, Merck KGaA, Germany
- Glycerol, Merck KGaA, Germany
- Glycine, Sigma-Aldrich Co., USA
- Hydrochloric acid, Merck KGaA, Germany
- Methanol,VWR International S.A.S.
- Methanol, anhydrous, Merck KGaA, Germany
- Methanolic HCl, SUPELCO, Sigma-Aldrich Co., USA
- Methyl iodide, Fluka Analytical, Sigma-Aldrich Co.
- Octyl β-D-glucopyranoside, Sigma-Aldrich Co.
- Penicillin (1000 U/mL)/Streptomycin (10 mg/mL), Sigma-Aldrich Co.

- Phosphate buffered saline (PBS), Invitrogen, Life Technologies Co.
- Phosphorus pentoxide, Merck
- Sephacryl S-100 HR, Pharmacia Biotech
- Sodium bicarbonate, Merck KGaA, Germany
- Sodium borodeuteride, Sigma-Aldrich Co.
- Sodium dodecyl sulfate, Sigma-Aldrich Co.
- Sodium hydroxide, VWR International, Norway
- Sodium sulfate, Merck KGaA, Germany
- Sodium thiosulfate, Merck
- Tetramethylethylenediamine, Bio-Rad Laboratories
- TMS is made with the following: 1 TMCS + 2 HDMS + 5 pyridin;
  TMCS - Chlorotrimethylsilane, Sigma-Aldrich Chemie GmbH, Germany.
  HMDS - Hexamethyldisilazane, Sigma-Adlrich Chemie GmbH, Germany.
  Pyridine, Merck
- Trifluoroacetic acid, Merck
- Tris-(hydroxymethyl)aminomethane, Chemi-Teknik AS, Norway
- Trisodium citrate, May & Baker LTD Dagenham, England
- Trizma hydrochloride, Sigma-Aldrich Co.

Appendix 2: Abbreviations

- AbM – *Agaricus blazei Murill*
- Ala – Alanine
- Asn – Aspargine
- CHAPS - 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
- CR – Complement receptor
- DCM – Dichloromethane
- DMEM – Dublecco's modified eagle medium
- DTT – Dithiothreitol
- EDTA – Ethylenediaminetetraacetic acid
- EIW – Ethanol insoluble water extract
- ESW – Ethanol soluble water extract
- EIW-A – Ethanol insoluble water extract, fraction A
- EIW-B – Ethanol insoluble water extract, fraction B
- FBS – Fetal bovine serum
- GC/GC-MS – Gas chromatography/Gas chromatography-mass spectrometry
- HPLC – High performance liquid chromatography
- IgE – Immunoglobulin E
- IL – Interleukin
- LC-MS – Liquid chromatography-mass spectrometry
- ^{1}H NMR – Proton nuclear magnetic resonance
- PAMP – Pathogen-associated molecular pattern
- PBS – Phosphate buffered saline
- PRR – Pattern recognition receptor
- RAW264.7 – monocyte/macrophage cell line, *Mus musculus*, BALB/c, acquired from ATCC
- RI – Refractive index
- SEC – Size exclusion chromatography
- SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis
- TFA – Trifluoroacetic acid
- TLC – Thin layer chromatography
- TLR – Toll like receptor
- TMS – Trimethylsilyl
- TNF – Tumour necrosis factor