Mannan-binding lectin (MBL) associated serine protease-3 (MASP-3):

Complex formation in serum and plasma, conditions required for the conversion of the zymogen form into a two-chain serine protease, and a search for substrates using recombinant material produced by stable expression in eukaryotic cell lines.

PhD Thesis

by

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Preface

This thesis is based on investigations carried out in the period January 2000 to December 2003 at the Department of Microbiology and Immunology, University of Leicester, England, under supervision of Professor Wilhelm Schwaeble.

As part of my studies, I had the pleasure to join the laboratory of Professor Jens Christian Jensenius at the Department of Medical Microbiology and Immunology, University of Aarhus, Denmark.

This thesis is a presentation of my work on mannan-binding lectin associated serine protease-3 (MASP-3) as well as an introduction to the human immune system.

Acknowledgements

I sincerely wish to thank my supervisor Professor Wilhelm Schwaeble for his excellent guidance during my PhD studies. I am indebted to him and have greatly appreciated his encouraging and supportive spirit. His enthusiasm has been an inspiration for me and created a dynamic working environment. I further wish to thank the internal examiner Dr. Cordula Stover, for always having good advice when needed.

I sincerely wish to thank Professor Jens Christian Jensenius and Dr. Steffen Thiel for providing me with the opportunity to work at the University of Aarhus and for their never-failing interest in my work. I also wish to thank Professor Shaun R. Coughlin for the opportunity to visit his laboratory at the University of California, San Francisco, US.

I would like to thank all my friends and colleagues at both Universities, especially Dr. Nick Lynch and Mr. Steve Hanson. I wish to thank Mette Møller-Kristensen for her positive qualities and for good suggestions and discussions. I wish to thank Dr. Tove Christensen for her help and friendship and Annette Hansen for her excellent technical guidance and personal nature.

I gratefully acknowledge the financial support by The Wellcome Trust for my PhD.

I thank my wife Katja and my children Aksel and Sofia for their support and love and whatever happens I intend to hold on to the three of you.

Abstract

The complement system is part of the innate immune system and is crucial for identifying invading microorganisms. The lectin pathway of complement activation is initiated through multimeric macromolecules which recognise pathogen-associated patterns and translate binding through activation of associated serine proteases that start a cascade of proteolytic events leading to bactericidal, opsonising and proinflammatory responses. Mannan-binding lectin (MBL) is one of the macromolecules mediating binding to specific carbohydrate structures common to a range of microorganisms. Three different mannan-binding lectin associated serine proteases (MASP-1/-2/-3) and a non-enzymatic protein of 19 kDa (MAp19) have been described. MASP-2 appears to mediate all processes required for complement activation while little or no complement-related functional activity was found to be mediated by MASP-1, MASP-3 or MAp19.

Functional and biophysical studies of MASP-3 relied on continuous and reliable supply of recombinant MASP-3. In this work production of recombinant MASP-3 in mammalian cells with subsequent affinity purification and characterisation of the recombinant MASP-3 was performed to obtain large quantities of homogeneous enzyme. The development of screening assays and assays for quantitative determination of MASP-3 levels were two other tools essential for the development of this thesis. As a result of this thesis MASP-3 levels in different body fluids were determined in healthy individuals using a quantitative assay. The assays were used to analyse the MASP-3 level in sample collections from patients suffering from Alzheimer disease and type-1 diabetes. The correlation of MASP-3 level and MBL genotype, H-/L-Ficolin concentration, age, BMI, acute phase and time of year were analysed.

The enzymatic activity of MASP-3 was analysed on chromogenic substrates and the results permitted a study of MASP-3 inhibition. Attempts were made to affinity purify potential MASP-3 substrates and ligands using beads coupled with recombinant MASP-3 and anti-MASP-3 antibodies. The influence of calcium on MASP-3 complex formation, dissociation, activation and stability was analysed.

List of abbreviations

AP:	alkaline phosphatase
ATIII:	antithrombin III
BCG:	bacillus calmette guerin
BMP:	bone morphogenic protein
bp:	base pair
C-:	carboxy-
CCP:	complement control protein
cDNA:	complementary DNA
CHO:	chinese hamster ovary cells
COS:	monkey kidney cell line
CR:	complement receptor
CRD:	carbohydrate recognition domain
CUB:	C1r/C1s/uEGF/BMP
Cx:	complement factor No. x
DAF:	decay-accelerating factor
ddH_2O:	double distilled water
dH_2O:	distilled water
DMEM:	Dulbecco's modified Eagle medium
DNA:	deoxyribonucleic acid
DNase:	deoxyribonucleoase
EBNA:	Epstein-Barr virus nuclear antigen
ECL:	enhanced chemiluminescence
EGF:	epidermal growth factor
ELISA:	enzyme linked immunosorbent assay
ER:	endoplasmic reticulum
EST:	expressed sequence tags
FX:	factor X
FCS:	foetal calf serum
GalNAc:	N-acetyl-D-galactosamine
GlcNAc:	N-acetyl-D-glucosamine
HEK:	human embryonic kidney
HRP:	horseradish peroxidase
Ig:	immunoglobulin
ITS:	insulin-transferrin-selenium
kb:	kilo basepairs
kDa:	kilodalton
LPS:	lipopolysaccharide
MAC:	membrane attack complex
MASP:	MBL-associated serine protease
MAp19:	MBL-associated protein of 19 kDa
MBL:	mannan binding lectin
MCP:	membrane cofactor protein
MDP:	muramyldipeptide
mRNA:	messenger RNA
MW:	molecular weight
N-:	amino-
NBT:	nitroblue tetrazolium
ND:	not determined
dNTP:	dideoxy nucleotide triphosphate
o/n:	over night
OD:	optical density
PAGE:	polyacrylamide gel electrophoresis
PAR:	protease activated receptor
PCR:	polymerase chain reaction
PNGase F:	N-glycosidase F
PPD:	purified protein derivative
PVDF:	polyvinylidene difluoride
RNA:	ribonucleic acid
RNase:	ribonuclease
rpm:	revolutions per minute
rRNA:	ribosomal RNA
RT:	room temperature
SCR:	short consensus repeats = CCP
SDS:	sodium dodecyl sulphate
SP:	serine protease
SRD:	scavenger receptor cysteine-rich domain
TRIFMA:	time-resolved immunofluorometric assay
tRNA:	transfer RNA
dTTP (T):	dideoxy thymidine triphosphate
uEGF:	sea urchin epidermal growth factor-containing protein
UV:	ultraviolet
v/v:	volume/volume
VWFA:	von Willebrand factor type A
w/v:	weight/volume

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

1.1 Immunology

Immunology is a branch of biomedical science that is devoted to the study of the body's responses to defend itself against infection, e.g. any microorganism or substance causing disease, and the consequences of such an immune response. Immunologists study the tissues, organs of the immune system (bone marrow, spleen, tonsils, thymus, lymphatic system), its specialised cells (e.g., B and T lymphocytes), and the molecular processes that constitute the immune system. The field of immunology has raised great public interest, due to the success of vaccination in the prevention of severe infectious disease, undesirable responses such as allergy, and autoimmune diseases (e.g. rheumatoid arthritis and systemic lupus erythematosus), which occur when the body recognise and reacts with an immunological response towards some of its own constituents. Immunity is the ability to recognise and dispose of foreign material that enters the body in the event of an infection, but also rejection or acceptance of transplants. The aim of these studies is to analyse aspects of the immune system (Delves and Roitt 2000).

1.2 The human Immune system

One of the body's self defence mechanisms is called the immune system. The system is comprised of proteins and cells interacting in a complex manner and it are often divided into the natural (also referred to as the innate) and the acquired immune system. The innate immune system provides basic means for first line of defence and destruction of pathogens. The acquired immune system has immunological memory, providing improved and enhanced immune responses on re-infection. The acquired immune response is the long-term defence system, where selected antigen-specific lymphocytes (B-cells and T-cells) respond to antigen. This system relies on the deletion of self-reactive lymphocytes, hereby generating a pool of naive lymphocytes. A lymphocyte is called naive until it has encountered its antigen. The encounter leads to binding of the antigen to a cell bound antigen-receptor, and subsequently to proliferation and differentiation of the cell. The secreted forms of the B-lymphocyte antigen-receptor are known as antibodies or immunoglobulins (Janeway and Travers 1997). The mechanisms involved in the natural immune system range from physical and chemical barriers to cells and proteins. The barriers function as non-specific protectors against

infections and they do not change with repeated exposure to infection. The barriers prevent the pathogen from entering the body, trapping microorganisms in mucous, or chemically neutralising it by extreme pH values e.g. in the stomach (pH~3). Secretions in the skin and mucous surfaces containing enzymes such as lysozyme also prevent infection. Organisms living in symbiosis often provide mutual immunological advantages and these microorganisms are referred to as the normal flora. Natural immunity is also delivered by cells such as macrophages and natural killer cells that can phagocytose and kill pathogens or virus-infected cells. Furthermore the natural immune system is mediated by soluble plasma components and includes proteins with both enzymatic and non-enzymatic activity. The response of the natural immune system has influence on the adaptive system and they are integrated in many aspects. There are two major protein systems in the natural immune system. They are known as the haemostatic and the complement systems respectively (Delves and Roitt 2000).

1.3 The complement system

The complement system is a vital part of the innate defence mechanism. This system involves at least 33 soluble plasma proteins, and a number of cell surface bound proteins. The complement system can distinguish self from non-self via recognition and regulatory proteins either found in plasma or as membrane-bound molecules on self-cell surfaces (Muller-Eberhard *et al.* 1988). The major functions of the complement system are to target microorganisms (opsonisation), and non-self components, recruitment of phagocytic cells to the site of infection, and direct killing of a target pathogen (Ross and Medof 1985). The complement system can be activated through three distinct pathways: the classical pathway, the lectin pathway, and the alternative pathway (see Figure 1.1). The activation of the complement system can have various effects: when C3 is cleaved into C3a and C3b, the C3b fragment is covalently bound to the surface of a pathogen, where it functions as an opsonin and can mediate phagocytosis.

The enzyme cascade cleaves a range of proteins, and some of the resulting peptide fragments can mediate inflammation and function as chemokines. C5a has the ability to attract phagocytic cells (Fearon and Locksley 1996). One result of activation of the complement system is the formation of the membrane-attack complex (MAC). This complex makes a pore

through the membrane of the pathogen, killing it by lysis. The peptides and cell fragments secreted as a consequence of lysis or phagocytosis are used by the adaptive immune system to signal proliferation of specific B-lymphocytes and development of new B-lymphocytes (Carroll 2000).

1.4 The classical pathway

The classical pathway is initiated by the binding of the C1 complex to charge clusters on target particles. The C1 complex consists of one C1q molecule and two serine proteases: two C1r and two C1s proteins ($C1qr_2s_2$) (Tschopp *et al.* 1980). The C1q molecule is composed of six heterotrimeric subunits consisting of a collagenous stalk and a globular head (Sim and Reid 1991). Upon binding of the C1 complex to targets, the serine protease C1r is converted from its proenzyme form to an active proteolytic enzyme, which can cleave and activate C1s. The activated C1s enzyme in the C1 complex can cleave C4 and C4b-bound C2. The result is the formation of a C3 convertase (C4b2b) which can cleave C3 and the subsequent formation of the C5 convertase (C4b2b3b) can furthermore cleave C5. The C5 convertase of the lectin and classical pathways is relatively labile with a half-life of 1.5 to 3 min. (Cooper and Muller-Eberhard 1970). The major C5 cleavage fragment (C5b) can initiate the assembly of the membrane attack complex (C5b6789) composed of C5b, C6, C7, C8, and several C9 molecules. This complex makes a pore through the membrane of the pathogen, killing it (Muller-Eberhard *et al.* 1986). See Figure 1.1 for details of complement activation by the classical pathway. C1q deficiency is strongly associated with systemic lupus erythematosus (Ratnoff 1996).

1.5 The lectin pathway

Binding of the MBL/MASP complex or ficolin/MASP complexes (introduced in section 1.9) to PAMPs (pathogen-associated molecular patterns) on the surface of a pathogen initiates the lectin pathway. The MBL complex consists of MBL oligomer, MBL-associated serine proteases (MASPs) and a non-enzymatic protein called MAp19 (Stover *et al.* 1999) or sMAP (Takahashi *et al.* 1999). There are three MASPs described in the literature: MASP-1 (Matsushita and Fujita 1992), MASP-2 (Thiel *et al.* 1997) and MASP-3 (Dahl *et al.* 2001). It has been established that MASP-2 is the enzyme responsible for the cleavage of C4 and C2

(Vorup-jensen *et al.* 2000). Comparing the efficiency of C4 and C2 cleavage by C1s and MASP-2 showed that MASP-2 cleaves these substrates with the higher efficiency (Rossi *et al.* 2001). MASP-1 was initially described as the C4 and C2 cleaving enzyme (Matsushita and Fujita 1992) and later as having C3 cleaving activity: this initial finding could have been due to the presence of MASP-2 in the preparation (Matsushita and Fujita 1995). There have been contradictory reports of alternative pathway activation by MASP-1 through direct cleavage of C3. Several publications claim to find direct cleavage of C3 by MASP-1 (Ogata *et al.* 1995; Matsushita M. *et al.* 2000; Dahl *et al.* 2001; Rossi *et al.* 2001) whereas other reports dismiss this activity as being unlikely (Wong *et al.* 1999; Petersen *et al.* 2000; Ambrus *et al.* 2003). Studies of the enzymatic activity of MASP-1 outside the complement system have shown some activity of MASP-1 in the haemostatic system. Thrombin-like substrate specificity towards factor XIII (FXIII) and fibrinogen has been reported (Hajela *et al.* 2002). The MASP-1/3 knock-out mouse reported by Fujita's group (Japan) in 2000 (Takahashi *et al.* 2000), has not been the central subject in any publication and thus has not contributed to elucidation of the substrate specificity of either MASP-1 or MASP-3. This group, however, did not observe a phenotype of even a partial deficiency of lectin pathway activity in absence of MASP-1 and MASP-3 under physiological conditions (Takahashi *et al.* 2001). The substrate of MASP-3 has not been defined.

There are three ficolins described in the literature: L-, H- and M-ficolin and complement activation has been shown for L-ficolin/MASP complexes, and H-ficolin/MASP complexes (Matsushita *et al.* 2000 and, Matsushita and Fujita 2001). Upon binding, the lectin pathway initiates activation of an enzyme cascade identical to that described for the classical pathway (See Figure 1.1).

1.6 The alternative pathway

Initiation of the alternative pathway is the result of binding of C3b to the surface of pathogens in the circulation. C3b and the serine proteases factor D and factor B are the components of this pathway. The bound C3b initiates the formation of the C3 convertase of the alternative pathway (C3bBb) and this rapidly deposits many molecules of C3b on the pathogen surface. The inherently labile C3 convertase is protected against decay by properdin which acts as a positive regulator of the alternative pathway (Wirthmueller *et al.* 1997). The C3 convertase

stabilised by properdin has a half-life at 37^{0}C of 10-34 min. (Medicus *et al.* 1976). The C5 convertase of the alternative pathway (C3bBb3b) can then be assembled (Figure 1) (Horstmann *et al.* 1985). The cleavage of C3 by C3bBb acts as an amplification loop for all three pathways. The enzyme cascades, which are activated by complement activation, are described in detail in Figure 1.1.

1.7 The terminal pathway of complement activation

The two C5 convertases; C4b2b3b and C3bBb3b cleave C5 into C5a and C5b, initiating the formation of the membrane attack complex (MAC). There are five different soluble plasma proteins involved in this complex: C5-C9. The formation of the MAC is controlled by S-protein and CD59 by blocking membrane insertion of the MAC complex (Morgan 1999).

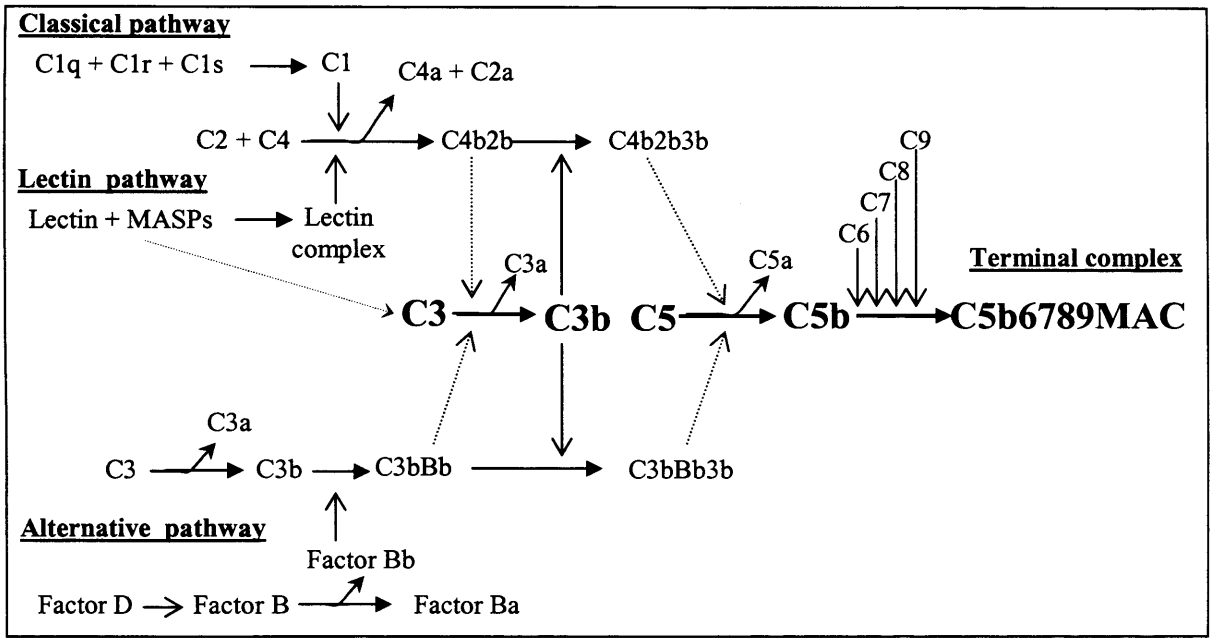

Figure 1.1. A schematic overview of the enzyme cascade initiated by the classical, lectin and alternative pathways of complement activation. The initiation of the individual pathways and the terminal complex are indicated in the text. C1 is the protein complex consisting of C1q, C1r, and C1s. The Lectin complex can consists of MBL/MASP or ficolin/MASP complexes. The terminal complex C5b6789MAC is also referred to as the membrane attack complex (MAC). The function of the C3 and C5 convertases are indicated with a punctured arrow (Modified from Law and Reid 1995).

The complement system is controlled at various points by inhibitors. C1-inhibitor is a member of the serine protease inhibitor (serpin) superfamily and inhibits the serine proteases of the classical pathway (Sim *et al.* 1979; Ziccardi 1981). Factor I inhibits the formation of the C3/C5 convertase by cleaving C3b and C4b in the presence of cofactors factor H and C4b-binding protein (C4BP), respectively (Sim *et al.* 1993; Villiers *et al.* 1985). Factor H also

controls the activity of the alternative pathway derived C3/C5 convertase by competing with factor B for C3b binding (Pangburn 2000). The serine protease inhibitor alpfa-2-macroglobulin, a serum glycoprotein, is a major plasma proteinase inhibitor with a wide specificity (Kan *et al.* 1985). MBL/MASP complex purified by affinity chromatography was found to contain alpha-2-macroglobulin bound directly to MASP, possibly through covalent bonds, indicating possible MASP inhibition by alpha-2-macroglobulin (Terai *et al.* 1995). The inhibitory effect of C1 inhibitor and alpha-2-macroglobulin on the classical and lectin pathway demonstrated that C1 inhibitor inhibited both pathways whereas alpha-2-macroglobulin inhibited neither (Petersen *et al.* 2000).

There are several complement receptors which mediate cellular responses to complement activity. The first of the receptors described was Complement receptor 1 (CR1) (Fearon 1979) recognised as a receptor of C3b and C4b fragments, and for C1q (Klickstein *et al.* 1997). Complement receptor 2 (CR2) has been reported as a receptor of C3d and iC3b as well as the Epstein-Barr virus glycoprotein gp350 (Fingeroth *et al.* 1984; Nemerow *et al.* 1985). Decay-accelerating factor (DAF) and membrane cofactor protein (MCP) both function on the surface of self-tissue regulating the amount of C3/C5 convertase complexes and C3b/C4b bound, respectively (Nicholson-Weller *et al.* 1982; Medof *et al.* 1984; Seya and Atkinson 1989).

1.8 The haemostatic system

The haemostatic system can be separated into three pathways: The clotting system, the fibrinolytic (plasmin) system, and the kinin system.

There are two interdependent pathways for blood coagulation: the extrinsic and the intrinsic pathways. Activation of the extrinsic pathway takes place when factor III (tissue factor, FIII) is released from ruptured tissue. In the presence of Ca^{2+}, FIII and factor VII (proconvertin, FVII) forms a complex capable of cleaving factor X (Stuart-Prower factor, FX), with factor VIII (anti-haemophilic factor, FVIII) as a cofactor (Figure 2) (Heimark *et al.* 1980; Shariat-Madar *et al.* 2002).

The intrinsic pathway depends on humoral proteins and endothelial surface changes for activation. Factor XII (Hageman factor, FXII) binds to endothelial surface when the structure and net-charge changes, e.g. due to injury which makes FXII cleave prekallikrein to kallikrein. High-molecular-weight kininogen (HMWK) acts as a binding site on the surface for both FXII and kallikrein, and kallikrein cleaves FXII into its activated form (FXIIa) which converts factor XI (plasma thromboplastin antecedent, FXI) into FXIa that also can bind to the HMWK complex. The final step of the intrinsic pathway is the cleavage of factor IX (Christmas factor) by FXIa.

The enzyme cascades of the two pathways merge with the cleavage of FX to FXa. The cleavage is mediated by interaction of FX with cofactor V (labile factor, FV). The FXa can then cleavage factor II (prothrombin, FII) to factor IIa (thrombin). Thrombin has a central role and many functions and enhancing effects in the system, but its main function is the cleavage of fibrinogen to fibrin. Fibrin is stabilised by active FXIII (fibrin stabilising factor, FXIII). The coagulation system is regulated to ensure restriction of coagulation to the site of injury. Antithrombin III (ATIII) and heparin inhibits the activated proteases FIIa, FIXa, FXa and FXIIa (Butenas and Mann 2002).

C1-inhibitor is a serpin (serine protease inhibitor) that, apart from regulating several complement proteases, regulates the activity of FXIa, FXIIa and kallikrein (Schapira *et al.*. 1985).

The activation of plasminogen to plasmin is the central mechanism of fibrinolysis. Activation of the fibrinolytic system counterbalances the fibrin production and it can be activated by the HMWK/FXIIa/FIXa/kallikrein complex, tissue plasminogen activator (t-PA) or urokinase

plasminogen activator (u-PA). Plasmin cleaves the unstable fibrin into small fragments, and can be inhibited by alpha-2-antiplasmin and alpha-2-macroglobulin (Figure 1.2) (Mullertz 1986).

Bradykinin is produced due to the activation of the kinin system, and is a degradation product of the HMWK/FXIIa/FIXa/kallikrein complex, arising from the cleavage of HMWK by kallikrein (Rojkjaer and Schmaier 1999).

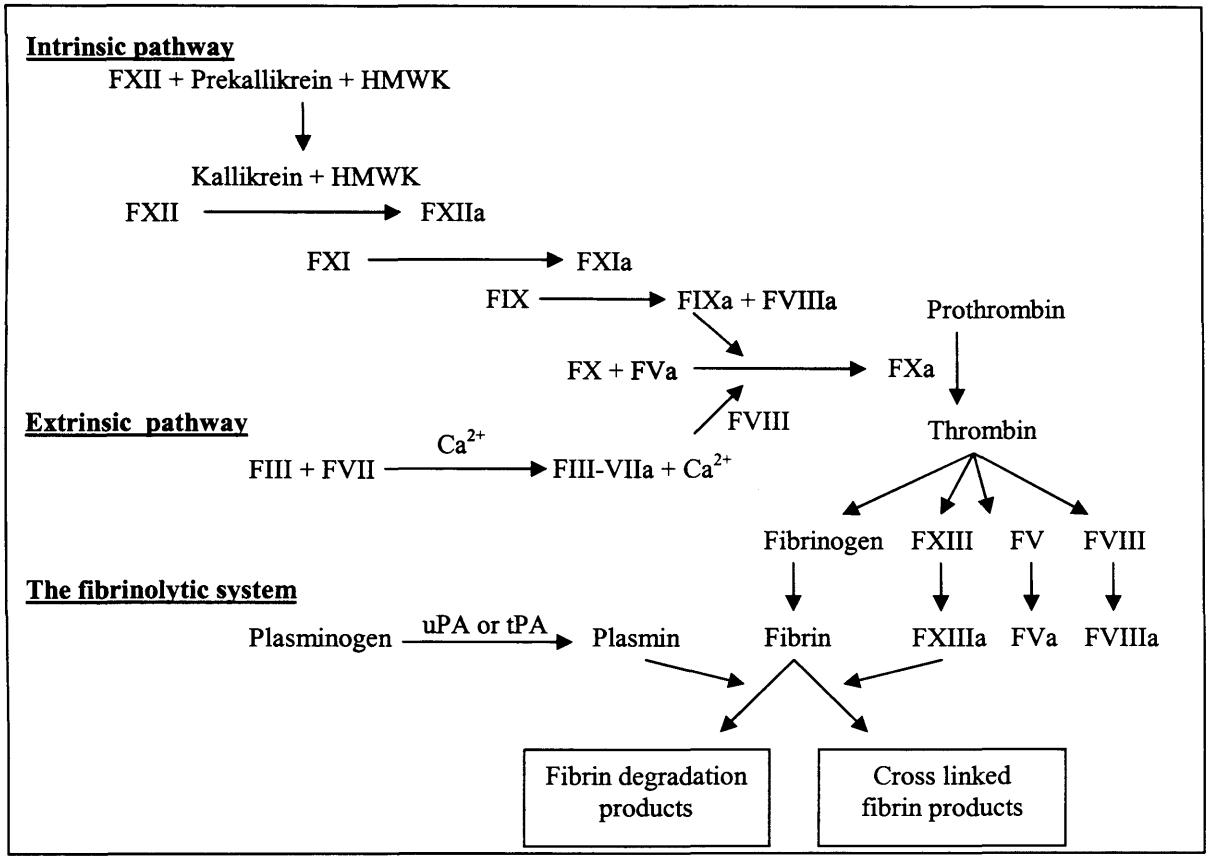

Figure 1.2. Schematic overview of the intrinsic and extrinsic pathway of coagulation and the fibrinolytic system. Factor V and VIII (indicated with highlights) are activated by thrombin. High-molecular-weight kininogen (HMWK) acts as a cofactor for the intrinsic pathway (Modified from Arif and Mufti 1998).

The function of plasma kallikrein is not restricted to the intrinsic pathway, but has been shown to also cleave the complement factors iC3b (Meuth *et al.* 1983) and C5 (Wiggins *et al.* 1981).

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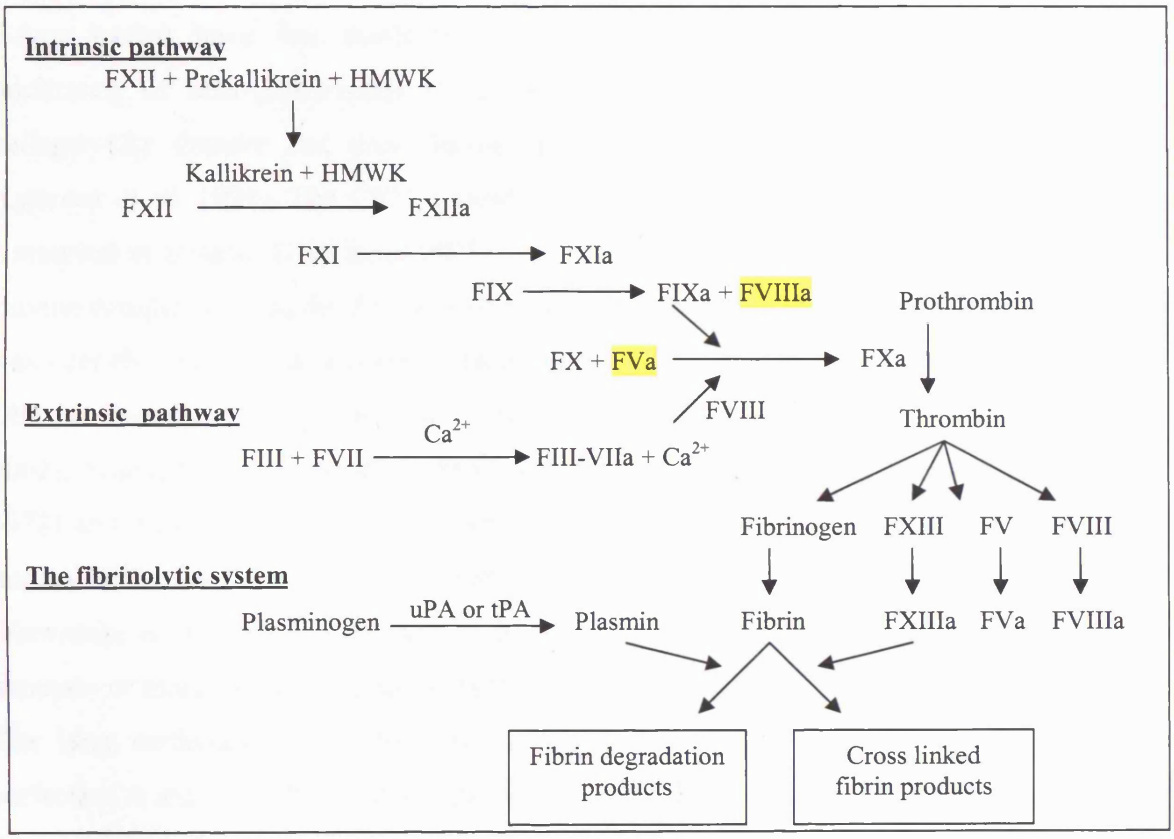

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1.9 Collectins and ficolins

Collagen forms a alpha-helical coil structure composed of Gly-Xaa-Yaa amino acids repeats. It has a wide distribution throughout the animal and plant kingdoms. A lectin is a molecule capable of recognising carbohydrate structures such as those present on the surface of pathogens. Lectins of the C-type have a carbohydrate recognizing domain (CRD) that is calcium dependent for binding to the sugar and this type is the most common in animals. The S-type lectins have free thiols from cysteine residues and a carbohydrate recognition preference of beta-galactosides (Drickamer 1988). Collectins are C-type lectins with a collagen-like domain and these lectins are found in vertebrates from avians to humans (Laursen *et al.* 1998). The CRD consists of ~90 aa folded in a structural pattern, which is conserved in general (Drickamer 1993).

Bovine conglutinin was the first animal lectin to be described (Bordet and Streng 1906) and it was later characterised as a collectin (Kawasaki *et al.* 1985).

Other collectins are only found in the bovidae family e.g. CL-43 and CL-46 (Hansen *et al.* 2002). Mannan-binding protein (MBP) was first isolated from rabbit liver (Kawasaki *et al.* 1978) and was later found to be a serum protein in rabbits (Kozutsumi *et al.* 1980). The human form of MBP was characterised a few years later and named: mannan-binding protein (Kawasaki *et al.* 1983). The name was later changed and is today usually referred to as mannan- or mannose-binding lectin, both abbreviated MBL.

The lung surfactants from different species were shown to contain the collectins lung surfactant A and –D (SP-A and SP-D) (White *et al.* 1985; Persson *et al.* 1988).

Several genes encoding different forms of SP-A were described via the characterisation of SP-A2 and a pseudo-gene (genes which are not transcribed): ΨSP-A (Katyal *et al.* 1992). These collectins were found to interact with different types of lipopolysaccharides (LPS) from bacteria. SP-A and SP-D have been shown to act as opsonins in the phagocytosis of some types of bacteria by alveolar macrophages (Pikaar 1995) and to bind influenza virus. (Malhotra *et al.* 1994; Hartshorn *et al.* 1994).

Recently, two human collectins were found by genomic analyses: CL-L1, which is present mainly in the liver as a cytosolic protein (Ohtani *et al.* 1999) and CL-P1, which is expressed in vascular endothelial cells and described as a scavenger receptor, a membrane-type collectin from placenta (Ohtani *et al.* 2001).

MBL is the only collectin known to activate complement and it is a central protein in the third pathway of complement activation; the lectin pathway (Ikeda *et al.* 1987). Ficolins have a similar domain structure to MBL but the CRD in MBL is replaced by a fibrinogen-like domain and the ficolins do not require Ca^{2+} for binding to their ligands. L- and H-ficolin are produced by the liver and found as serum proteins. L-ficolin and H-ficolin are associated with MASPs and MAp-19, and can activate the lectin pathway of complement. Furthermore, L-ficolin can act as an opsonin (Matsushita *et al.* 2002; Matsushita and Fujita 2000).

M-ficolin has been found on peripheral blood monocytes as a membrane-bound protein. It is capable of binding N-acetyl-D-glucosamine but there has been no demonstration of complement activation through the lectin pathway or MASP association. It is speculated that M-ficolin may potentially mediate monocyte adhesion by acting as a phagocytic receptor (Teh *et al.* 2000). The structure and relative size of the trimeric subunits of collectins and ficolins is shown in Figure 1.3.

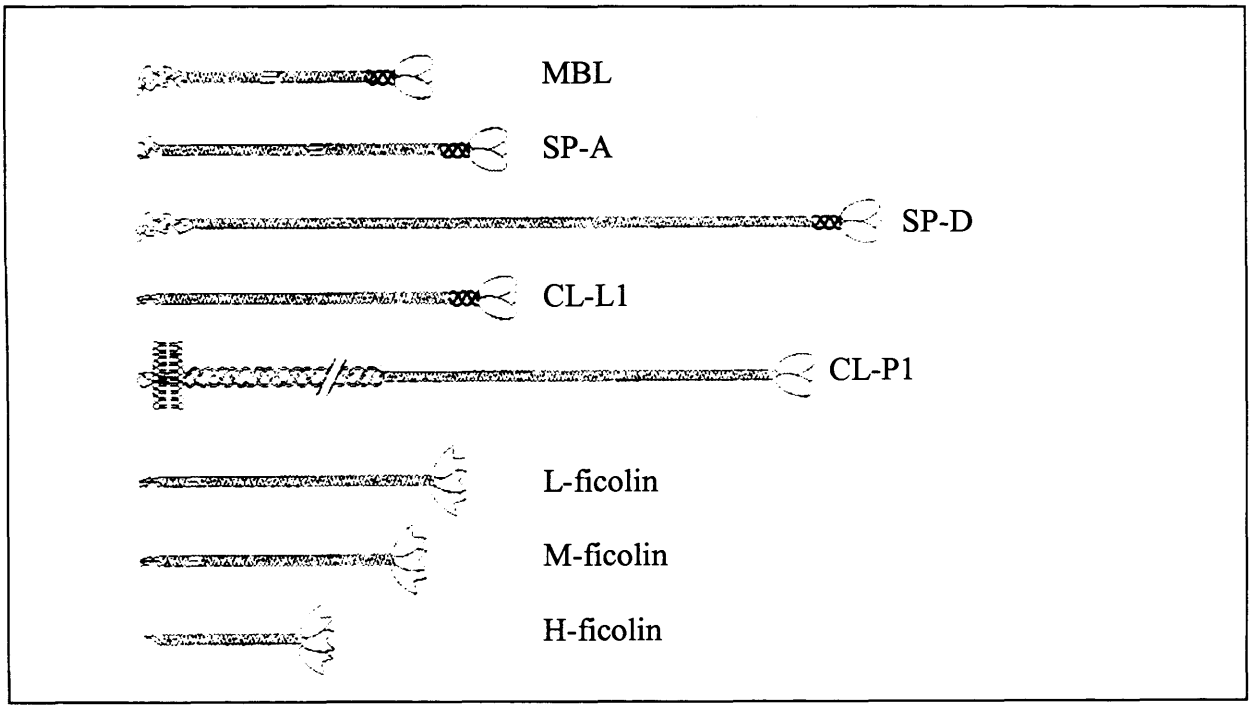


Figure 1.3. The structural subunits of human collectin and ficolins contain three polypeptides stabilized by 2-3 disulfide bridges at the N-terminal (left side) and a triple-helix collagen-like region which ranges from 33aa (H-ficolin) to 177aa (SP-D). The C-terminal (right side) of the proteins contains the recognition domains of the polypeptides. The subunits are drawn approximately to scale except for CL-P1 which contains a 378aa long alpha-helical coiled-coil structure following its membrane spanning domain. The figure is modified from (Holmskov *et al.* 2003).

1.10 The structure of MBL

The human MBL precursor protein contains a signal peptide, a cysteine-rich domain, a collagen-like domain, a 'neck' region, and a carbohydrate-binding domain, and is encoded by four exons (Ezekowitz *et al.* 1988). A recombinant rat MBL-A CRD was crystallised in complex with an oligosaccharide indicating the participation of calcium in the CRD structure (Weis *et al.* 1992). The folding of the trimeric subunits and subsequent coordination of CRDs allow for optimal binding to specific carbohydrates (Weis and Drickamer 1994; Sheriff *et al.* 1994). The trimeric subunits can form larger oligomers containing 2-6 subunits (Lu *et al.* 1990). The gene coding for MBL is located on chromosome 10q11, within the same gene cluster as SP-A and SP-D (see Figure 1.4). By comparing the structure of the MBL gene to the structure of SP-A and SP-D it was concluded that the MBL gene evolved by recombination of an ancestral collagen gene with a gene encoding carbohydrate recognition (Sastry *et al.* 1989; Kolble *et al.* 1993). Promoter region consensus sequences were considered consistent with the suggestion that MBL is regulated as an acute-phase protein synthesised by the liver (Taylor *et al.* 1989).

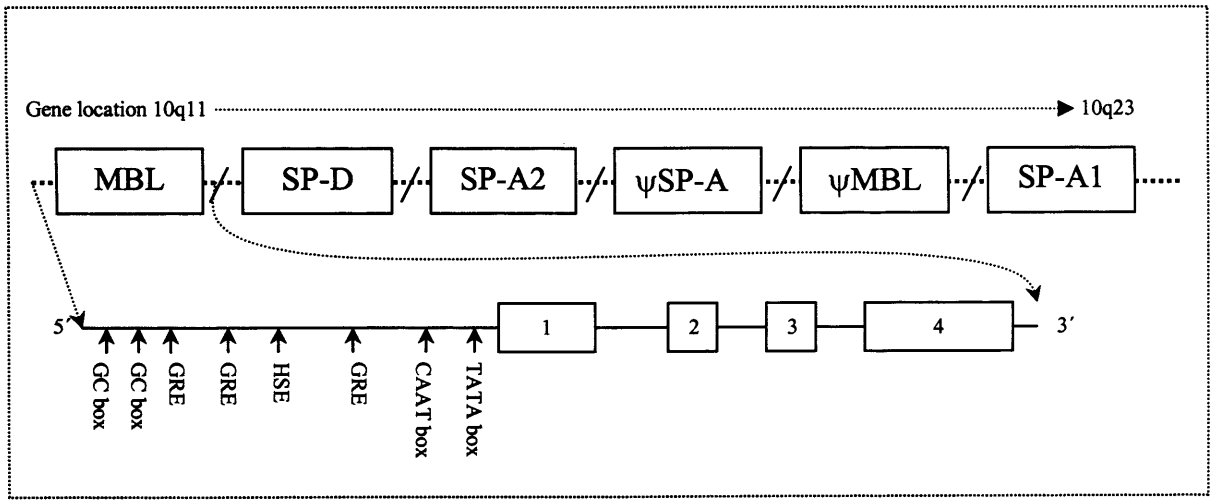


Figure 1.4. Schematic depiction of the human chromosome 10q11-23 encoding MBL, SP-D, SP-A2, ψSP-A, ψMBL and SP-A1. The genes are interrupted by other genes over 35000 kb and are not drawn to scale. The abbreviation ψ (pseudo) is used for the recently described ΨSP-A (Hoover and Floros 1998) and ψMBL (Guo *et al.* 1998).

The MBL gene organisation is shown with indication of the four exons and the promoter region elements. The transcription elements consists of a TATA box (-38 bp), a CAAT box

(-79 bp), three glucocorticoid responsive elements (GRE: -736, -656 and -245 bp) and a heat shock consensus sequence (HSE: -592 bp) (Sastry 1989). Two forms of MBL have been described in rodents, designated as A and C forms (Mizuno *et al* 1981; Oka *et al.* 1988).
Both murine MBL forms can serve as recognition molecules of the lectin pathway system, although mouse MBL-A was estimated to be approximately five fold more efficient. The higher concentration of the mouse MBL-C was taken into account in this estimation (Liu *et al.* 2001). When comparing the two mouse MBL forms (moMBL-A and moMBL-C) with human MBL (huMBL) and the human pseudo MBL by primary amino acid sequence alignment, it seems likely that ψhuMBL represents the dysfunctional gene for the human equivalent of murine MBL-A form (Table 1.1).

	huMBL	ψhuMBL	moMBL-A
ψhuMBL	60	--------	--------
moMBL-A	57	70	--------
moMBL-C	59	51	51

Table 1.1 describes the percentage amino acid identity between the mouse and human MBL forms. The alignment suggests a homology between ψhuMBL with moMBL-A and, huMBL with moMBL-C.

The human MBL gene has been characterised in great detail. Three mutations in exon 1 give rise to amino acid changes and thereby changes in the primary structure of MBL. The wild type alleles have been designated type A, while mutated alleles have been designated B, C, D. (Sumiya *et al.* 1991; Lipscombe *et al* 1992; Lipscombe *et al.*1992 (b))
The mutations D, B and C in exon 1 occur at codon 52, 54 and 57, respectively (see Figure 1.5 below) (Madsen *et al.* 1994).

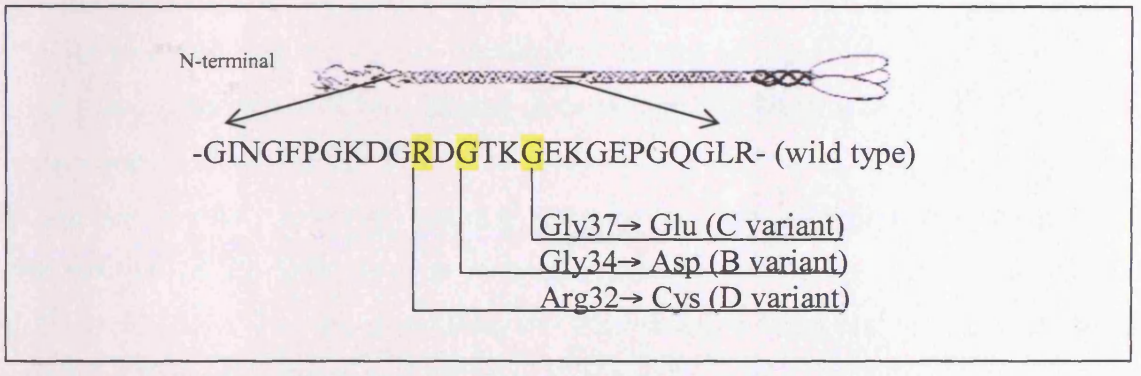


Figure 1.5. The first part of the collagen-like structure of MBL is encoded by exon 1. Three mutations give aa changes in position 32, 34 or 37. The amino acid substitution in the B and C variants interrupts the Gly-Xaa-Yaa repeats. The substitution in variant D, encoding a cysteine instead of an arginine residue, generates aberrant disulphide bonds. All three substitutions cause low plasma concentration of functional MBL.

The mutation frequency varies between ethnic groups. In the Caucasian population the frequency of wildtype/B/C and D variants are ~80/11/3 and 6%, in Africans ~69/2/28 and 1%, in Asians 87/13/0 and 0% and in South Americans 50/45/5 and 0% respectively (Madsen *et al.* 1998). The plasma level of MBL is also regulated at the transcription level. Two promoter polymorphisms at position -550 (H/L variants) and -221 (X/Y variants) substitute a guanine with a cytosine base in both cases. A third polymorphism at position +4 in the 5′ untranslated region of exon 1 substituts a cytosine with a guanine base (P/Q variants) (Madsen *et al.* 1995). The promoter haplotypes HY, LY and LX have a dominant effect on the concentration of MBL, promoting high, intermediate and low plasma levels of MBL respectively. The plasma concentration of MBL may vary up to 6-fold between individuals with identical genotype (Steffensen *et al.* 2000) and 3 fold between mice of the same inbred strain (Liu *et al.* 2001).

1.11 Serine proteases

Protein cleavage is called proteolysis and can be performed by the proteins named proteases or peptidases. Proteolytic cleavage of proteins takes place both intracellularly and extracellularly (Stroud 1974).

The three most important criteria currently used for classifying the proteases are:

1. The type of reaction catalysed.

2. The nature of the catalytic site.

3. The evolutionary relationship between the 3-dimensional structures of enzymes.

The criterium "reaction catalysed" divides the proteases into 2 groups: the exoproteases and the endoproteases. The exoprotease group consists of the enzymes that cleave 1-3 amino acids off a substrate and the endoprotease group consists of enzymes which cleave the substrate into two peptides. The subdivision of proteases using enzyme nomenclature (EC) according to the amino acid composition of the active site in the different proteases gave rise to 5 subgroups: serine, cysteine, aspartic, and metallo proteases and a group with unknown catalytic mechanisms. The serine, cysteine and aspartic proteases have a serine, cysteine and aspartic acid amino acid in the active site of the protease, respectively, whereas the metallo proteases make use of a metal ion in the catalytic mechanism. The evolutionary relationship between proteases reflects important similarities in the secondary and tertiary structure as well as catalytic and biological functions of the proteins (Barrett 1994). Serine proteases are described in further detail below.

The serine proteases are among the best-studied groups of proteolytic enzymes. They are found in many different life forms, ranging from viruses and bacteria to eukaryotic cells. The serine proteases have a catalytic site called "the catalytic triad" composed of the three amino acids His, Asp and Ser. The arrangement of the catalytic triad residues is the main parameter for the function of the serine proteases, and is used for sorting the enzymes into families (Warshel *et al.* 1989). The serine proteases are divided into 6 clans, and the clans are divided into families. The clans are named SX were S stands for serine and X (A, B, C, D, E or F) denotes the different clans. The different families are named SY, where S stands for serine, and Y (1,2,3,4...) denotes the family number. The name of the first described or found type of

protease is often used as a family name. As an example, clan: SB, family: S8 is often referred to as the subtilisin family and SA, S1 is the chymotrypsin or trypsin family (Rawlings and Barrett 1994). Many serine proteases are composed of several different domains and are therefore termed mosaic proteins. The serine protease domain is situated at the C-terminal part of the sequence and contains the active site of the enzyme, while the N-terminal domains are involved in the interactions with substrates and other proteins. The pro-enzymes are synthesised with a signal peptide that connects them to the secretory pathway (Stroud 1974, and Krem *et al.* 1999). The organisation of the active site and the 3-dimensional structure of the protein have proven to be important factors in relating proteins of different evolutionary origin to clans and families. X-ray crystallography has shown structural similaries of proteins from the same family. Folding twists, turns, helixes and β-sheets can be identical even for proteins with only 40% similarity in the primary structure. This makes the classification a powerful tool for predicting the function of a given protein (Doolittle 1985). Structure-function relationship between enzymes has proven to be the best way to characterise enzymes comprehensively. The serine protease domains contain identical folds consisting of several antiparallel β- sheets forming 2 β-barrels and 2 α helixes (Figure 1.6). The knowledge of the amino acids in the direct vicinity of the catalytic triad is higher than the more distal contacts, which may also be important for the structure-function correlation (Perona and Craik 1995).

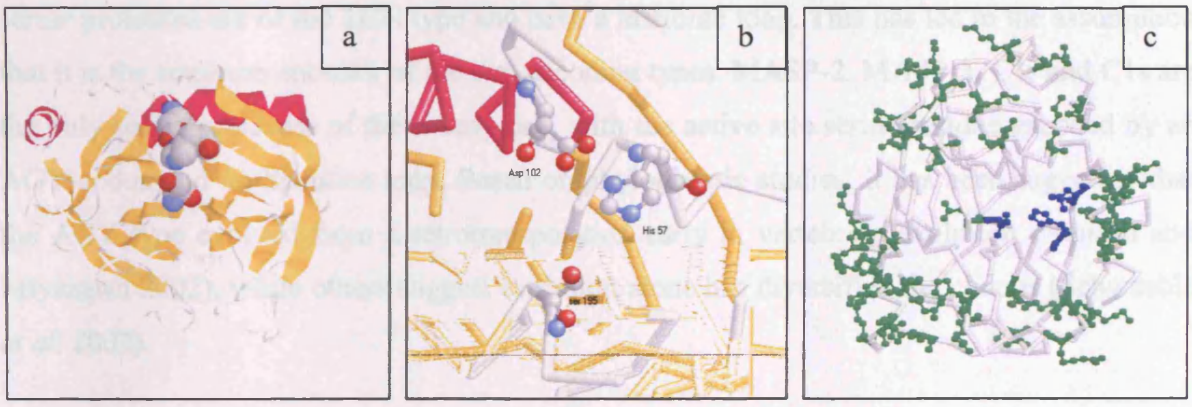


Figure 1.6. A model of the serine protease domain of trypsin. The structure in 6a shows the 2 β-barrels (in yellow) and the α-helix (red) surrounding the catalytic triad (space filled amino acids). 6b shows the catalytic site in "slab mode". 6c demonstrates that all charged groups (in green) are on the surface of the molecule except for the catalytic triad Ser195, His57, and Asp102 (in blue) found at the centre of the two β-barrel domains. Pictures were made in Rasmol 2.6 from the Brookhaven file: 1TRN.

1.12 Serine proteases of the complement and coagulation system

The chymotrypsins are the most intensively studied of the protease families, due to the important roles of this enzyme family and the availability of the proteins.

Serine proteases are the essence of the complement and the coagulation systems. These proteins mediate the enzymatic cascades, or have important control functions (Figures 1.1 and 1.2). There are at least nine serine proteases directly involved in the complement system and twelve in the haemostatic system. The twenty-one proteins can be subdivided into groups according to features such as domain structure, primary, secondary tertiary, quaternary, and genomic structure (see appendix 1 for detailes). The serine proteases of the classical and lectin activation pathway are similar in many aspects, and a known feature of one protein has been used as a model for others. An example of this is the case of protein crystal structures. One feature is the so called histidine loop which is found in most serine proteases in animals. It is a disulphide bridge between two cysteine residues on each side of the active site histidine residue. Also the encoding of the active site serine residue has been found suitable for classification. Serine has 6 synonymous codons: AGY (Y denoting T or C) or TCN type (N denoting A, T, G and C). Two mutations are required to get from one to the other encoding form (Endo *et al.* 1998). In lower animals, such as Drosophila melanogaster, the majority of serine proteases are of the TCN type and have a histidine loop. This has led to the assumption that it is the common ancestor of the two encoding types. MASP-2, MASP-3, C1r and C1s are the only serine proteases of the twenty-one, with the active site serine residue encoded by an AGY codon and no histidine loop. Based on phylogenetic studies, it has been suggested that the AGY type evolved from a retrotransposition early in vertebrate evolution (Nonaka and Miyazawa 2002), while others suggest evolution alone has diversified the codons (Schwaeble *et al.* 2002).

1.13 Serine proteases of the lectin pathway

The MASP proteins are mosaic proteins composed of six domains, an N-terminal CUB domain (CUB1) followed by a EGF-like domain, another CUB domain (CUB2), two CCP domains (CCP1 and CCP2) and a serine protease (SP) domain. A small sequence (12-15 residues) called the link region separates the CCP2 domain from the SP domain. This domain structure is unique for C1r, C1s, MASP-1, MASP-2 and MASP-3. There are no other known proteins, from any organism, with this domain structure. The zymogen proteins become activated after a cleavage of the Arg-Ile bond in the linker region, leading to a two-chain structure held together by a disulphide bridge. The conformational change upon cleavage enables the catalytic site to "switch on" and substrate cleavage is then possible (Freer *et al.* 1970). The pattern of disulphide bridges between cysteine residues have been solved for C1r and C1s (Arlaud *et al.* 1987, and Hess *et al.* 1991).

Due to the homology of the primary structure of C1r/C1s and MASP primary sequence, the disulphide bridges in the MASP's have been modelled similarly (Figure 1.7).

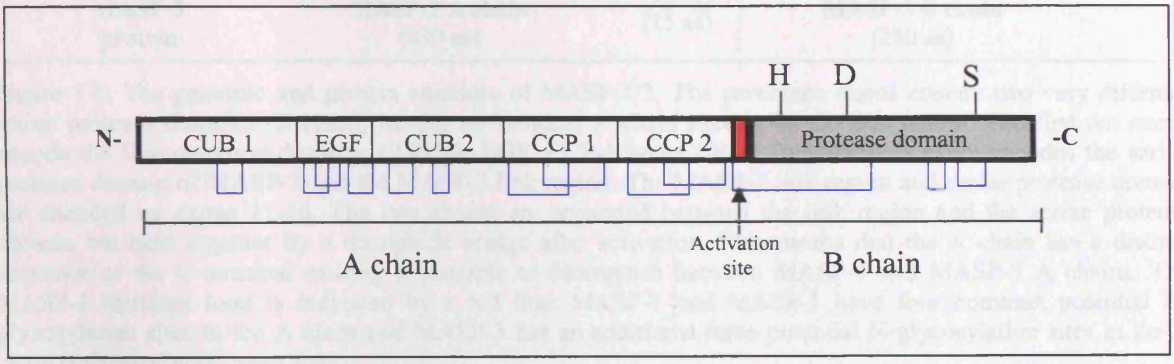


Figure 1.7. The schematic organisation of common features for C1r/s and MASP proteins such as: domain structure, disulphide bridges (blue lines), catalytic triad (H, D and S) and activation site (modified from Wong *et al.* 1999).

1.14 The MASP proteins

Two genes encode the four proteins associated with the MBL and ficolins of the lectin pathway. Human MASP-1 and MASP-3 are encoded by seventeen exons on chromosome 3q27 and MASP-1 is an alternatively spliced form of the gene (Figure 1.8) (Takada *et al.* 1993, and Dahl *et al.* 2001).

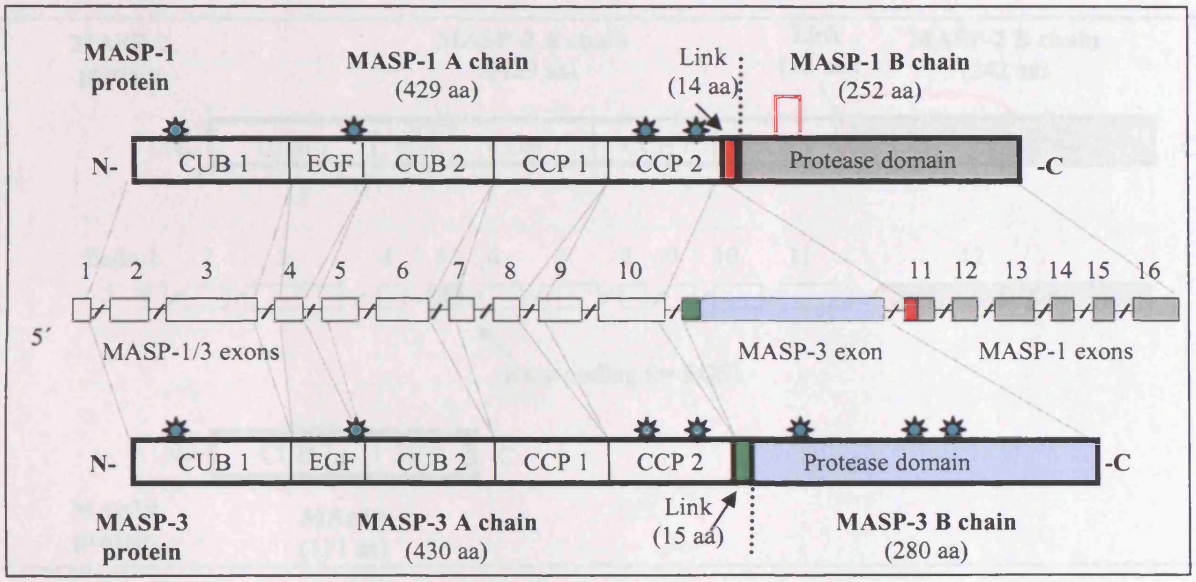


Figure 1.8. The genomic and protein structure of MASP-1/3. The seventeen exons encode two very different serine protease domains (B chain) having an identical A chain except for the link region. The first ten exons encode the five common domains CUB1/2, EGF, CCP-1 and CCP-2. The MASP-3 exon, encodes the serine protease domain of MASP-3 and the MASP-3 link region. The MASP-1 link region and serine protease domain are encoded by exons 11-16. The two chains are separated between the link region and the serine protease domain but held together by a disulphide bridge after activation. This means that the A chain has a distinct sequence at the C-terminal making it possible to distinguish between MASP-1 and MASP-3 A chains. The MASP-1 histidine loop is indicated by a red line. MASP-1 and MASP-3 have four common potential N-glycosylation sites in the A chain and MASP-3 has an additional three potential N-glycosylation sites in the B chain (indicated with green stars).

The exons of human MASP-1 and MASP-3 encode polypeptide chains of 695 and 725 residues including the signal peptide of 19 residues in both cases. The calculated molecular weight without glycosylation of MASP-1 is 79,268 Da and 81,873 Da for the MASP-3 protein (including the signal peptide in both cases). The observed weights on Western blotting of non-activated MASP-1 and MASP-3 are 90 kDa and 94 kDa under reducing conditions, respectively (Figure 1.10).

MASP-2 and MAp19 (MBL associated protein of 19 kDa) are encoded by twelve exons on chromosome 1p36 (Stover *et al.* 1999). MAp19 is also known as sMAP (small MBL-associated protein) and was characterised almost simultaneously by two groups. It is encoded by 5 exons of which the first four are identical with MASP-2. The fifth exon encodes only four amino acids and a stop codon (Figure 1.9) (Stover *et al.* 1999b; Takahashi *et al.* 1999).

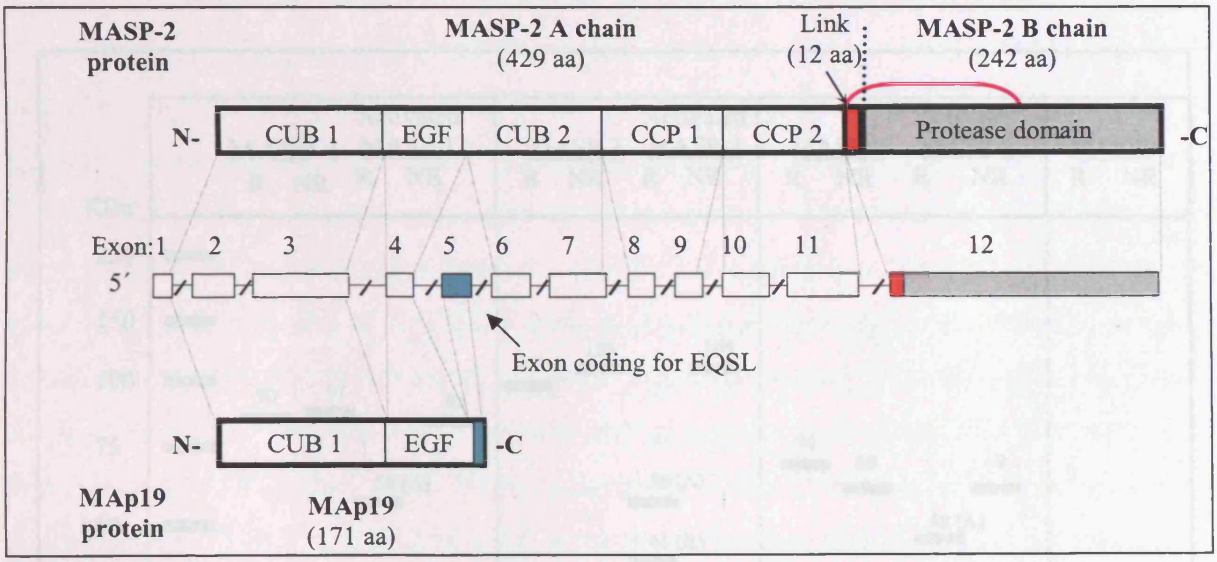


Figure 1.9. The genomic and secondary protein structure of MASP-2 and MAp19. The exon structure encoding MASP-2 is similar to that of MASP-3 with 10 exons encoding the A chain and a single exon encoding the linker region (12 residues) and the serine protease domain. The alternative splicing form makes use of the small fifth exon, encoding the amino acids EQSL. MAp19 has the first two domains of the A chain (CUB1 and EGF) and then C-terminal sequence EQSL encoded by exon 5.

The exons of human MASP-2 and MAp19 encode polypeptide chains of 683 and 171 residues including the signal peptide of 15 residues (Figure 9). The calculated molecular weight of MASP-2 is 75,685 Da and 20.629 Da respectively for MAp19 (including the signal peptide in both cases). The observed weights on Western blotting are 74 kDa (non-activated) and 20 kDa under reducing conditions (Figure 1.10) (Schwaeble *et al.* 2002).

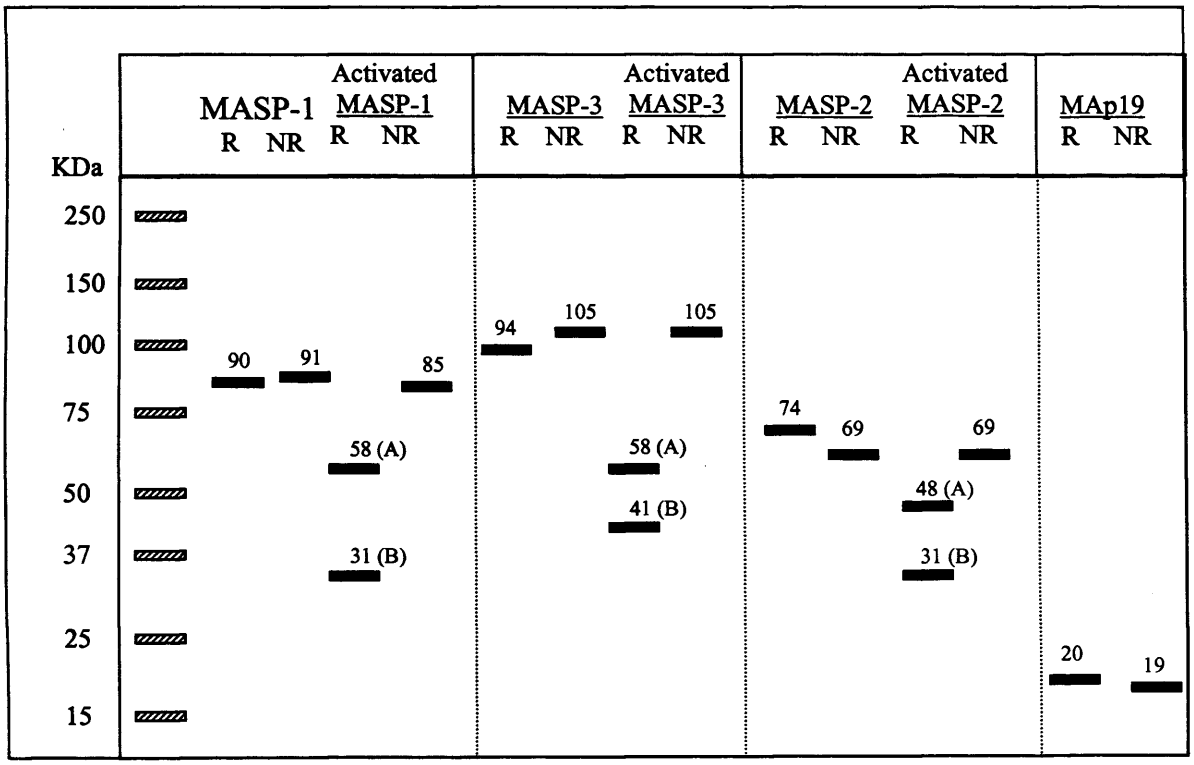


Figure 1.10. Line drawing of the observed mobility of MASP and Map19 when subjected to SDS-PAGE under reduced (R) and non-reduced (NR) conditions. The molecular weight of each protein and peptide are indicated as well as a Mw marker. A and B denotes the A and B chain of activated protein, separated under reducing conditions.

1.15 The CUB domain

The CUB domain has its name from its initial description in C1r/C1s, sea urchin epidermal growth factor-containing protein, and human bone morphogenetic protein-1. The CUB domain is predicted to have a barrel shape of anti-parallel beta-sheets. (Bork and Beckmann 1993). The domain is found in complement proteins and in many extracellular proteins, mainly involved in developmental processes. It is composed of approximately 110 residues and the domain is not found in plants, prokaryotes or insects (Day *et al.* 1993). Four cysteine

residues form 2 disulphide bridges (Cys1-Cys2, Cys3-Cys4) which are conserved in most CUB domains (Romero *et al.* 1997). The CUB1 domain of C1r/s and the MASPs are the exception from this pattern by only having the Cys3-Cys4 bridge.

The formation of the C1s homodimer is mediated by the negatively charged CUB1 domains bridged by a Ca^{2+} ion (Illy *et al.*1991).

The CUB1 domain also plays a crucial role in the binding of MASP to MBL, and it has been shown that the recombinant EGF-CUB2 peptide does not bind to MBL (Wallis and Dodd 2000; Chen and Wallis 2001). The CUB2 domain is has the four cysteine residues of generally described CUB domains. The crystallisation of the recombinant MASP-2 CUB1-EGF-CUB2 peptide provided plausible models of dimerisation of MASP-2 and interaction between MASP-2 and MBL (Feinberg *et al.* 2003). The finding of a MASP-2 deficient patient illustrated the importance of the CUB1 domain. A mutation in codon 120, normally encoding an aspartic acid residue, was mutated to encode a glycine residue. This resulted in low plasma levels of MASP-2, possibly due to stability changes, and a lack of complex formation with MBL (Stengaard-Pedersen *et al.* 2003). Alignment of the CUB1 and CUB2 domains from C1r/s, MASP-1/3 and MASP-2/MAp19 gives the percentage identity of the primary amino acid sequence (table 1.2). The areas surrounding the Asp120 residue are highly conserved between the 4 different sequences. This implies the possibility of a similar functional effect on MASP-1/3 if the mutation should be found in these proteins.

1.16 The EGF domain

A calcium-binding Epidermal Growth Factor-Like Domain (EGF-like domain) is present in a large number of membrane-bound and extracellular (mostly animal) proteins. The tertiary structure was determined as two beta-hairpins (Rao *et al.* 1995).

The EGF domain participates in the binding of Ca^{2+} to proteins and the domain is crucial for numerous protein-protein interactions. It has six conserved cysteine residues forming three disulphide bridges (Cys1-Cys3, Cys2-Cys4 and Cys5-Cys6) and contains 45-50 residues. Apart from the complement system proteins, it is found in proteins associated with blood coagulation, fibrinolysis, cell adhesion and neural development (Campbell and Bork 1993). The importance of the EGF domain in complex formation has been described for both C1r/s

and MASP-1/-2 (Arlaud *et al.* 2001; Wallis and Dodd 2000). The interaction between the CUB1, EGF and CUB-2 domains in terms of quaternary structure and functionality has recently been investigated by crystallisation of the protein fragments (Feinberg *et al.* 2003). Alignment of the EGF domains from C1r/s, MASP-1/-3 and MASP-2/MAp19 gives the percentage identity of the primary amino acid sequence (Table 1.2).

1.17 The CCP domain

The complement control protein domain (CCP domain) is a common motif in complement proteins and is always found in repeats of two or more CCP domains. The domain is also called the short consensus repeat (SCR). It is composed of approx. 60 residues, and contains 4 conserved cysteine residues forming two disulphide bridges (Cys1-Cys3, Cys2-Cys4). The CCP motif is common for the regulatory components of the complement system. The number of CCP domains varies from two (in the serine proteases) to thirty (in complement receptor 1, CR1). CCP domains are thought to bind to C3, C4 and their fragments (Reid *et al.* 1986). The tertiary structure of CCP domains from factor H determined by nuclear magnetic resonance (NMR), was described as a hydrophobic core surrounded by five beta-strands (Norman *et al.* 1991). The three-dimensional structure of CCP2 from C1s was obtained by crystallisation of the recombinant CCP2-SP peptide. The results showed a very stiff domain with strong interactions to the SP domain on the opposite side of the groove of the catalytical triad (Gaboriaud *et al.* 2000). Alignment of the CCP domain sequences shows the homology between the proteins (table 1.2). Including the link region and comparing the conserved residues with the three-dimensional structure of CCP2-SP, it is clear that the contact area of CCP2 and the serine protease domain is conserved between C1r, C1s and the MASPs (Gaboriaud *et al.* 1998).

1.18 The Serine Protease domain

The C-terminal serine protease domain (SP domain) is the functional domain with enzymatic activity through its catalytic triad. The domain has evolved over billion of years from simple digestive proteases to part of specialised mosaic proteins (Patthy 1985). The primary sequence surrounding the active site serine residue and 30-40 residues downstream from it,

are essential and sufficient for functional prediction (Krem *et al.* 1999). It is composed of 240-280 residues, and contains several conserved cysteine residues forming disulphide bridges. The crystal structure of C1s SP domain showed the core as two six-stranded beta-barrels with several surface loops and a C-terminal alpha helix. This folding structure has been found to be the general pattern of trypsin-like serine proteases (Gaboriaud *et al.* 2000).
The site of substrate binding is determined by the tertiary structure and the formation of hydrogen bonds orientates the substrate for cleavage by the catalytic site (Perona and Craik 1997). Upon activation of the serine protease, the new N-terminal interacts with an aspartic acid residue preceding the active site serine residue. This interaction forms a substrate specific binding site called the pocket.
The active site serine residue is denoted number 1 (S1). The residue preciding the serine is called S2 and so forth. The cleavage site in the substrate is generally described as the proteolytic site (P site, Pn....-P2-P1-P1′-P2′-...Pn′) and cleavage occurs between residues P1-P1′ (Schechter and Berger 1967). The cleavage of a substrate occurs when the active site serine makes a nucleophile attack on the orientated substrate. The three residues of the catalytical triad all contribute to the reaction mechanism (Warshel *et al.* 1989).
Alignment of the SP domains from C1r/s, MASP-1/-3 and MASP-2/MAp19 gives the percentage identity of the primary amino acid sequence (Table 1.2).

	MASP-2	MASP-3	C1r	C1s
MASP-1	50 / 54 / 50 36 / 44 33	100 / 100 / 100 100 / 100 **32**	40 / 54 / 41 27 / 43 29	42 / 38 / 37 33 / 43 26
MASP-2		50 / 54 / 50 36 / 44 38	40 / 45 / 46 35 / 44 35	39 / **28** / 45 33 / 38 35
MASP-3			40 / 54 / 41 27 / 43 38	42 / 38 / 37 33 / 43 35
C1r				41 / 43 / 37 28 / 37 43

CUB1 / EGF / CUB2
CCP1 / CCP2
Linker + SPD

Table 1.2. The percentage identity between the six domains of C1r/s, MASP-1, -2 and -3.
The table should be read as shown in the left corner. The homology between MASP-1 and MASP-3 linker and serine protease domain (SP) and the homology between C1r and C1s EGF domain are exemplified (blue and red). It is clear that the homology is not evenly distributed between the domains. The MASP-1 linker sequence and serine protease domain has a lower homology than the others.

1.19 Glycosylation

Glycosylation is the attachment of carbohydrates to proteins, and it is the most common modification of eukaryotic proteins. The bound carbohydrates lengthen the life time of a protein by decreasing the clearance rate in serum, and it makes the protein more soluble. The characteristic sequences of potential N-glycosylation sites are: -Asn-Xaa-Ser- or -Asn-Xaa-Thr-. The Xaa residue can vary, but is never a proline residue, and proline cannot immediately follow the Ser or Thr residue (Bause E. 1983). O-glycosylation primarily occurs in the Golgi apparatus where N-acetylgalactosamine groups are bound to Ser and Thr residues of certain proteins. The collagen region of some proteins contains hydroxy-Lys and hydroxy-Pro residues, which are modified by O-glycosylation. There is no apparent sequence consensus system to determine O-glycosylation (Jentoft 1990). There are several potential N-glycosylation sites in C1r (four sites), C1s (two sites), MASP-1 (four sites) and MASP-3 (seven sites). The four potential N-glycosylation sites in MASP-1 are located in the A chain, but not in the link region and are therefore also present in MASP-3. There are no potential N-glycosylation sites in MASP-2. The N-glycosylation of C1r and C1s has been shown to influence the quaternary structure of these proteins and plays a role in homologous (C1s-C1s) and heterologous (C1r-C1s) interactions (Aude *et al.* 1988).

1.20 Background on MASP-3 prior to this PhD thesis

An unknown protein band of 42 kDa was observed in a lectin preparation from human plasma on silver stained SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel-electrophoresis made by Annette Hansen). The band was isolated, digested using trypsin, and the peptides were sequenced. The peptide sequences WQALIVVE and EHVTVYL were used to design degenerate primers for PCR on a human liver cDNA library. Two cDNA transcripts of 4.1 kb and 3.0 kb were obtained in the vector pEAK8 and used to transfect E.coli (XL1 blue) for amplification. The company Pangene (CA, USA) conducted the capture of the two clones. The cDNA transcripts of 4.1 kb represented the entire open reading frame (ORF) of MASP-3. The construct was used to transfect a human embryonic kidney cell line expressing the Epstein-Barr virus nuclear antigen (HEK293 EBNA) for expression. The cell supernatant was

harvested after six days and purified on a Mono-Q ion exchange column. The purified material was 95 % pure when analysed on silver stained SDS-PAGE but the yield was low. Recombinant MASP-3 was shown to compete with MASP-2 for the same binding site on MBL hereby leading to a competitive inhibition of C4 activation through MASP-2 as MASP-3 does not cleave C4. No enzymatic activity of recombinant MASP-3 towards the complement components C2, C3, C4, C5, FB or MASP-2 was found (Dahl *et al* 2001).

1.21 Lectin/MASP complexes

MASPs have been shown to associate with MBL, L-ficolin (Matsushita *et al.* 2000) and H-ficolin (Matsushita *et al.* 2002). There have been no reports of MASP or MAp19 association with any other collectins or M-ficolin. The stoichiometry of the complexes is not fully characterised.

The "MBL complex" (ie material isolated by binding to immobilised mannan) has been shown to consist of MBL, MASP-1, 2, 3 and MAp19, and MBL can form oligomeric structures composed of different numbers of subunits. Oligomeres comprised of 2, 3, 4, and 6 subunits have been observed but other forms possibly exist (Holmskov *et al.* 2003).

The binding site for MASPs on MBL is suggested to be in the first part of the collagen-like domain and binding is calcium dependent (Wallis and Drickamer 1999). This hypothesis was supported by the functional characterisation of the MBL B allotype that has a mutation in codon 54. The mutation changed the Gly 34 residue, of the fifth collagen repeat to an Asp residue, and MBL could not associate with MASPs (Matsushita *et al.* 1995). The collagen-like structure is similar for a large number of proteins and the fact that 33% homology is given in the Gly-Xaa-Yaa repeat raises the question whether MASP only associate with MBL and the ficolins (Wallis *et al.* 2004).

MASPs form homodimers through interactions of CUB1-EGF-CUB2, but there are contradicting reports about calcium dependence of the dimer formation (Chen and Wallis 2001; Thielens *et al.* 2001). An interesting question in this respect is the potential formation of a MASP-1/3 dimer. Since the three domains are identical between MASP-1 and MASP-3, absence of this hetero dimer would mean that the serine protease domain would play an important part in the formation of the dimer.

Using recombinant rat proteins it was found that the smallest MBL complex consists of an MBL dimer and one MASP-1 or one MASP-2 dimer. No complex formation of MASP-1 and MASP-2 monomer on an MBL dimer was observed. Trimeric and tetrameric MBL oligomers were mainly associated with MASP-1 and the larger complexes were determined as tetrameric MBL with two associated MASP dimers (Chen and Wallis 2001). In another report it was shown by using normal human plasma derived MBL/MASP complexes that MASP-1/Map19 was mainly associated with the smaller MBL oligomers whereas MASP-2 and MASP-3 were associated with the larger forms (Dahl *et al.* 2001). The complexes are very stable and high salt concentration as well as a calcium chelating agent are required for dissociation (Thiel *et al.* 2000).

1.22 Lectin pathway and disease

Knowledge of the lectin pathway of complement activation has evolved at increasing speed since it was published more than sixteen years ago, that Ra-reactive factor can activate complement (Ikeda *et al.* 1987). Two years later it was found that low serum levels of MBL correlated with the susceptibility to infection (Super *et al.* 1989). This defect of opsonisation was later linked to a mutation in codon 54 of the MBL mRNA (Sumiya *et al.* 1991).
The finding explained why serum of a patient, suffering from recurrent bacterial infections, lacked the ability to opsonise baker's yeast (Miller *et al.* 1968).
Other studies followed and papers have been published showing that plasma levels are genetically determined (Sumiya *et al.* 1991; Lipscombe *et al.* 1992; Madsen *et al.* 1994). The association between MBL levels and susceptibility to frequent infections have been described for both children (Super *et al.* 1989; Garred *et al.* 1995) and adults (Summerfield *et al.* 1995). Numerous reports on diseases associated with deficiency of lectin pathway proteins and many clinical studies have been published and reviewed. Polymorphisms, oligomeric variants, associated and influencing proteins increase the complexity of determining deficiency. It is not just a question of quantity but also quality. Over the years experimental methods have improved and are still being developed. New assays for MBL, ficolins and the associated proteins are constructed and some are now commercially available. The recent description of MASP-2 polymorphisms, MASP-3, and the ficolins makes the picture even more complex.

From my point of view the two most important clinical papers so far are the first description of MBL and MASP-2 association with infection (Super *et al.* 1989; Stengaard-Pedersen *et al.* 2003). The clinical data regarding MBL are very well reviewed in Kilpatrick (2002) and Turner and Hamvas (2000).

1.23 The aim of this thesis

The goal of my PhD was to enable investigation of human MASP-3 by establishing the relevant protocols and produce the necessary materials. One of the first essential tasks was to produce large quantities of recombinant MASP-3. The goal was to produce enough enzymatic active recombinant MASP-3 to supply both internal use and external collaborators. Another major focus of my studies was the establishment of a microtiter plate-based assay for quantification. This development was dependant on the availability of useful antibodies, thus the existing antibodies would be tested and novel would have to be designed. The optimal result would be the establishment of an assay capable of quantifying low levels of MASP-3 directly in plasma and preferably also in other body fluid samples.
In order to contribute to the characterisation of MASP-3 experiments were designed to evaluate the enzymatic capability, inhibitor interactions, glycosylation and tissue distribution of MASP-3. The association and dissociation of the MBL/MASP-3 complex was taken under investigation as well as 3 dimensional structural data.

2. Cloning and expression of recombinant MASP-3

2.1 MASP-3 cDNA Constructs and recombinant protein production

Strategies for purification of proteins from plasma often lead to a final preparation that is contaminated with other proteins. In order to avoid these contaminants, a system for recombinant expression of MASP-3 was designed. Yeast and *E.coli* (*Eschericia coli*) expression systems are used for production of large quantities of recombinant proteins. The *E.coli* bacterium is not capable of controlling the formation of disulphide bridges and the consequence of this is protein aggregation or formation of inclusion bodies (Georgio 1996).

Transfected yeast strains such as *Pichia pastoris* are capable of producing recombinant human proteins, which are correctly folded, but the oligomeric carbohydrate structure is different from that of mammalian cells (Cregg *et al.* 1993). The glycosylation pattern of yeast has a tendency to consist of multiple mannose carbohydrates and this could alter the function of the recombinant protein especially with regards to MBL. Due to the high level of glycosylation and a complex disulphide bridge structure of the MASP-3 protein these systems were excluded. A mammalian expression system was chosen instead, which allowed for optimal folding and post-translational modifications. Previous work by Steen Vang Petersen and Thomas Vorup Jensen with MASP-1 and MASP-2 constructs provided by Wilhelm Schwaeble had shown that the CHO-K1 cell expression system failed to produce detectable amounts of recombinant proteins, while COS7 and HEK293 cells successfully produced the recombinant human proteins.

The HEK293 EBNA cells were chosen for transient expression of MASP-3 and stable cell lines were obtained by selection.

2. Cloning and expression of recombinant MASP-3

2.2 Materials and methods

Unless otherwise noted, experiments were conducted at room temperature (RT).
All cell work was preformed with maximum precaution for a sterile environment, and unless otherwise noted buffers for DNA work were made with distilled water (dH_2O) and autoclaved before use. Buffers used for protein work were made with Millipore water and buffers for Western blotting did not contain NaN_3 since it would interfere with horseradish peroxidase on labelled antibodies.

2.2.1 MASP-3 constructs

Human MASP-3 cDNA was amplified in the pEAK8 vector using previously cloned material (Dahl *et al.* 2001). cDNA preparations from 2 litre bacterial cultures were made with the two cloned constructs: clone 4.1 kb and clone 3.0 kb. Preparation and purification were performed using the protocols and material supplied in the kit (Qiagen Mega and Giga kit, Cat. No. 19781). The constructs were further analysed by PCR using the "Expand Long Template" PCR system (Boehringer Mannheim, Cat. No. 1681834) or Ready-to-go beads (Amersham, Cat. No. 27-9555-01). DNA sequencing kit (Perkin Elmer, Cat. No. 402079) was used for sequencing the products and the guidelines from the manufacturer for mixtures were used. PCR products were analysed on agarose gels made with concentrations of 0.8-2% agarose (Gibco, Cat. No. 15510-027), depending on the size of the PCR product. The solid agarose was mixed with TAE buffer (40 mM Tris-acetate and 1 mM EDTA adjusted to pH 8) and boiled until dissolved. The solution was cooled to ~55°C and mixed with 0.5 µg/ml Ethidium bromide (Sigma, Cat. No. E8751). The ethidium bromide binds to the DNA and could be visualised upon illumination with UV light and a photograph was taken either with a polaroid MP4 camera or with a digital camera (Flour-S). PCR product was added to 1/10 volume loading buffer (35% Ficoll 400 (Amersham, Cat. No. 17-0300-10), 0.25% w/v Bromphenol blue (Merck, Cat. No. 8122) and 0.25% w/v xylene cyanol (Amersham, Cat. No. US23513)) before loading onto the gel in the sample wells.

In one well, a DNA marker was loaded, and the gel was run at a maximum of 120 volt, for a suitable time to separate the fragments. The weight marker used was made by digesting pBR327 (a kind gift from Tove Christensen, University of Aarhus, Denmark) with Hinf I (Roche, Cat. No.779652). The fragment sizes of this marker were: 154, 221, 298, 452, 517 and 1631 bp. Other DNA markers used were the DNA weight marker II (Boehringer Mannheim, Cat. No. 1218590) and DNA weight marker VII (Boehringer Mannheim, Cat. No. 1669940). Primers for PCR are shown in figure 1 in the result section of this chapter and were purchased from DNA Technology Aarhus, Denmark.

2.2.2 Polymerase chain reaction (PCR)

PCR reactions were performed using Ready-to-go PCR beads (Amersham, Cat. No. 27-9555-01) with 10 pico-moles of each primer (forward and reverse) and 1 ng DNA. PCR reactions were in general performed using a temperature programme of 2 min. denaturation at 94°C, 10 cycles of 94°C (20 sec.), 50°C (30 sec) and 72°C for 2 min., 20 more similar cycles where the 2 min. at 72°C was prolonged by 20 sec. after each cycle. The PCR reactions were cooled to 4°C after the final cycle.

2.2.3 Transformation of HEK293 EBNA cells

HEK293 EBNA cells (Invitrogen, cat. No. R620-07) were maintained at 37°C in humidified air with 5% CO_2 in 150 cm^2 culture flasks (TTP AG., Cat. No. 9075). All buffers and media additives were sterile-filtered using 0.45µm filters (Sartorius, Cat. No. 16555) or autoclaved. The medium used for cell culturing was DMEM-12 (Biological Industries, Cat. No. 01-055-1A) with 5% heat inactivated (56°C for 30 min.) foetal calf serum (FCS from Gibco, Cat. No. 10106-169), 10 mM glutamax (Gibco, Cat. No. 35050-038) and supplemented with ITS (Insulin-transferrin-selenium. Gibco, Cat. No. 51300-044). The antibiotics; penicillin (20.000 U/l) and streptomycin (40 mg/l) (Roche, Cat. No. 1074440) were used in the medium until the cells were transformed, and then omitted. Cells were grown to 80-90% confluence in 150 cm^2 culture flasks with 35 ml medium and then split in four 150 cm^2 flasks with 4 x 10^6 cells per flask. Prior to transfection the

cells were washed three times with 10 ml medium and left for 3h in 35 ml basic medium without FCS and Glutamax.

A preparation of 450 µg (300 µl) purified pEAK8 vector containing the cDNA encoding MASP-3 was sterilized by incubation with 45 µl diethylether (Merck, Cat. No. 100929) for 2h at RT with open lid. To three 10 ml tubes were added 180 µl 2.5 M $CaCl_2$ (Merck, Cat. No. 102378), 67 µl MASP-3 plasmid and 1433 µl buffer A (150 mM NaCl (Merck, Cat. No. 6404), 1 mM EDTA (Sigma, Cat. No. ED4SS), 10 mM Tris-HCl (Calbiochem, Cat. No. T-8133) adjusted to pH 7.12). To each tube was added 1680 µl buffer B (50 mM HEPES (Gibco, Cat. No. 11344-025), 250 mM NaCl, 1.5 mM Na_2HPO_4 (Merck, Cat. No. 106559) adjusted to pH 7.12) and left for 15 min. before it was added to the cells in 35 ml basic medium without FCS and Glutamax.

A mock transfection using pEAK8 vector without MASP-3 cDNA was performed in parallel. The cells were stored in 37°C incubator for 2 h and then washed three times with 10 ml basic medium and left in 35 ml basic medium overnight. The four cultures were washed and incubated with medium containing 10 mg/l puromycin (Sigma, Cat. No. P7255). After 3 days the mock transfected cells were dead and the selection pressure was decreased to 1 mg/l puromycin. At this time the cells were pooled and split into 5 new flasks and left for nine days in the incubator. The cells were washed and pooled before the majority was frozen in basic medium containing 10 % v/v DMSO (Sigma, D5879) and 10 % v/v FCS. The cells were frozen in three steps in an isolated container: 2h 4 °C, overnight at -20 °C and then at -80 °C for storage. The selected cells were continuously cultured and 240 wells (ten 24-well plates) in parallel were seeded with 0.75 cells/well in 300 µl basic medium plus 10 mg/l puromycin. Two clones were isolated (named Aksel 17.9.01 and Aksel 3) and re-cloned in two 24-well plates with 750µl basic medium and 0.25 cells/well. When samples of supernatants were collected for analysis, a maximum of 50% of the medium was replaced. The re-cloning led to 4 and 6 clones respectively that were amplified in 75 cm^2 flasks. The cell cultures were harvested and frozen. Clones 17/9, 3.2, 3.3, 3.4, 3.5 and 3.6 were chosen for further analysis of MASP-3 production.

When the cultures were 80% confluent the cells were split and ½ of the cells were frozen n medium containing 10 % v/v DMSO and 10 % v/v FCS.

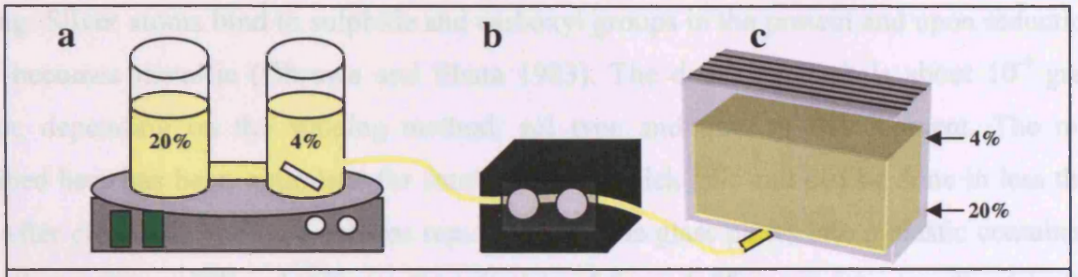
2.2.4 Analysis of supernatants from HEK293 EBNA cells

MBL/MASP/MA19 protein complex was purified from human plasma by the Danish State Serum Institute (MO12) and recombinant MASP-3 (rMASP-3) from a previous transient expression (Dahl *et al.* 2001) were used as positive controls. The supernatant from the mock transfection was used as negative control.

2.2.5 SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel-electrophoresis)

The size of a given protein can be estimated by forcing it through a matrix using electrophoresis (Weber and Osborn 1969). SDS is a negatively charged detergent which, when bound to the protein, gives it a uniform charge distribution. The protein-SDS complex will under electrophoresis be mobile in the gel proportionally to the molecular size. Using a standard marker containing proteins of know sizes, the size of the sample protein can be estimated. The disulphide bridges, protein complexes and secondary protein structures could be disrupted by pre-treatment of the samples using reducing agents, SDS and heat. The protein samples were analysed under reducing or non-reducing condition. Each sample was added 25% sample buffer (1% (v/v) glycerol, 3% w/v SDS, 8% (v/v) 25 mM Tris, 48% (w/v) Urea, 0.001% v/v Bromphenol blue and 10% 0.6 M Dithiotreitol (DTT, Sigma Cat. No. D9779) for the reduced sample and 10% 1.4 M iodoacetamide (IAA, Sigma, Cat. No. I6125). IAA was added to the samples after they had been boiled to prevent reformation of disulphide bridges. The matrix composition determines the velocity of the proteins in the current. In a fine matrix only small proteins can be forced to pass through the gel. In a coarser matrix the small proteins and peptides pass all the way through the gel, whereas large proteins and complexes are separated and contained in the gel. The amount of acrylamide in the gel determines the matrix permeability and is referred to as the percentage content. The advantages of different gel permeabilities can be combined in a gradient gel, with a low percentage content of acrylamide in the top of the gel and an increasing percentage content through the gel. Gradient gels of 4-20% acrylamide have a good separation of proteins with a size of 20-400 kDa. The physical size of a gel determines the resolution and the volume/numbers of

samples one can analyse at a time. Four types of gels were made: 170 x 150 x 1.5 / 0.75 (mm) and 110 x 50 x 1.5 / 0.75 (mm) depending on the number and volume of the samples. To make the gradient SDS-PAGE gels, two connected cylinders, a magnetic mixer, a pump and a multi gel container was used. Figure 1 shows the setup and the protocol for making SDS-PAGE gels.



PAGE (17 x15 cm) 2 x 0.75 mm + 8 x 1.5 mm

Material to mix:	4%	20%
Acrylamide 30%:	20.16 ml	100.68 ml
Lower Tris 4X:	37.80 ml	37.80 ml
H_2O / Glycerol:	91.32 ml (H_2O)	10.8 ml (Glycerol 99%)
2 min. under vacuum at 25 °C		
SDS 10%:	1.524 ml	1.524 ml
APS 10%:	0.2028 ml	0.2028 ml
TEMED :	0.02028 ml	0.02028 ml

Figure 1. The equipment and the protocol for making gels. The left cylinder (a) contains the 20% acrylamide and the right cylinder the 4% acrylamide. The pump (b) moves the mixed solution to the multi gel chamber (c) so the first gel solution to enter will be 4% and the final drop will be 20%. By adjusting the content of acrylamide other gradients can be produced. The lower Tris buffer x4 contained 18.2% w/v Tris in dH_2O, pH 8.9

A stacking gel with loading wells was formed as a top gel with 1-20 wells depending on the amount required. The top gel was made using 12.3 ml dH_2O, 5.6 ml upper Tris buffer (6.1 % w/v Tris in dH_2O pH 6.7), 3 ml acrylamide (30%), 170 µl APS (10%), 210 µl SDS (10%) and 18 µl TEMED (Sigma, Cat. No. T8133). A positive and a negative control were included on each PAGE if such controls were available. The gel electrophoresis was performed using electrophoresis buffer (pH 8.9, 0.025 M Tris, 0.19 M Glycine (Sigma, Cat. No. G7126) and 0.1% w/v Sodium Dodecyl Sulphate ((SDS) Sigma, Cat. No. L3771)) in a chamber from Hoefer Scientific Instruments with water cooling to 15°C

2.2.6 Silver staining

Proteins can be visualised by staining after electrophoresis. One method extensively used is silver staining. Silver atoms bind to sulphide and carboxyl groups in the protein and upon reduction the silver becomes metallic (Ohsawa and Ebata 1983). The detection limit is about 10^{-9} gram of protein, depending on the staining method, gel type and time of development. The method described here has been optimised for small 0.75 mm thick gels and can be done in less then 30 min. After electrophoresis the gel was removed from the glass plates into a plastic container. All incubations were at RT and with gentle agitation of the gel. The proteins were fixed in the gel using 95 ml 50% v/v acetone (Merck, Cat. No. 10013) with 5 ml 20% (v/v) trichloroacetic acid ((TCA) Merck, Cat. No. 100810) and 50 µl formalin (Sigma, Cat. No. F1635) for 10 min. The gels were washed in water 3 x 5 sec. and 1 x 2 min. before incubation with 100 ml 50% v/v acetone for 10 min. The acetone was replaced with 100 ml water with 200 µl 10% w/v sodium thiosulphate (Merck, Cat. No. 106516) for 1 min. After a wash (3 x 5 sec.) in water, the gel was incubated with 0.27 g silver nitrate (Merck, Cat. No. 101510) and 250 µl formalin in 100 ml water for 10 min. The gel was rinsed in water and the silver precipitated by adding 100 ml water containing 2 g sodium carbonate (Merck, Cat. No. 106392), 50 µl 10% w/v sodium thiosulphate and 50 µl formalin. The gel was incubated until optimal staining was achieved (1-5 min.), rinsed with water and the reaction was stopped with 1% acetic acid (Merck, Cat. No. 100063) in water for 5 min. The gel was preserved in 50 ml water with 25% v/v ethanol and 1.7 ml glycerol (Sigma, Cat. No. G7757), (Wallevik and Jensenius 1982). Other silver staining protocols have a higher sensitivity, but they are often more time consuming (up to 20 fold).

2.2.7 Western blotting

Western blotting is, in simple terms, a copy of the SDS-PAGE gel for analysing a specific protein using a specific antibody. The proteins separated in the SDS-PAGE gel after electrophoresis were transferred to a polyvinylidene difluoride membrane (PVDF, Amersham, Cat. No. RPN303F) by applying voltage perpendicularly to the gel (Towbin *et al.* 1979). The membrane was pre-incubated in methanol and washed in transfer buffer (25 mM Tris, 192 mM Glycine, 0.1% w/v SDS, 20% v/v Ethanol pH 8.3) before use. The membrane was placed on the gel in a chamber containing transfer buffer and 500 Vh (total voltage hours) was applied. After transfer the blot was removed from the gel and blocked in TBS (Tris Buffered Saline, pH 7.4, 140 mM NaCl, 10 mM Tris (Calbiochem, Cat. No. T-8133) and 0.1% v/v NaN_3)/ 0.1% v/v Tween 20, Merck, Cat. No. 8222184) for 30 min. The blot could then be used for immunoblotting or stored at –20 °C.

2.2.8 Molecular weight markers

The molecular weight of proteins analysed were compared with standard markers. The standard marker proteins are usually recombinant proteins produced in bacterial expression systems. The typical marker used in these experiments had a molecular weight span from 10 – 250 kDa. When larger proteins were to be estimated, single proteins such as IgM were used. The most extensively used markers were the pre-stained (BioRad, Cat. No. 161-0373) and unstained precision protein marker (BioRad, Cat. No. 161-0363). The pre-stained marker was used for Western blots and the unstained marker for PAGE gels destined for staining.

2.2.9 Polyclonal anti-MASP-3 antibodies

The single most powerful tool for analysing novel proteins is the specific antibody. It has therefore been crucial for the work with MASP-3 to produce and characterise specific anti-MASP-3 antibodies. The basic structure of immunoglobulin was suggested by R. Porter in 1962 (Fleischman *et al.* 1962). The molecule was found to be comprised of two large and two smaller chains denoted heavy (50 – 75 kDa) and light chain (25 kDa). The heavy chains are bound

together by disulphide bridges and a light chain is bound to each heavy chain by disulphide bridges. This gives the molecule a shape that looks like a Y. The two ends (the top of the Y) are called the variable regions and this region determines the specificity of the immunoglobulin. The site on the antigen recognised by the antibody is called an epitope and this site is usually a conformational determinant (structural epitope) comprising different parts of the antigen brought together by the tertiary structure. Linear determinants are rarely found but when they are seen it is mostly in the terminal part of the antigen (Colman 1988).

Small molecules and peptides are seldom immunogenic and no immune response will be seen after immunisation of animals with a 15-30 amino acid sequence. The peptide will however be immunogenic if coupled to a larger protein. This protein is called the carrier and the peptide is called the hapten. One example of carrier protein is tuberculin purified-protein derivative (PPD). Primary structure information on novel proteins is often in the from of peptide sequence and these will have to be coupled to a carrier protein for production of antisera. To get an enhanced response from an immunised animal one can use an adjuvant preparation for priming and activating the immune system of the animal. This contains killed bacteria such as Mycobacterium tuberculosis or bacillus calmette-Guerin (BCG). Lipopolysaccharide (LPS) from different sources or the synthetic compound muramyldipeptide (MDP) are examples of other adjuvants used to activate the immune system. Animals are immunised several times (boosted) and serum is collected for analysis. It is important to collect a sample of serum before starting immunisation to be able to compare the effect of immunisation.

2.2.10 Crosslinking of peptide to carrier protein

2.5 mg PPD (Statens Serum Institut DK, Cat. No. RT-47) was diluted in 2.5 ml 50 mM phosphate buffer pH 8. 2 mg MBS (m-maleimidobenzoly-N-hydroxysuccinimide ester, Sigma Cat. No. A4503) was added and the solution incubated for 30 min. at RT. The solution was dialysed overnight, RT against 4 l of PBS (Phosphate Buffered Saline, pH 7.4, 140 mM NaCl (Merck Cat. No. 6404), 2.7 mM KCl (Merck, Cat. No. 116101), 1.5 mM KH_2PO_4 (Merck, Cat. No. 104873) and 8.1 mM Na_2HPO_4 (Merck Cat. No. 106559)).

Buffer was replaced and dialysis continued 4 h at RT, for removal of excess MBS. The buffer was replaced by 4 l of 50 mM phosphate buffer pH 7 at 4°C. After adding 1.5 mg peptide and incubating 3h at RT, the PPD-peptide conjugate was formed.

2.2.11 Immunisation procedure

Priming was done by subcutaneous injection of 0.2 ml BCG (Bacillus Calmette Guerin, State Serum Institute DK, Cat. No. 2645). Immunisation was performed with 35 µg PPD-conjugated peptide in 0.5 ml 145 mM NaCl + 6.7% v/v $Al(OH)_3$, mixed with 0.5 ml Freund's adjuvant (Difco, Cat. No. 0638). Before and after priming with BCG, blood samples were taken from each animal to be used later as pre-immunisation controls. Pre-immunisation, immunisation, and boosting were performed at intervals of three weeks. Blood samples were taken 2 weeks after each immunisation. Several MASP-3 peptides were designed and used for immunisation (or the service of animal handling was purchased from Genosphere, France). Below are listed the peptides used for immunising rats or rabbits for production of polyclonal anti MASP-3 antisera.

Antibody name	Peptide sequence	Useable on Western blot	Useable in assays	Targets chain
Rat95	$_1$GQPSRSLPSLVKR	Yes	No	A
Rat97	$_1$IIGGRNAEPGLFPWQALI	Yes	No	B
Rabbit 7931	$_1$WVWEQMGLPQSVVEPQVER	Yes	Yes	B
Rabbit 7932	$_1$WVWEQMGLPQSVVEPQVER	Yes	Yes	B
Mouse 1E2	*	Yes	Yes	A

Table 2.1. The amino acid sequence of the peptide used rat and rabbit immunisations are written from the N-terminus (1) towards the C-terminus. The Mouse 1E2 antibody is a monoclonal anti MASP-1/MASP-3 antibody with an A-chain epitope. The antibody was a kind gift (Fujita T, Fukushima, Japan) but is now commercially available (Hycult Biotechnology, The Netherlands, Cat. No. HM2092). The four peptide raised antibodies could be used for Western blotting of both reduced and non-reduced samples while the monoclonal 1E2 antibody only worked on non-reduced samples.

Several animals were immunised with each of the four peptides to increase the chances of raising an immune responds in at least one animal of each immunisation. In the case of Rat96

(immunised as rat95) and rat98 (immunised as rat97) no specific anti MASP-3 antibodies could be detected using Western blotting with the individual pre-sera as control. Two chickens were also immunised using the rabbit 7931/7932 peptide but in neither case were there any specific anti MASP-3 antibodies produced (data not shown). The Peptide used to raise antibodies in rabbit 7931 and 7932 were further used for the production of monoclonal anti MASP-3 antibodies in mouse and rat (see chapter 3.4.2 for details).

2.2.12 Immunoblotting

This technique was used to analyse proteins and protein complexes on Western blots. The optimal results are achieved with specific and characterised antibodies. When analysing proteins, specific detection using polyclonal antiserum is often difficult because the antiserum may contain antibodies which react with other proteins on the blot. The problem could be partly solved by adding aggregated Ig, human serum albumin ((HSA) State Serum Institute Cat. No. 440511) or other possible interfering proteins to the buffer. The unspecific antibodies would then be excluded from binding to the Western blot. Primary antibody was diluted 500 - 5000 fold (depending on the antibody) in 25 ml TBS/Tween 20 (Tris Buffered Saline, pH 7.4 with 0.1% v/v Tween 20). After incubation at RT, minimum 3 h, the membrane was washed 3 x 5 min. in TBS/Tween 20 followed by incubation with a labelled secondary antibody diluted 2000 - 4000 fold in 25 ml TBS/Tween 20. The membrane was washed 3 x 5 min. in TBS/Tween 20 and was ready for development. Different methods were used to detect bound antibodies. The secondary antibody could be HRP (horseradish peroxidase)-labelled and the blot be developed using ECL (chemiluminescent substrate). HRP oxidises luminol in the presence of H_2O_2 and the reaction results in light emission. The primary antibody could be biotin labelled, which can be detected with HRP-labelled streptavidin. When using this method, HRP-labelled streptavidin replaces the secondary antibody. Due to the price of ECL, efforts were made to produce the substrate in-house. The "homemade ECL" consisted of two separate solutions, which was mixed 1-1 immediately before use. A stock preparation of 100 ml of solution 1 contained: 1 ml of 250 mM luminol (Fluka, Cat. No. 09253) in DMSO, 0.44 ml 90 mM coumaric acid (Sigma, Cat. No. C-9008) in DMSO and 10 ml of 1 M Tris pH 8.5 in distilled water. A stock preparation of 100 ml of

solution 2 contained: 10 ml 1 M Tris pH 8.5 and 64 µl hydrogen peroxide (30 %) in distilled water. An equal volume of solution 1 and 2 was mixed prior to development of the Western blot. Stock solutions were kept in dark containers and at 4°C. The two separate ECL solutions were stabile for more than 3 months.

The emitted light could be detected on photosensitive film or with a special camera setup such as the KODAK Image Station 1000. Western blots were sometimes developed several times with different antibodies, either by washing in TBS/Tween 20 and then adding new antibody or by stripping the blot. Stripping blots of antibodies for redevelopment using another primary antibody were done by incubating the blot for 45 min. 70°C, pH 6.9 in a TBS solution containing 62.5 mM Tris, 2% w/v SDS and 7.75% v/v β-mercaptoethanol. After incubation the membrane is washed overnight in TBS. The stripping of the blot led to decrease in sensitivity upon repeated Western blotting.

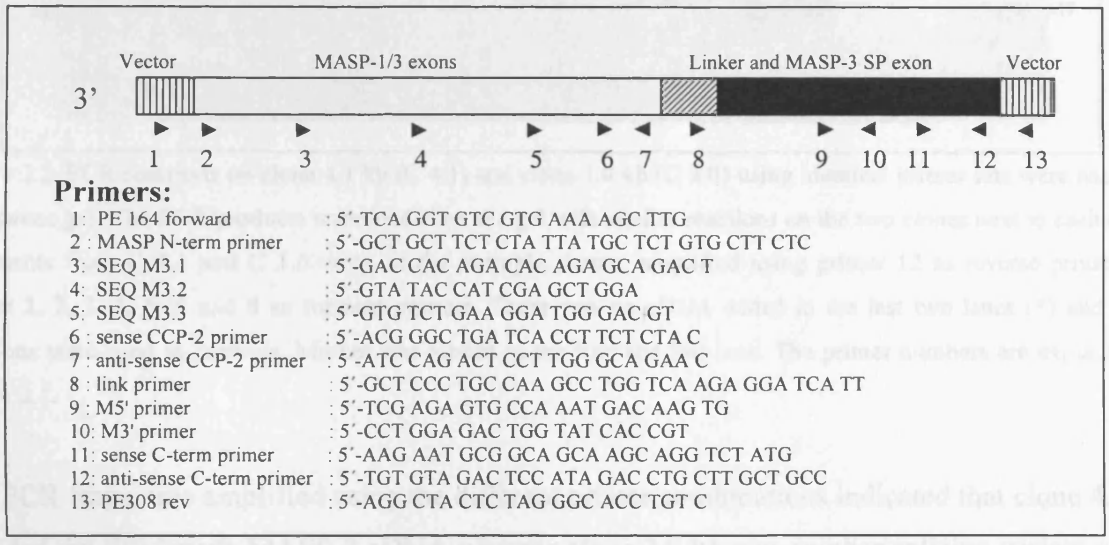
A more crude way was to incubate the blot 5 min. in buffer containing 0.1% azide. This would destroy the HRP but not the bound antibodies, thus reuse of a given Western blot was only possible if the previously bound antibodies were taken account of.

Antibodies conjugated to alkaline phosphatase (AP) were detected by a colour reaction where nitroblue tetrazolium (NBT) oxidises 5-bromo-4-chloro-3-indolyl phosphate (BCIP) which is hydrolysed by AP (Blake *et al.* 1984).

2.3 Results

2.3.1 PCR on MASP-3 clones 4.1 kb and clone 3.0 kb

Transformed *E.coli* with cDNA encoding MASP-3 were cultured and the plasmids composed of the pEAK8 vector and MASP-3 clone 4.1 kb or 3.0 kb were purified (the difference between the two clones is shown in Figure 2.2). PCR reactions using the different primers listed below in combinations on the 4.1 kb and 3.0 kb clones were analysed on agarose gels.



Primers:

1: PE 164 forward	: 5'-TCA GGT GTC GTG AAA AGC TTG
2 : MASP N-term primer	: 5'-GCT GCT TCT CTA TTA TGC TCT GTG CTT CTC
3: SEQ M3.1	: 5'-GAC CAC AGA CAC AGA GCA GAC T
4: SEQ M3.2	: 5'-GTA TAC CAT CGA GCT GGA
5: SEQ M3.3	: 5'-GTG TCT GAA GGA TGG GAC GT
6 : sense CCP-2 primer	: 5'-ACG GGC TGA TCA CCT TCT CTA C
7 : anti-sense CCP-2 primer	: 5'-ATC CAG ACT CCT TGG GCA GAA C
8 : link primer	: 5'-GCT CCC TGC CAA GCC TGG TCA AGA GGA TCA TT
9 : M5' primer	: 5'-TCG AGA GTG CCA AAT GAC AAG TG
10: M3' primer	: 5'-CCT GGA GAC TGG TAT CAC CGT
11: sense C-term primer	: 5'-AAG AAT GCG GCA GCA AGC AGG TCT ATG
12: anti-sense C-term primer	: 5'-TGT GTA GAC TCC ATA GAC CTG CTT GCT GCC
13: PE308 rev	: 5'-AGG CTA CTC TAG GGC ACC TGT C

Figure 2.1. The cDNA inserts encoding MASP-3 were analysed using primers 1 to 13 in different combinations. The schematic drawing represents clone 4.1 kb in the pEAK8 vector. The vector sequence, A-chain, linker and serine protease domain (SP) encoding area are indicated with vertical lines, white, cross hatching and black respectively.

The two constructs were analysed using different primer set combinations to clarify the difference in size. PCR products were analysed by electrophoresis in a 1% w/v agarose gel. The DNA separated in the agarose gel was visualised by UV light and photographed.

The result of the PCR experiments strongly indicated the cDNA composition of the two constructs as shown in Figure 2.2 with primer No.12 as reference point.

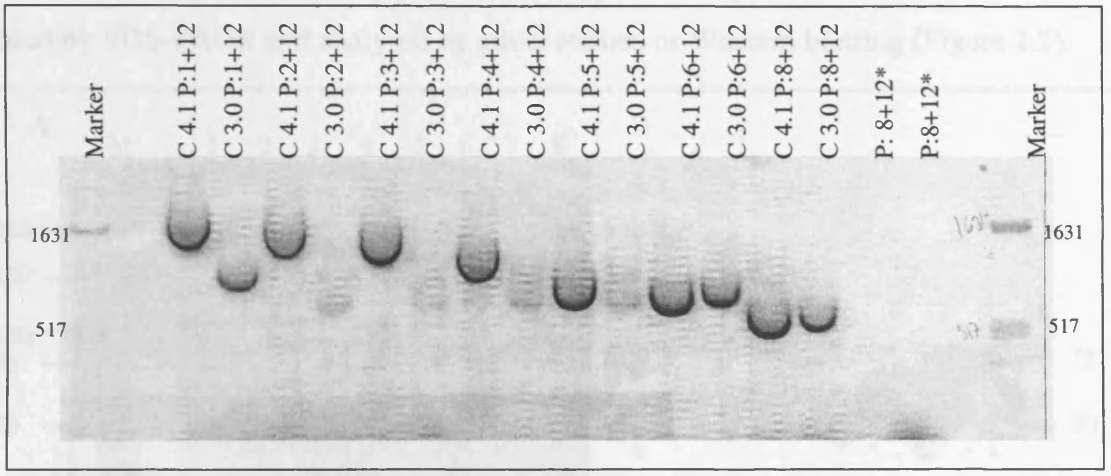


Figure 2.2. PCR reactions on clone 4.1 kb (C 4.1) and clone 3.0 kb (C 3.0) using identical primer sets were analysed on agarose gel. The PCR products were loaded on the gel with similar reactions on the two clones next to each other. Fragments from C 4.1 and C 3.0 were, in the example shown, amplified using primer 12 as reverse primer and primer 1, 2, 3, 4, 5, 6 and 8 as forward primers. There was no cDNA added in the last two lanes (*) and these reactions were used as controls. Marker was loaded in the first and last lane. The primer numbers are explained in Figure 2.1.

The PCR fragments amplified using the different primer combinations indicated that clone 4.1 kb encoded the full length MASP-3 cDNA whereas clone 3.0 kb was another splicing variant of the MASP-1/3 gene. This variant would, according to the PCR results, encode the CCP2, linker region and serine protease domain. PCR experiments conducted using other primer combinations supported this conclusion (data not shown).

2.3.2 Expression of recombinant MASP-3 in HEK293 EBNA cells

Transient expression in HEK293 EBNA cells of recombinant MASP-3 was preformed using the two constructs encoding either full length MASP-3 (clone 4.1 kb) or clone 3.0 kb in the pEAK-8 vector. Mock transfections using the empty pEAK8 vector as control were conducted alongside the others. The first three transfections failed to produce any detectable amounts of recombinant

MASP-3. After amplification and purification of the two constructs a new transfection attempt was made, resulting in expression of recombinant MASP-3 encoded by clone 4.1 kb. There was no expression detected from HEK293 EBNA cells transfected with the construct encoding clone 3.0kb and transfection was not repeated. The supernatant proteins of the cell cultures were separated by SDS-PAGE and analysed by silver stained or Western blotting (Figure 2.3).

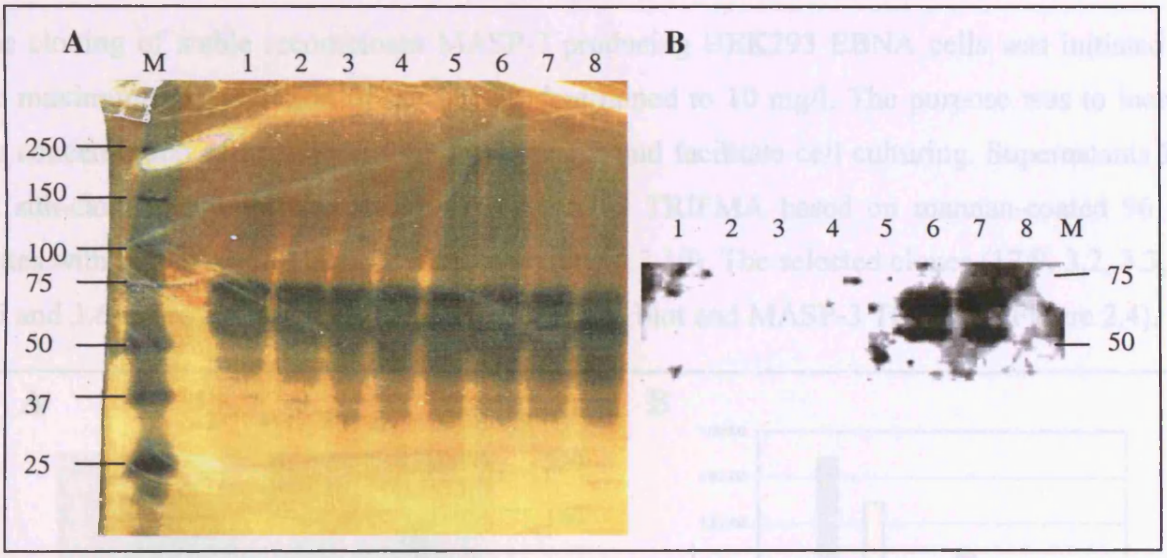


Figure 2.3. Supernatants from cell cultures were analysed by SDS-PAGE silver staining and Western blotting. Supernatant from HEK293 EBNA cells transfected with clone 4.1, encoding full length MASP-3 and mock transfected cell culture were analysed. Two 6-18% SDS-PAGE gradient gels were loaded with 2 µl (silver stained) or 15 µl (Western blot) prestained protein marker in lane M. In lane 1, 2, 3, 4, 5, 6 and 7 were loaded 80 µl supernatant from day 0, 1, 2, 3, 4, 5 and 6 respectively, after transfection with MASP-3 construct. Lane 8 was loaded with 80µl supernatant from mock transfected cell culture incubated 6 days. All samples were reduced before separation. The two gels were either silver stained (A) or Western blotted (B). The Western blot was developed with Rat anti MASP-3 linker region and HRP-labelled Rabbit anti rat IgG antibody as secondary antibody.

The cell cultures contained 2% heat inactivated FCS, which was visualised by silver staining of supernatant from day 0 after transfection (Figure 2.3-A). An increasing production of protein by the cells could be seen during the 6 days of incubation, but there were no clear difference in protein composition between MASP-3 construct and mock transfected cells. A small amount of recombinant MASP-3 could be detected by Western blotting after 5 and 6 days (Figure 2.3-B). The identification of two bands of ~55 and 65 kDa may be explained as a result of MASP-3 A chain degradation with a 10 kDa N-terminal fragment of activated MASP-3 being cleaved of (see chapter 4). Recombinant MASP-3 could be detected in supernatants incubated 1, 2, 3 and 4 days

after purification by ion exchange chromatography. The production level of recombinant MASP-3 was very low and in range of nanograms of recombinant MASP-3/ml of cell culture supernatant.

2.3.3 Stable clone selection

The cloning of stable recombinant MASP-3 producing HEK293 EBNA cells was initiated and the maximum concentration of puromycin determined to 10 mg/l. The purpose was to increase the concentration of MASP-3 in the supernatants and facilitate cell culturing. Supernatants from all sub-cloned cultures were tested in a MASP-3 TRIFMA based on mannan-coated 96 well plates with bound recombinant MBL (see chapter 4.2.10). The selected clones (17/9, 3.2, 3.3, 3.4, 3.5 and 3.6) were analysed on SDS-PAGE Western blot and MASP-3 TRIFMA (Figure 2.4).

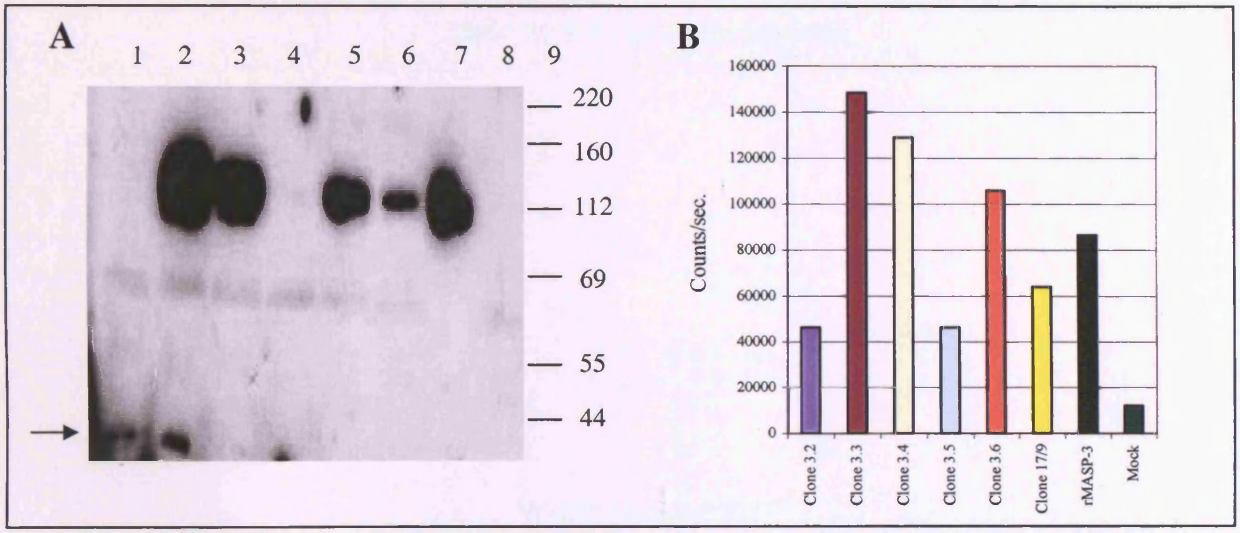


Figure 2.4. Single cloned cell culture supernatants were analysed by SDS-PAGE and Western blotting (A) and TRIFMA (B). The SDS-PAGE analysed by Western blotting were loaded with 20 µl supernatant from clone 3.2, 3.3, 3.4, 3.5, 3.6 and 17/9 in lane 1-6 respectively. Lane 7 contained 20 µl of recombinant MASP-3 preparation as positive control and supernatant from mock transfected HEK293 EBNA cells (20 µl) was loaded in lane 8. A volume of 12 µl Rainbow marker was loaded in lane 9 (bands indicated with lines). The Western blot was developed using rabbit 7932 anti-MASP-3 B chain C-terminal (see table 2.1 for details) followed by HRP-labelled goat anti-rabbit IgG as secondary antibody. All samples on the Western blot were analysed under reducing conditions. Activated MASP-3 B chain was detected and is indicated with an arrow. B: The supernatants were tested in the mannan/MBL based MASP-3 TRIFMA and one of the dilutions (1/8) is depictured in Figure 4-B. The MASP-3 level in supernatants from clone 3.2, 3.3, 3.4, 3.5, 3.6 and 17/9 are represented by the blue, purple, ivory, cyan and coral, respectively. Recombinant MASP-3 purified on a Resource Q column is represented by the black bar and the mock supernatant with a green bar.

The Western blot in Figure 2.4-A was exposed long enough to identify the 110 kDa band representing MASP-3 in supernatant from clone 17/9. The recombinant MASP-3 from clone 3.2 and 3.5 were found to be 100% activated, while the other clone supernatants mainly contained non-activated protein. The intensity of the bands on the Western blot was found to reflect the level determined by TRIFMA (Figure 2.4-B).

2.3.4 Purification and biotinylation of rabbit 7932 antibodies

IgG from five ml of the polyclonal rabbit 7932 anti human MASP-3 antiserum (see table 2.1 for details) was purified on protein G beads and the protein concentration of the eluted fractions estimated by OD. The fractions containing more than 0.1 mg protein/ml were pooled and stored at 4°C. The protein concentration of the pool was estimated to be 3.9 mg/ml in a total volume of 12 ml. IgG from pre-immunisation sera from rabbit 7931 (see table 2.1 for details) and 7932 were purified on protein G beads and stored at 4°C. The IgG fraction of rabbit 7932 antibody was tested on mannan coated plates with 1 µg recombinant MBL/ml and dilutions of recombinant MASP-3. The specificity of the antibody was tested by inhibition with the C-terminal peptide in dilutions (Figure 2.5).

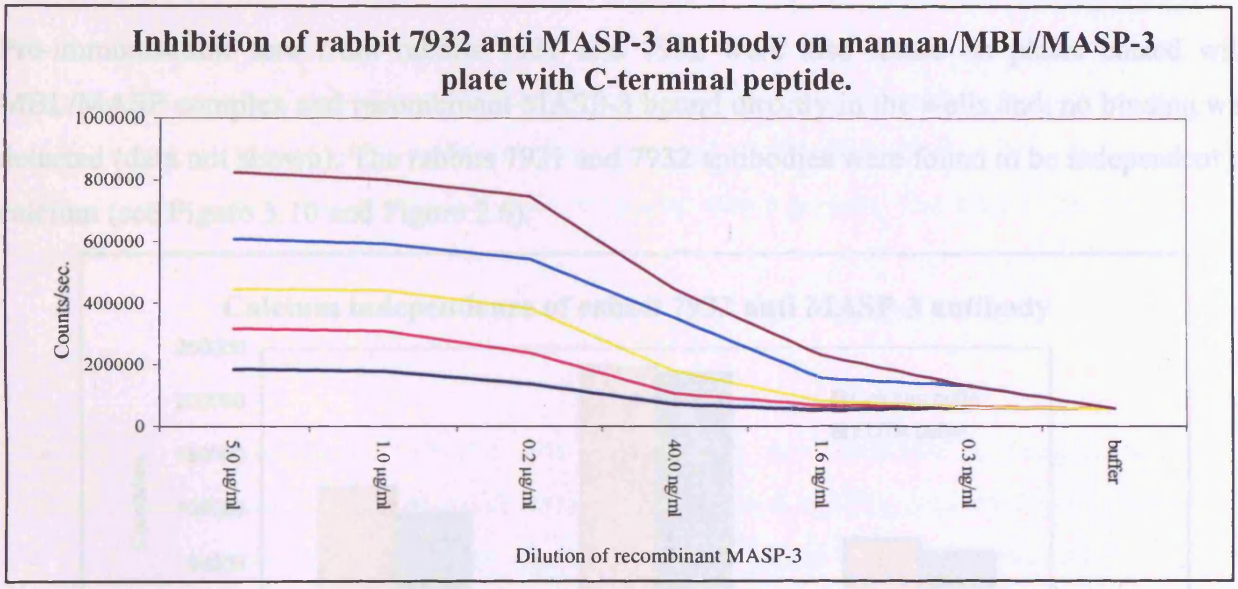


Figure 2.5. The specificity of the rabbit 7932 anti MASP-3 antibody was measured on dilutions of recombinant MASP-3 bound to MBL in a TRIFMA assay (chapter 3.2.7). The binding of the antibody to MASP-3 could be inhibited by incubation of the peptide together with the antibody in a dose dependent manner. The peptide concentration range was 0 (purple curve), 80 ng/ml (green curve), 0.4 µg/ml (yellow curve), 2 µg/ml (red curve) and 10 µg/ml (blue curve). The recombinant MASP-3 concentration range was 0.3 ng/ml-5 µg/ml. The amount of counts/sec. measured by the fluorimeter reflected the amount of Eu^{3+} -labelled antibody bound to the solid phase in the wells.

Anti-MASP-3 antibody was biotinylated using three concentrations of biotinylation agent, BNHS: 167, 56 and 28 µg/mg protein. The biotinylated anti MASP-3 antibodies were tested on a plate coated with 0.25 µg recombinant MASP-3/ml (Figure 2.6 next page).

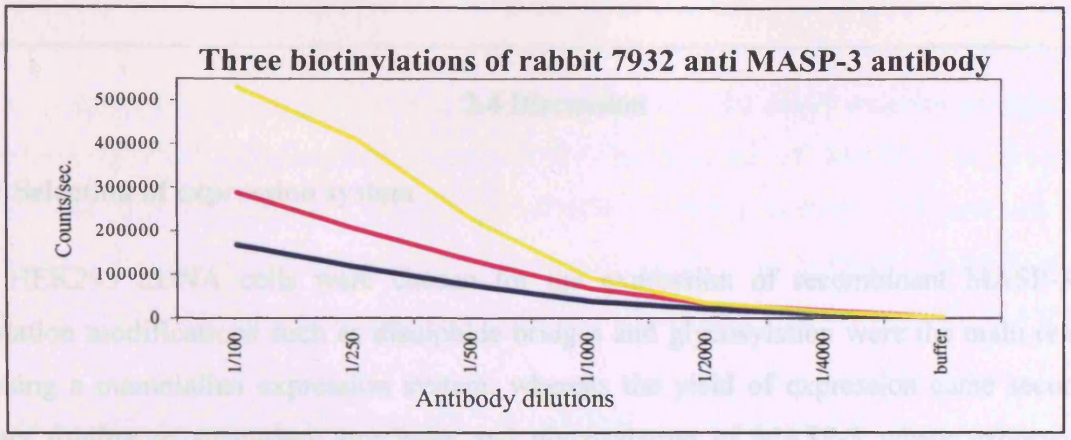


Figure 2.6. The three biotinylated antibody preparations were compared on a plate coated with recombinant MASP-3. The biotinylated antibody dilutions ranged from 1/100- 1/4000. The blue, red and yellow curve represents 28, 56 and 167 µg BNHS/mg protein, respectively. The background/signal ratio of bound antibody that was measured was dependent on the level of BNHS coupled to the antibody. Future biotinylations of rabbit 7932 and 7931 were performed using the recommended 167µg BNHS per mg protein (see chapter 3.2.8).

Pre-immunisation sera from rabbits 7931 and 7932 were also tested on plates coated with MBL/MASP complex and recombinant MASP-3 bound directly in the wells and, no binding was detected (data not shown). The rabbits 7931 and 7932 antibodies were found to be independent of calcium (see Figure 3.10 and Figure 2.6).

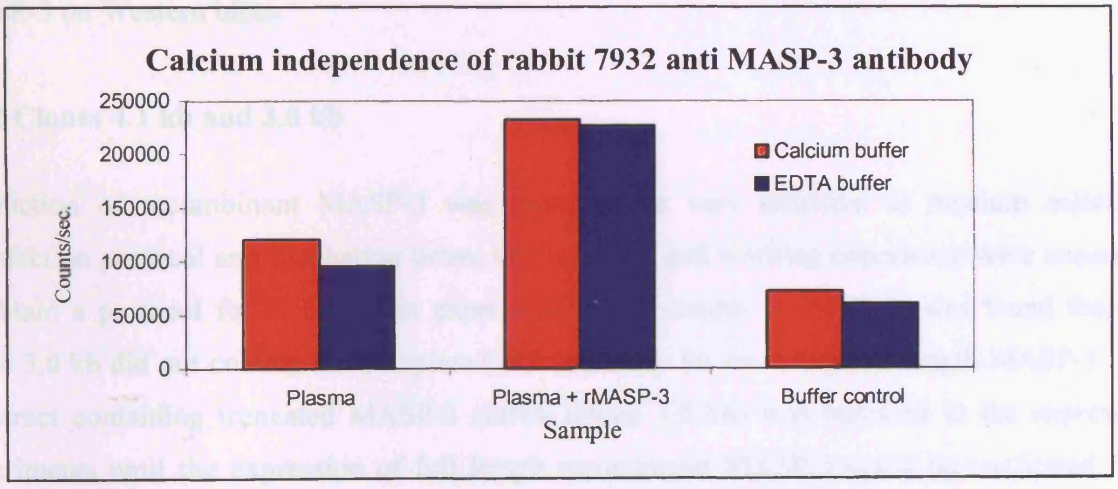


Figure 2.6. The Figure shows plasma samples diluted 1/50 in TBS/tween 20 buffer with either 5 mM calcium (red bars) or 5 mM EDTA (blue bars) and +/- recombinant MASP-3 (10 ng/ml sample). The MASP-3 levels were evaluated using the MASP-3 sandwich TRIFMA assay (chapter 4.2.1) except that the plates were coated with 5 µg/ml 1E2 monoclonal anti MASP-3 A chain antibody and that there was no additional NaCl , HSA or IgG added to the incubation buffer. Buffer controls were included to evaluate the background signalling.

2.4 Discussion

2.4.1 Selection of expression system

The HEK293 EBNA cells were chosen for the expression of recombinant MASP-3. Post-translation modifications such as disulphide bridges and glycosylation were the main reason for choosing a mammalian expression system, whereas the yield of expression came second. The correct folding of secondary structures and glycosylation of MASP-3 where expected to be important features for functional protein analysis. Extensive purification of the supernatants on columns was anticipated to be necessary to obtain a MASP-3 concentration high enough for experiments on potential substrates. Previous experience with the expression of MASP proteins in the mammalian system also favoured this. The number of anti-MASP-3 antibodies had increased over the last five years. The antibodies produced were all peptide derived polyclonals and only the antibodies derived against the C-terminal of MASP-3 (Rabbit 7931 and 7932, see table 2.1 and Figure 4.1 for details) could recognise native protein. The others could recognise MASP-3 on Western blots.

2.4.2 Clones 4.1 kb and 3.0 kb

Production of recombinant MASP-3 was found to be very sensitive to medium selection, transfection protocol and incubation times. Optimisation and working experience were necessary to obtain a protocol for recombinant expression of full length MASP-3. It was found that the clone 3.0 kb did not contain the complete ORF necessary for encoding full length MASP-3. The construct containing truncated MASP-3 cDNA (clone 3.0 kb) was included in the expression experiments until the expression of full length recombinant MASP-3 could be confirmed. The focus of the project at this time was to produce full length MASP-3 and since there had been no protein expression detected from cells transfected with the truncated MASP-3, further attempts to produce the variant were abandoned. A truncated MASP-3 protein (tMASP-3) recombinant protein would have been an important observation and would have encouraged research for identification of tMASP-3 in human serum. The tMASP-3 protein would not be associated with

MBL due to lack of the first three domains of the A chain and novel isolation protocols would have been essential (Wallis and Dodd 2000). The amount of MASP-3 in the different preparations was initially analysed by SDS-PAGE Western blotting. The method was not quantitative and MASP-3 concentrations could only roughly be estimated. Inter-experimental comparison of MASP-3 supernatants of preparations should only be done with this in mind.

2.4.3 Selection of stable MASP-3 producing cell line

Selection of stabile MASP-3 producing cell lines was initiated with a survival experiment to determine the selection pressure necessary. The consequence of using of the maximum puromycin concentration (10 mg/l) lead to a selection of the strongest and most reliable cell lines. The cell lines had individual characteristic growth rates and stability. Clone 3.3 was rapidly dividing with a cell cycle of 6-10h, while clone 17/9 divided very slowly and had a cell cycle of >24h. There were extremely few dead cells in the cell culture of clone 17/9, while a normal turnover of cells was seen in the other cell cultures. This diversity was an advantage and gave the stable cell lines an additional feature. Cells from clone 3.3 could be put in culture and be ready for recombinant MASP-3 production within 4-6 days, while clone 17/9 was easy to maintain for long periods with a minimum of maintenance. The additional clones were not further characterised. The cells were adapted to a low percentage of FCS during the time of culturing and no FCS was added during the production of MASP-3 containing supernatants for purification.
The TRIFMA experiment facilitated the analysis of the supernatants and made it possible to evaluate a larger number of samples. The silver stained SDS-PAGE and the Western blot was still used in experiments where a physical visual analysis was desirable.

2.4.4 Polyclonal anti-MASP-3 antibodies

The obtained antibodies made it possible to distinguish between the A and B chain of MASP-3 and between MASP-1 and MASP-3 A chains using Rat95 anti MASP-3 link region antibody. Furthermore all the polyclonal antibodies were capable of recognising MASP-3 under both

reduced and non-reduced conditions making it possible to distinguish between activated and non-activated MASP-3 as well as different parts of the protein.

In conclusion, four polyclonal and two monoclonal anti MASP-3 antibodies were produced by immunising with MASP-3 peptides. The B chain specific antibodies rabbit 7931 and 7932 recognising the C-terminal part of MASP-3 were found to bind to native MASP-3. The antibodies were found to be independent of calcium. This enabled the development of MASP-3 specific assays with the sandwich TRIFMA assay (see chapter 4.2.1) as one of the major achievements.

3. Purification of recombinant MASP-3

3.1 Purification of MASP-3 and assay development

Recombinant human MASP-3 was expressed transiently in HEK293 EBNA cells, using cDNA encoding full length MASP-3 in the pEAK8 vector, and purified by ion exchange chromatography. MASP-3 containing supernatant from cells cultured for six days was passed onto a 1 ml Mono Q column and bound protein was eluted with a salt gradient. Fractions were analysed by SDS-PAGE Western blotting and fractions containing MASP-3 were pooled.

The purification resulted in a preparation of approximate 95% pure MASP-3 estimated from a silver staining of a SDS-PAGE. The protocol was developed prior to this thesis (Dahl *et al.* 2001).

The purification method was simple and effective, however the volume of supernatant produced by stable MASP-3 producing cell lines clone 3.3 and 17/9, could not be passed through small columns within reasonable time. The Mono Q column available had a maximum loading volume of 0.1 ml/min and this was only when the machinery was working optimal. When the stable cells were ready for culturing, a daily turnover of 500 - 750 ml supernatant (containing ~3 μg MASP-3/ml) was easily processed. This called for another way to purify MASP-3 from supernatants.

3.2 Material and methods

SDS-PAGE and Western blotting were generally performed as described in chapter 2.

3.2.1 Affinity chromatography matrix

The matrix used in affinity chromatography determines the type of proteins purified. Different types of beads have specific properties of purification: recovery, stability, selectivity, molecular weight fractionation range and resolution. By coupling ligands to beads, another property is added to the matrix. The ligand can be e.g. carbohydrates, peptides or proteins. In the case of MASP, the natural ligand is MBL and recombinant MBL was obtained as a gift from the company NatImmune, DK. Another option was to couple anti-MASP-3 antibodies to beads, but this would only be reasonable if the antibody recognized MASP-3 in its native conformation. Antisera from rabbit 7931 and 7932 were capable of recognizing recombinant MASP-3 in complex with MBL, as shown in chapter 4.2, thus they could potentially be used for purifying MASP-3 on beads. The immunoglobulins from the polyclonal sera were purified before being coupled to the beads. The purified immunoglobulin could then be further processed and specific anti-MASP-3 antibody extracted by purification on beads coupled with recombinant MASP-3.

3.2.2 Production of recombinant MASP-3

The selected clones were cultured in 150 cm^2 flasks in 35 ml DMEM-12 supplemented with 10 mM glutamax, 2% heat inactivated fetal calf serum (FCS) and an insulin-transferrin-selenium solution (ITS).
The cell cultures were maintained at 37°C in humidified air with 5% CO_2 and split when 80 - 90% confluent into ten 150 cm^2 flasks. When these cultures were 60-80% confluent, the cells were rinsed with medium without FCS and incubated with 50 ml supplemented DMEM-12 without FCS. The supernatant was renewed every 24h for 4-6 days. The cultures were then terminated or kept in FCS-containing medium for later use. The protocol resulted in the production of 4-6 litres of supernatant containing recombinant MASP-3. Non-glycosylated

recombinant MASP-3 was produced by including 0.5 mg tunicamycin/ml in the culture medium. The culture supernatant was centrifuged 10.000 g for 10 min. and the supernatant were pooled and stored at 4°C. The first two batches of supernatant were dialysed against TBS/Tween 20 (Tris Buffered Saline, pH 7.4 with 0.1% v/v Tween 20) with 5 mM Ca^{2+} until the pH indicator in the medium was removed. This was done at 4°C using 4 l buffer for 500 ml supernatant placed on a magnetic stirrer. The buffer was changed three times over a 16h period and supernatants were pooled before the purification was started.

3.2.3 Purification of immunoglobulin from polyclonal antisera

Protein G from streptococcus sp. binds to immunoglobulins from a variety of animals. Immunoglobulins from the polyclonal rabbit 7932 and 7931 were purified on protein G Sepharose 4 beads (Amersham Cat. No. 17-0618-01) using the protocol below.
Five ml protein G beads were rinsed using 10 ml of 0.1 M glycine pH 2.5 and washed in 4 times 10 ml PBS (Phosphate Buffered Saline, pH 7.4, 140 mM NaCl (Merck Cat. No. 6404), 2.7 mM KCl (Merck, Cat. No. 116101), 1.5 mM KH_2PO_4 (Merck, Cat. No. 104873) and 8.1 mM Na_2HPO_4 (Merck Cat. No. 106559) with 10 mM EDTA. The 5 ml protein G beads were incubated overnight at 4°C on a rotary stirrer with agitation with 5 ml PBS/10 mM EDTA and 5 ml of rabbit serum. The beads were packed in a column and the effluent collected. The column was washed with twenty column volumes of PBS/10 mM EDTA until UV absorption $\leq$ 0.005 mg/ml (A_{280}). Bound IgG was eluted in 1 ml fractions using 0.1 M glycine pH 2.5. Fractions were collected in eppendorf tubes containing 50 µl 1M Tris pH 8.5 to increase the pH. Optical density (OD) was measured and the fractions containing protein were pooled.

3.2.4 Coupling of proteins to Sepharose beads

CNBr-activated Sepharose 4B beads (Amersham Cat. No. 17-0490-01) was conjugated with specific proteins for the construction of a purification matrix. The dry beads were washed 3 times for 10 min. in 1 mM HCl on a sintered glass filter. The ligand for coupling was diluted in

coupling buffer (0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3) and OD_{280} of the solution measured. Activated beads were incubated with the ligand for 2h at RT on a rotor. The unbound ligand was collected and the beads washed in coupling buffer. Remaining coupling sites on the beads were blocked by incubation with ethanolamine (1 M, pH 9) for 2h at RT on a rotor. The matrix was washed with three cycles of buffers that were high and low in pH (0.1 M acetate pH 4 and 0.1 M Tris pH 8, both buffers containing 0.5 M NaCl). The product was stored in TBS buffer at 4°C. The OD_{280} of the supernatant containing unbound ligand was measured and the protein concentration compared with that of the starting material. This comparison gave an estimate of how much of the ligand was bound to the beads. Recombinant MBL was coupled to CNBr-activated Sepharose 4B beads in concentrations ranging from 0.1 - 0.25 mg MBL/ml beads and used for affinity purification of recombinant MASP-3.

The IgG fraction of the rabbit 7932 anti-MASP-3 antiserum and recombinant MBL were coupled to CNBr activated Sepharose 4B beads to be used for affinity purification of recombinant MASP-3. A volume of 2 ml purified rabbit 7932 IgG, containing 3.9 mg/ml of protein was coupled to 5 ml CNBr-activated Sepharose 4B beads. OD_{280} was measured before and after and the coupling efficiency was estimated to 71%. Assuming all the bound protein was immunoglobulin, the concentration would be 1.1 mg immunoglobulin/ml beads.

The recombinant MBL was coupled to the beads in a lower concentration due to the large size of the molecule. In one experiment 1 mg recombinant MBL was coupled to 4 ml CNBr-activated Sepharose 4B beads. OD_{280} was measured before and after coupling and an estimated 82% of the MBL was bound to the beads. The concentration of MBL on the beads was calculated to be 205 µg/ml.

3.2.5 Ion exchange chromatography

The recombinant MASP-3 was initially purified by ion exchange chromatography as the sole purification step of supernatants. This was done on either a 1 ml Mono Q column (Amersham Cat. No. 17-0546-01) or a 6 ml Resource Q coloumn (Amersham Cat. No. 17-1179-01). Supernatants affinity purified on MBL-derivatised beads were further purified and concentrated by ion-exchange.

The purification system consisted of two peristaltic pumps integrated in a pump controller, mixer, column holder, UV detector, chart recorder and a fraction collector. The supernatant was passed onto the column in a tube by pressure produced by the pump and the protein content determined by the UV detector. The normal flow rate was 0.1 ml/min. By controlling the flow from the two pumps a gradient of two buffers could be made. The buffers were mixed before entering the column. The effluent and eluates could be collected on in a fraction collector in tubes of a suitable volume.

3.2.6 Affinity purification of MASP-3

The recombinant MBL conjugated CNBr-activated Sepharose 4B beads were used in the same system as described above. The normal flow rate on this column was 1 ml/min. The time consuming process of passing the large volume supernatants on the column, using a peristaltic pump, was reduced when incubations of beads with supernatant in bottles was introduced. These incubations were done by taking a volume of beads and mixing it with a volume of supernatant for 1.5h at 4°C and then packing the beads into a column. Binding capacity of the beads was estimated and an excess volume of MASP-3 containing supernatant diluted 1-1 in washing buffer (TBS/Tween 20 with 5 mM Ca^{2+}) was used to saturate the beads.

The beads were extensively washed on the column and eluted with a single step buffer change to washing buffer containing 1 M $MgCl_2$. The eluted material was collected in 3 - 5 ml fractions and stored at 4°C until analysed for MASP-3 content. The MASP-3 containing fractions, from several purifications, were pooled and dialysed before being loaded on the Mono Q ion exchange column.

Several experiments were preformed using protein G-purified rabbit 7932 IgG conjugated to CNBr-activated Sepharose 4B. These experiments were done on a pilot scale in 1.5 ml reaction tubes.

3.2.7 TRIFMA (time-resolved immunofluorometric assay)

TRIFMA is an assay similar to ELISA (enzyme-linked immunosorbent assay) and can be exploited in the same manner.

The enzyme-coupled antibodies in the ELISA are replaced by a Eu^{3+}-labelled antibody in the TRIFMA. By adding an enhancement buffer (Wallac Oy, Turku, Finland), the Eu^{3+}-ion separates from the complex and produce fluorescence upon excitation (Hemmilä *et al.* 1984). The emitted fluorescence was assessed on a fluorimeter (Wallac 1232 Delphia^{TM} fluorimeter, Finland). Maxisorp 96 wells microtiter plates (Nunc, cat. No. 437958) were coated with mannan by incubation with 100 µl/well of a solution containing 10 µg mannan /ml diluted in coating buffer for 3h at RT. The plate was blocked with 200 µl/well TBS (defined chapter 2.2.12) containing 1mg HSA/ml, incubated 30 min. at RT and washed in TBS/Tween 20 containing 5 mM Ca^{2+}. Mannan was prepared as described in the paper by Najakajima and Ballou (1974). The wells were incubated with 100µl of a solution containing 1 µg recombinant MBL/ml of TBS/Tween 20 with 5 mM Ca^{2+} overnight at 4°C. The dilution buffer used for dilution samples and antibodies was a TBS/Tween 20/5mM Ca^{2+} with 100 µg heat aggregated normal human IgG (ΔnIgG, State Serum Institute. Cat. No. 418293)/ml and 100 µg heat aggregated HSA (ΔHSA)/ml. The wells were washed three times in TBS/Tween 20/5 mM Ca^{2+} buffer before storage at 4°C. Samples were diluted in dilution buffer and 100 µl/well were incubated for 4h at RT. The wells were washed three times in TBS/Tween 20/ 5 mM Ca^{2+} and the MBL bound MASP-3 was detected by incubation with 100 µl diluted anti MASP-3 antiserum as primary antibody. Rabbit 7932 and 7931 immunised with the C-terminal peptide of MASP-3 were found to bind native MASP-3 (see Figure 2.6) and were used diluted 1/1000 - 1/3000 in buffer. Several secondary Eu^{3+}- and biotin-labelled antibodies were tested and for this assay 100 µl of biotinylated goat anti-rabbit antibody (Amersham Cat. No. RPN480) was preferred and used at 1/8000 dilution in dilution buffer.

After 4h incubation, the plate was washed three times in TBS/Tween 20/5 mM Ca^{2+} and incubated 1h with 100 µl Eu^{3+}-streptavidin (Delfia. Cat. No. 1244-360) diluted 1/2000 in TBS/Tween 20 with 25 µM EDTA. After a final wash the amount of bound Europium could be measured by adding 200 µl enhancement buffer to each well followed by incubation for 5 min. on a shaker and measured on a fluorimeter (Wallac). A preparation of recombinant MASP-3 or a standard pool of sera were diluted and used as reference in all analyses to be able to compare experiments, and samples were tested in duplicate. A buffer control (non-sense) was also included on each plate to evaluate background signal.

The TRIFMA described above was the preferred analysis for estimation of recombinant MASP-3 in the various fractions and supernatants. The purification of recombinant MASP-3 opened the possibility of testing another type of assay: the inhibition assay. The general protocol for the MASP-3 inhibition TRIFMA was as follows. Plates with 96 wells were coated with 100 µl of solution containing 0.25 µg recombinant MASP-3/ml PBS for 1h at RT and then blocked with 200 µl of TBS buffer containing 1 mg HSA /ml for 30 min. at RT. The plates were washed three times in TBS/Tween 20 before use or storage at 4°C. Samples was diluted in buffer (TBS/Tween 20 with 5 mM Ca^{2+}, 1 M NaCl and 5% v/v normal rabbit serum) and a fixed amount of biotinylated rabbit 7932 anti MASP-3 antibody was added to each dilution. 100 µl of each sample were incubated on the plates for 2h at RT and then washed three times in TBS/Tween 20/Ca^{2+} buffer. The amount of bound biotinylated antibody was detected by incubating each well with 100µl Eu^{3+}-streptavidin diluted 1/1000 in TBS/Tween 20 containing 25 µM EDTA for 1h at RT. The wells were washed three times in TBS/Tween 20/Ca^{2+} buffer and finally incubated with 200 µl enhancement buffer for 5 min. at RT and measuremed on a fluorimeter.

3.2.8 Biotinylation of protein

Covalently bound biotin can bind to the protein avidin that can be labelled with a detection marker such as HRP or Eu^{3+}. Proteins were biotinylated using the protocol bellow. An estimated amount of protein was dialysed against PBS (Phosphate Buffered Saline, pH 7.4,) two times 1h and overnight with agitation at RT. The protein was then dialysed against PBS with a pH of 8.5 adjusted with a 5% w/v Na_2CO_3 for 3h at RT. The dialysed protein solution was added to 167 µg

biotin-N-hydroxysuccinimide (BNHS from Sigma, Cat. No. H1759)/mg of protein and incubated for 4h at RT on rotor. The excess BNHS was removed by dialysis (for 1h then overnight) against TBS (defined chapter 2.2.12) at RT with mixing. The biotinylated protein solution was centrifuged at 10000g for 30 min. and the supernatant stored at 4°C (Guesdon *et al.* 1979).

3.3 Results

3.3.1 Purification of recombinant MASP-3

The initial purifications of recombinant MASP-3 were done as described in chapter 3.2.5. Ion exchange fractions were subsequently analysed for recombinant MASP-3 content by SDS-PAGE Western blotting and silver staining (Figure 3.3).

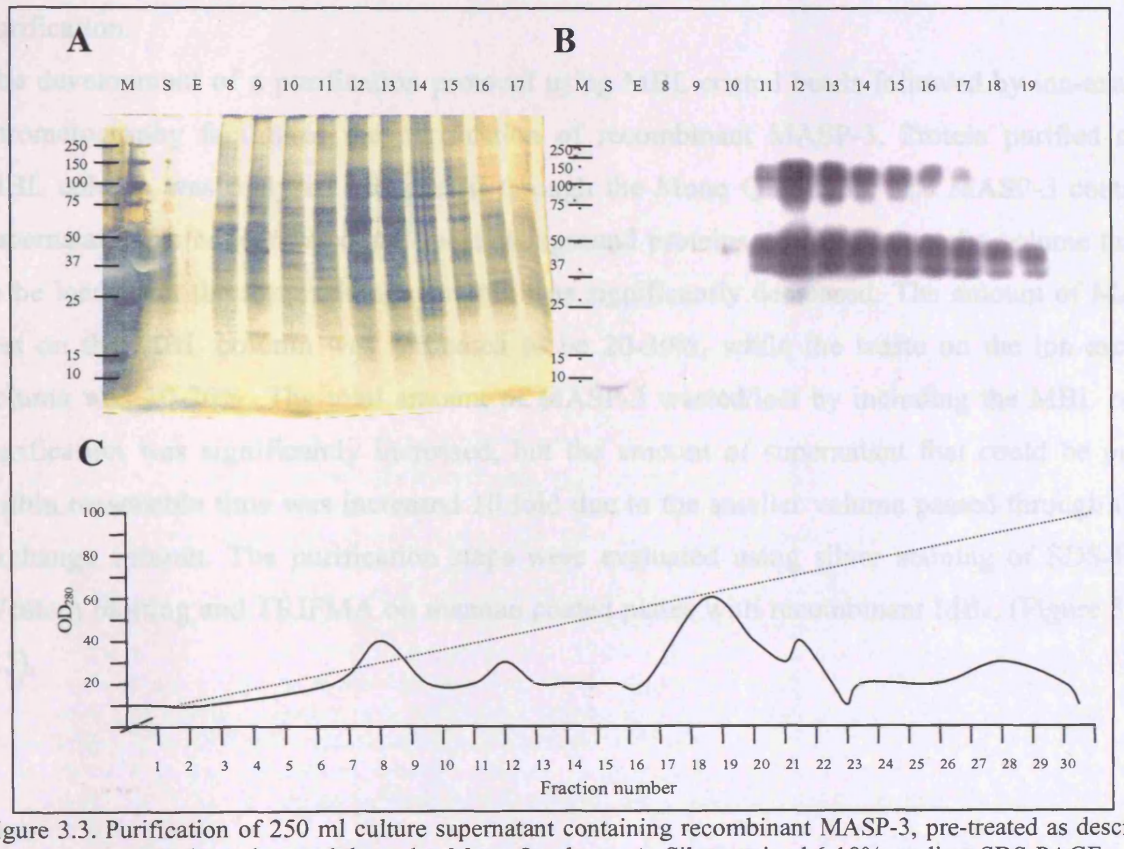


Figure 3.3. Purification of 250 ml culture supernatant containing recombinant MASP-3, pre-treated as described in material and methods, and passed through a Mono Q column. A: Silver stained 6-18% gradient SDS-PAGE was used for analysing the purification of recombinant MASP-3 on a Mono Q ion exchange column. Lane M was loaded with 2 µl BioRad unstained protein marker. Lane S was loaded with 20 µl MASP-3 supernatant and lane E with 20 µl effluent from the Mono Q column. Lane 8-17 represents the fraction number of the 1 ml fractions eluted using a salt gradient. A 10 µl volume of each fraction was loaded on the gel. B: A similar SDS-PAGE was used for Western blotting and developed using rabbit 7932 anti-MASP-3 B chain antibody (see table 2.1 for details) followed by detection with HRP labelled goat anti-rabbit antibody. Fraction 18 and 19 were included on the Western blot and lane M was loaded with 12 µl prestained BioRad protein marker otherwise the two experiments were identical and all samples were reduced before electrophoresis. C: A schematic representation of the protein from 200 ml MASP-3 culture supernatant from the Mono Q column with a NaCl gradient (dashed line). The OD_{280} was represented as a percentage of range 1 equal to 1 mg HSA/ml at 100%.

The silver stained SDS-PAGE in Figure 3.3-A was developed for a long period of time (10 min.) to visualise the purity of the preparation. The 110 kDa band identified in Figure 3.3-B represented non-activated MASP-3 and the ~45 kDa band represented the B chain from activated MASP-3. The bands recognised as ~90 and ~35 kDa are thought to be non-glycosylated MASP-3. The recombinant MASP-3 was estimated to be 70% activated on the basis of the proportion of full length and B chain detected on the Western blot. The faint band in lane S represents activated MASP-3 B chain in the culture supernatant, indicating that MASP-3 was activated before purification.

The development of a purification protocol using MBL-coated beads followed by ion-exchange chromatography facilitated the purification of recombinant MASP-3. Protein purified on the MBL column was dialysed and passed through the Mono Q column. The MASP-3 containing supernatant was concentrated and most background proteins excluded, thus the volume that had to be loaded on the ion-exchange column was significantly decreased. The amount of MASP-3 lost on the MBL column was estimated to be 20-30%, while the waste on the ion-exchange column was 10-20%. The total amount of MASP-3 wasted/lost by including the MBL column purification was significantly increased, but the amount of supernatant that could be purified within reasonable time was increased 10 fold due to the smaller volume passed through the ion exchange column. The purification steps were evaluated using silver staining of SDS-PAGE, Western blotting and TRIFMA on mannan coated plates with recombinant MBL (Figure 3.4 and 3.5).

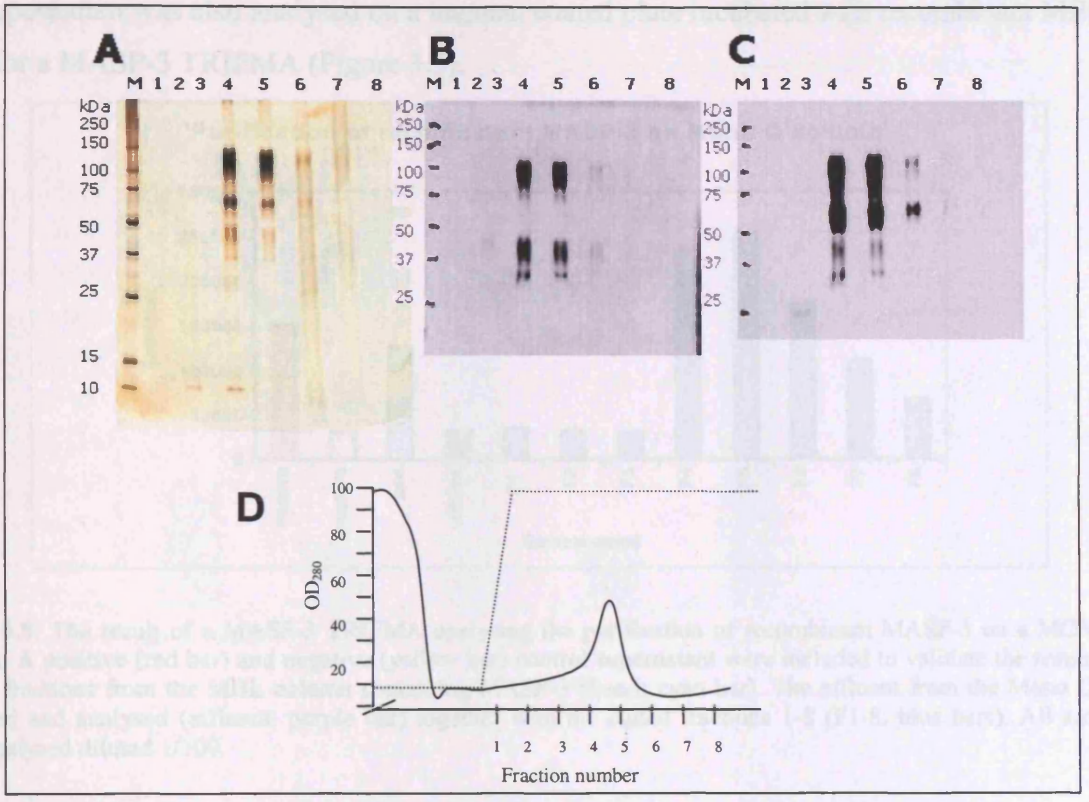


Figure 3.4. Two 6-18% gradient SDS-PAGE gels were loaded with BioRad marker and fraction 1-8 from the purification of recombinant MASP-3 on MBL beads with subsequent Mono Q ion exchange. One SDS-PAGE was loaded with 2 µl unstained marker and 2.5 µl of each fraction and silver stained after electrophoresis (A). The other gel was loaded with 12 µl pre-stained marker and 2.5 µl of each fraction and Western blotted (B and C). The Western blot was developed using rat 97 anti-MASP-3 B chain antiserum, which bound the 110 kDa non-activated and 42 kDa activated MASP-3 B chain (B). The blot was rinsed in TBS/Tween 20 with 0.1% azide and re-developed with rat 95 anti MASP-3 link region recognizing the 110 kDa non-activated and 68 kDa activated A chain of MASP-3 (C). In both cases a HRP-labelled rabbit anti rat antibody was used as secondary antibody. The MASP-3 content in fractions 4 and 5 were estimated to be >95% and estimated to be 40% activated from the silver staining and the Western blot. D A schematic representation of the protein from 25 ml pooled MASP-3 containing fractions from the Mono Q column concentrated on a rMBL column with a $MgCl_2$ gradient from 0.1 to 1M concentration in TBS/Tween 20/ 5mM Ca^{2+} buffer (dashed line). The OD_{280} was represented as a percentage of range 0.1 equal to 0.1 mg HSA/ml at 100%.

By comparing the two purification methods it was clear that the MBL column facilitated the process of MASP-3 purification by reducing the concentration of contaminant proteins in the supernatant prior to the ion exchange chromatography. The reduction of non-specific proteins also resulted in a higher resolution profile of MASP-3 elution from the ion exchange column.

The supernatant was also analysed on a mannan coated plate incubated with recombinant MBL as basis for a MASP-3 TRIFMA (Figure 3.5).

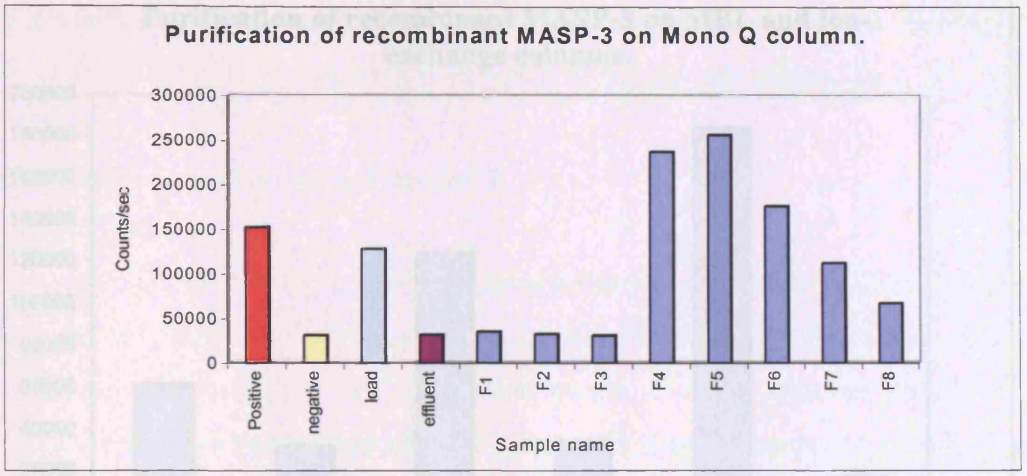


Figure 3.5. The result of a MASP-3 TRIFMA analysing the purification of recombinant MASP-3 on a MONO Q column. A positive (red bar) and negative (yellow bar) control supernatant were included to validate the result. The pooled fractions from the MBL column containing MASP-3 (Load: cyan bar). The effluent from the Mono Q was collected and analysed (effluent: purple bar) together with the eluted fractions 1-8 (F1-8: blue bars). All samples were analysed diluted 1/100.

The result obtained by TRIFMA was similar to the SDS-PAGE experiment. The four control supernatants should have been included in the SDS-PAGE experiment to make the perfect comparison. Most MASP-3 purification experiments were analysed using the MASP-3 TRIFMA leading to evaluation in a quantitative manner. The recombinant MASP-3 batch 19.2.02 was purified from 4½ litres culture supernatant by twenty-one purifications on MBL beads and nine purifications on the MONO Q column. The result was a 12 ml batch that was estimated to be >95% pure MASP-3 with a concentration of 250 µg/ml determined by OD, silver-stained SDS-PAGE and MASP-3 TRIFMA.

A summary of the purification of batch 19.2.02 was made using the TRIFMA assay (Figure 3.6).

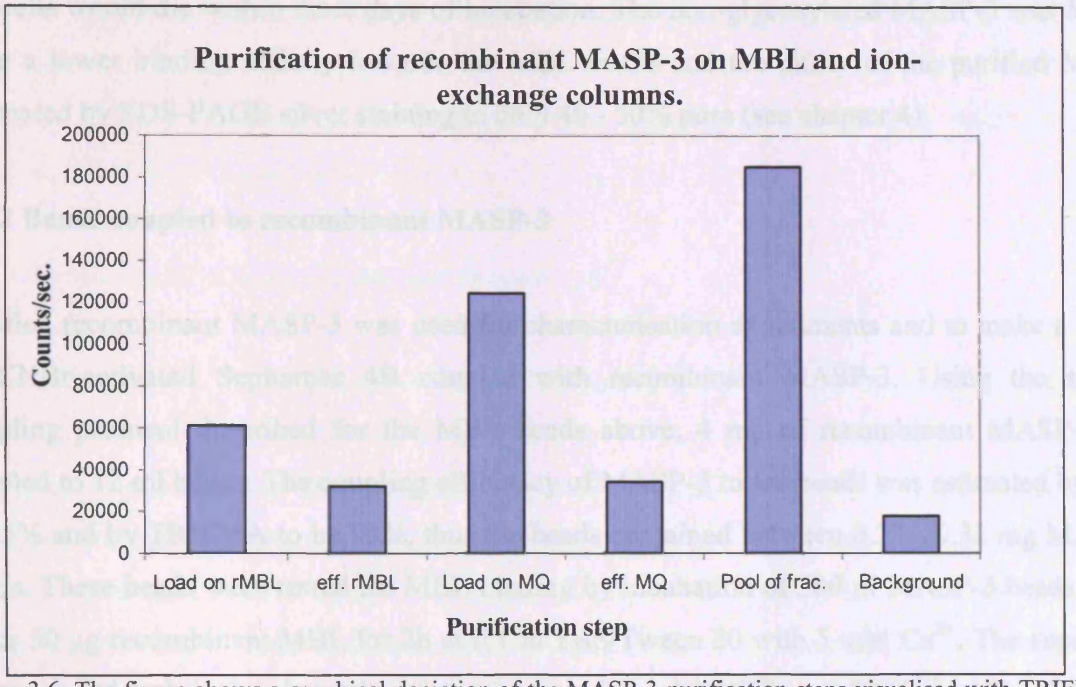


Figure 3.6. The figure shows a graphical depiction of the MASP-3 purification steps visualised with TRIFMA. The dialysed recombinant MASP-3 supernatant diluted 1-1 with the washing buffer (see chapter 3.2.6) loaded on the MBL column (Load on rMBL) and the effluent collected (eff. rMBL). The fractions eluted from the MBL column were pooled and loaded on the Mono Q ion-exchange column (Load on MQ) and the effluent collected (eff. MQ). The fractions from the Mono Q ion-exchange column containing recombinant MASP-3 were pooled (Pool of frac.) and all the samples were compared to the washing buffer (Background) in the MASP-3 TRIFMA.

The stable cell lines could produce a high amount of recombinant MASP-3 (2-4 µg recombinant MASP-3/ml supernatant per day), which made the purification protocol the limiting step of MASP-3 production. To further enhance the recombinant MASP-3 production, supernatants were centrifuged, supplied with 5 mM Ca^{2+} and purified by incubation with a volume of MBL coated beads for 2h at 4°C on a rotor. The beads were washed on a column and eluted with washing buffer containing 1 M $MgCl_2$. MASP-3 containing fractions from related purifications were pooled and concentrated on a ion-exchange Resource Q column. The purification on columns was performed at RT and the purification profile was similar to the one shown in Figure 3.6 when analysed by MASP-3 TRIFMA, except that the effluent after the MBL beads contained more MASP-3. The recombinant MASP-3 was, using any of the described methods, purified as partially activated protein as judged from silver stained SDS-PAGE or Western blots.

The addition of tunicamycin had a negative effect on the production of recombinant MASP-3 and the cells would die within three days of incubation. The non-glycosylated MASP-3 was found to have a lower binding affinity towards the MBL beads and the purity of the purified MASP-3 estimated by SDS-PAGE silver staining to only 40 - 50% pure (see chapter 4).

3.3.2 Beads coupled to recombinant MASP-3

Purified recombinant MASP-3 was used for characterisation experiments and to make a column of CNBr-activated Sepharose 4B coupled with recombinant MASP-3. Using the standard coupling protocol described for the MBL beads above, 4 mg of recombinant MASP-3 were coupled to 12 ml beads. The coupling efficiency of MASP-3 to the beads was estimated by OD_{280} to 75% and by TRIFMA to be 95%, thus the beads contained between 0.23 - 0.32 mg MASP/ml beads. These beads were tested for MBL binding by incubation of 500 µl MASP-3 beads with 0, 10 or 50 µg recombinant MBL for 2h at RT in TBS/Tween 20 with 5 mM Ca^{2+}. The supernatant was removed and the beads washed three times with 1 ml TBS/Tween 20/Ca^{2+} buffer. The beads were eluted using 200 µl of washing buffer with 1 M $MgCl_2$ (Figure 3.7).

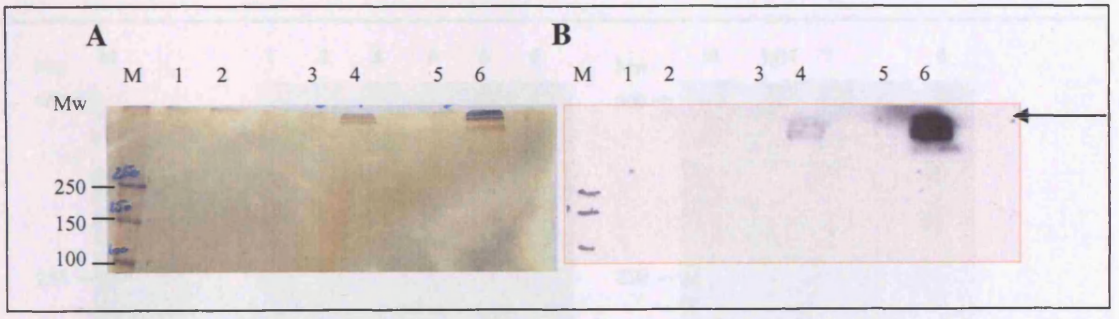


Figure 3.7. Two 6-18% gradient SDS-PAGE were loaded with marker BioRad in lane M and the supernatant from the incubation with 0, 10 and 50 µg recombinant MBL in lane 1, 3 and 5 respectively. The eluate from the three incubations were loaded in lane 2, 4 and 6 next to the supernatant of each experiment. One SDS-PAGE was silver stained (A) and the other was analysed by Western blotting (B). The gel intended for silver staining was loaded with 2 µl unstained protein marker and 20 µl of each fraction. A volume of 12 µl pre-stained protein marker was used on the gel that was Western blotted. The Western blot was developed using mouse monoclonal anti-human MBL (hybridoma 131-1, State serum institute, Denmark) followed by HRP-labelled rabbit anti-mouse antibody as secondary antibody. MBL was detected above the 250 kDa marker (right arrow).

The beads coated with recombinant MASP-3 were found to bind MBL. The experiment was repeated several times, including empty CNBr-activated Sepharose 4B beads as control. The conclusion was that the coupling for MASP-3 to the beads mediated binding of both recombinant MBL and MASP depleted plasma purified MBL (MASP depleted MO12 made by Annette Hansen, Denmark, data not shown).

3.3.3 Protein analysis on 4% polyacrylamide gel

To analyse the MBL bound to the MASP-3 beads, effort was put into developing a novel SDS-PAGE experiment, capable of separating molecules with an Mw between 200 and 900 kDa. The optimal gel for separating different oligomeric forms of MBL was a 4% polyacrylamid gel made using a matrix of 55% v/v H_2O, 15% v/v lower and upper tris buffer (see methods chapter 2.2.5), 14% v/v acrylamide (30% solution), 1% glycerol, 0.7% SDS, 0.4% APS and 0.07% TEMED. The gels were fragile after electrophoresis and they could only be stored for two weeks at 4°C. The different oligomeric forms of plasma-derived MBL and recombinant MBL were separated and analysed by silver staining or Western blotting (Figure 3.8).

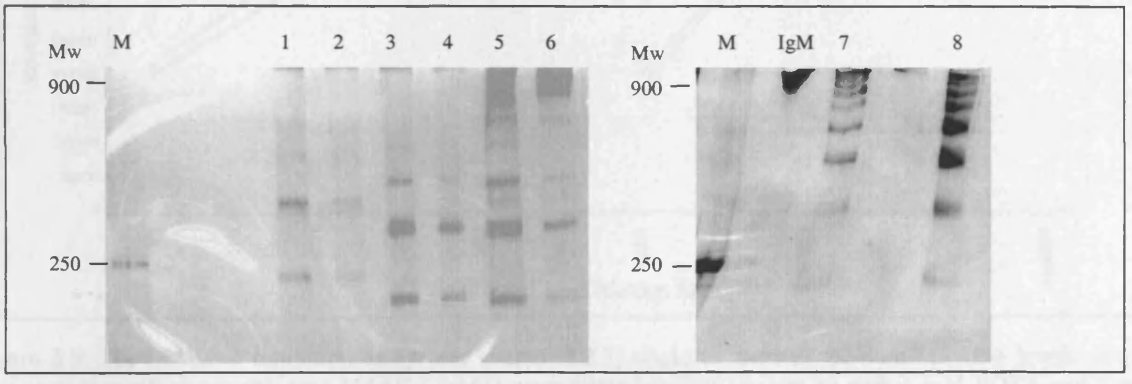


Figure 3.8. Two separate experiments of MBL electrophoresis on 4% SDS-PAGE. The gels were loaded with 1 µl unstained protein marker from BioRad in lane M and 500 ng human IgM in lane IgM. Lane 1 and 2 were loaded with 200 and 100 ng boiled MO12 (State serum institute, Denmark). Lane 3 and 4 were loaded with 200 and 100 ng MO12, not boiled. Lane 5 and 6 were loaded with 400 and 200 ng MASP depleted MO12, which were not boiled. Lane 7 and 8 were loaded with 1 and 4µg recombinant MBL, which were boiled. The gels were silver stained after electrophoresis.

The gels were analysed by Western blotting and developed with hybridoma 131-1 anti-MBL antibodies (not shown). At least five distinct bands were detected on the separated MO12 preparation and eight bands were seen in the separation of recombinant MBL. The method was intended to facilitate evaluation of MASP and MAp19 binding to different MBL oligomeric structures but no direct binding of MASP to the Western blots was detected.

3.3.4 MASP-3 inhibition TRIFMA

The establishment of the MASP-3 inhibition assay offered a novel method for analysing samples containing MASP-3. The qualities of the assay encouraged extensive optimisation. The results obtained had to be correlated in an opposite manner to the other types of MASP-3 assays. Low counts/sec in the TRIFMA was equal to high levels of MASP-3 in the sample (Figure 3.9).

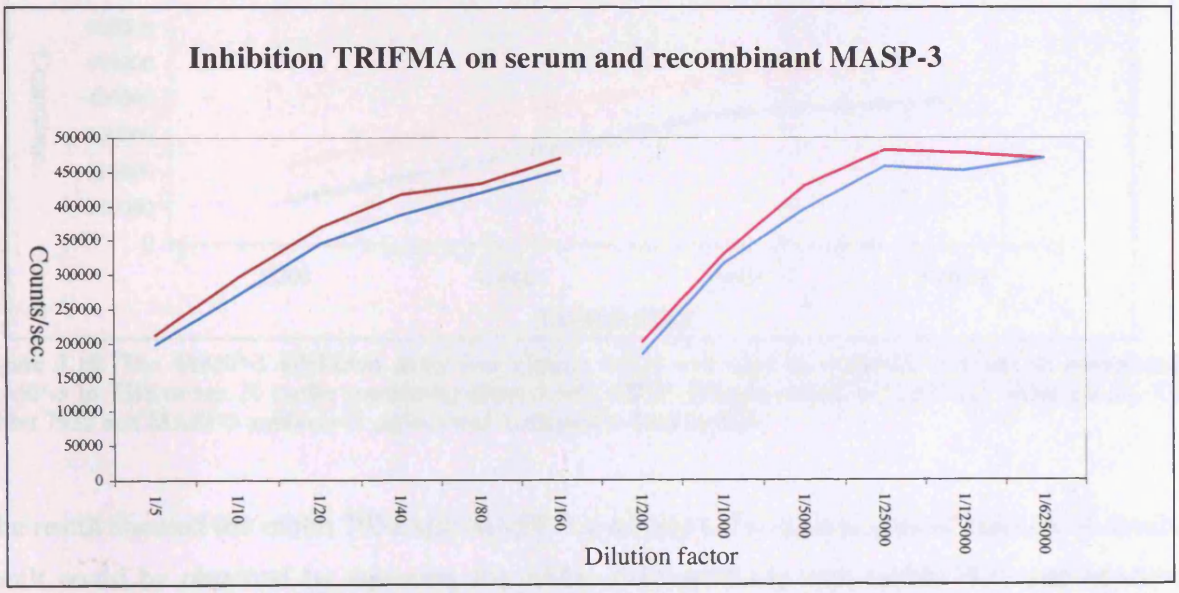


Figure 3.9. The MASP-3 inhibition assay (see chapter 3.2.7) could be used to measure MASP-3 levels. A pool of four sera or purified recombinant MASP-3 (rM3) were diluted in TBS/Tween 20 with 5 mM EDTA and 5 or 20% normal rabbit serum (nRs) and biotinylated rabbit 7932 (1 µg/ml). The MASP-3 levels in human serum samples could be detected in material diluted less then 1/80 (coral (5% nRs) and blue (20% nRs) curve) and in the purified recombinant preparation diluted less then 1/25000 (purple (5% nRs) and cyan (20% nRs) curve). The amount of counts/sec was not influenced by addition of normal rabbit serum.

High salt concentrations would lower the signal and the background but the ratio between the two was unchanged. The background signal of a similar experiment made in TBS/Tween 20 with 5

mM EDTA and 1M NaCl was in the range of 100.000 counts/sec. By changing the MASP-3 coating on the plate or rabbit 7932 antibody concentration in the buffers no improvement in the sensitivity of the assay was accomplished. The assay was abandoned due to high background and low sensitivity and a sandwich assay developed.

A MASP-3 inhibition assay using microtiter plates coated with 1 µg rabbit 7932 anti MASP-3 antibody (see table 2.1 for details)/ml and biotinylated recombinant MASP-3 added to the sample dilutions gave very low counts between 800 and 3000 and was only tried once (data not shown). The inhibition assays and the rabbit 7932 anti MASP-3 antibody were functional in buffer containing either EDTA or Ca^{2+} in moderate amounts (Figure 3.10).

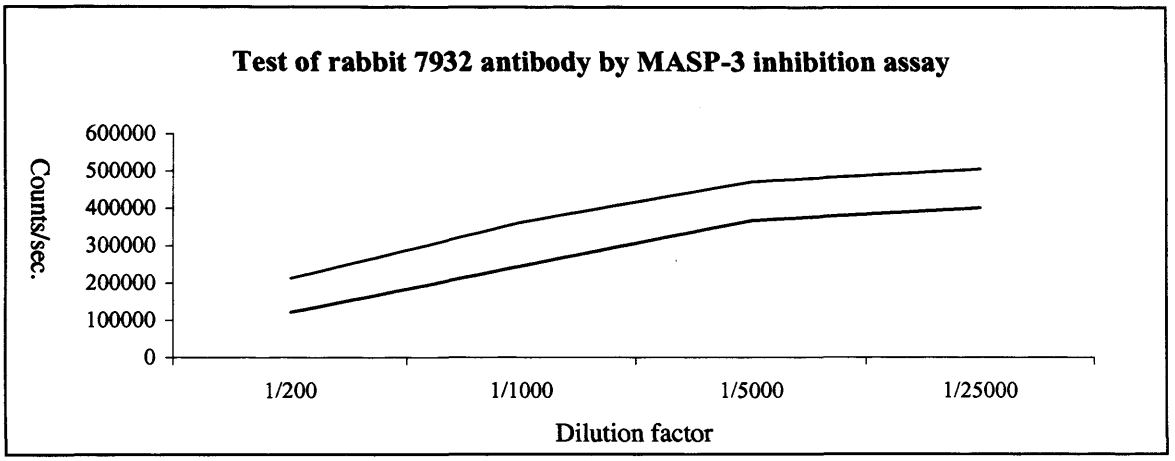


Figure 3.10. The MASP-3 inhibition assay (see chapter 3.2.7) was used to evaluated dilutions of recombinant MASP-3 in TBS/tween 20 buffer containing either 5 mM EDTA (Purple curve) or 5 mM ca^{2+} (blue curve). The rabbit 7932 anti MASP-3 antibody (1 µg/ml) was functional in both buffers.

The result showed the rabbit 7932 anti MASP-3 antibody to be independent of calcium. A similar result could be obtained by replacing the rabbit 7932 antibody with rabbit 7931 anti MASP-3 antibody (see table 2.1 for details). A marked difference in the signal/background ratio was observed to be influenced by the dilution buffer.

3.4 Discussion

3.4.1 Purification of recombinant MASP-3

The initial purification of recombinant MASP-3 by ion-exchange chromatography was ideal for small scale preparations of 5-75 ml supernatant. For large scale purification, the method was too time consuming and unreliable. The development of a new purification method, which could comply with the increase in recombinant MASP-3 production and consumption, was essential. With the recombinant MBL coupled to beads the purification protocol was improved. The purification of MASP-3 on recombinant MBL coated beads was found to be the method improvement necessary to meet these demands. Another advantage of including the MBL column was that it increased the chances of obtaining correctly folded MASP-3.

By establishing stable cell lines producing recombinant MASP-3, more MASP-3 could be produced and future availability of MASP-3 was secured.

The recombinant MASP-3 was produced without serine protease inhibitors because we wished to examine enzymatic activity. The presence of inhibitors in the culture supernatant could have ruined the results of MASP-3 characterisation experiments. A study concerning inhibition of the enzymatic activity of MASP-3 was performed at a later stage (Chapter 4.2.2 and 4.3.8).

Recombinant MASP-3 produced with 0.5 mg tunicamycin /ml in the culture supernatant was purified using the recombinant MBL coated beads and ion-exchange chromatography. The preparation was only 40-50% pure as estimated from silver stained SDS-PAGE. The non-glycosylated recombinant MASP-3 (chapter 3.3.2) was not pure enough to be used for crystallisation. The preparation was used for the characterisation of MASP-3 in general (Chapter 4).

Purified recombinant MASP-3 was, apart from being used within the research group, also given as gifts to other scientists and collaborators working with different aspects of immunology.

Development of the 4% SDS-PAGE for separating molecules with a molecular weight between 200 and 900 kDa was important in the process of examining the apparent difference in MASP and MAp19 association with MBL. The gel could separate the different oligomeric forms of MBL better then any other published method.

3.4.2 MASP-3 TRIFMA

Continual improvements and changes of the MASP-3 TRIFMA were necessary to increase the sensitivity of the assay. The MASP-3 TRIFMA based on mannan-coated microtiter plates incubated with recombinant MBL was limited to Ca^{2+} containing buffers to ensure MBL binding to the surface. The mannan/MBL based MASP-3 assay had a functional dimension in terms of the MBL derived lectin pathway of complement activation. The samples could be incubated on mannan coated microtiter plates +/- addition of recombinant MBL in the incubation buffer. The subsequent detection using anti-MASP-3 antibody would reveal both potential MBL and MASP-3 deficiencies.

It was expected that the protein complexes containing MASP-3 would have to be separated in order to obtain conclusive results from serum samples, therefore assays that could be performed in buffer containing EDTA and a high salt concentration were constructed.

The inhibition TRIFMA was developed with the expectation of obtaining a fast, simple and sensitive assay for measuring MASP-3 levels in samples. The first two objectives were achieved since the assay could be performed within three hours from loading the samples to measuring the plate on the fluorimeter. The results from the assay were reproducible but the relative sensitivity of the assay was not as high as required for measuring MASP-3 levels in body fluid samples. Effort was made to optimise the assay but still the sensitivity was low. The most likely explanation would be that the amount of specific anti-MASP-3 antibody in the preparation of immunoglobulins from rabbit 7932 was low. This could also explain the high background signal, which could not be diminished even with normal rabbit serum in the incubation buffer.

A sandwich assay using monoclonal anti-MASP-3 A chain antibody (1E2 from Teizo Fujita, Japan) for catching and biotinylated rabbit 7932 anti MASP-3 antibody for development was the last MASP-3 TRIFMA to be established (chapter 4.2.1). The assay was the preferred method at the end of the study and used for sample screening and MASP-3 quantification in general (this is described in chapter 5).

The purification of immunoglobulin from polyclonal antiserum on protein G beads was standard procedure and without complications. The biotinylation of protein G purified rabbit 7932 anti-MASP-3 antibody was done in order to rationalise the MASP-3 assay by excluding the tertiary

biotinylated anti-rabbit antibody. Three concentrations of biotin-N-hydroxysuccinimide were tested to ensure that the specificity of the antibody was not destroyed by the maximum recommended concentration.

The MASP-3 beads were used to affinity purify a small amount of specific anti-MASP-3 antibody from biotinylated rabbit 7932 antibody. The result indicated the potential of the MASP-3 beads for purification of the specific antibody from the polyclonal antiserum, but large-scale affinity purification was not performed due to initiation of monoclonal antibodies production (made under contract by the German company Davids Biotechnologie, Regensburg).

Immunisation of rats and mice with the C-terminal peptide of MASP-3 and recombinant MASP-3 B chain was initiated for production of monoclonal anti-MASP-3 antibodies. Monoclonal anti-MASP-3 antibodies against the C-terminal of MASP-3 were ready for introduction in December 2003 after the ending of my own laboratory work. It was introduced by Steffen Holst Holmvard, Denmark who was able to replace the polyclonal with the monoclonal in the TRIFMA assays and obtain similar results (data not shown).

4. Structure and functional aspects of MASP-3

4.1 Structure and functional aspects of MASP-3.

Characterisation of recombinant and plasma derived MASP-3 in terms of structure and function were essential studies in this thesis. The general structure was known from previous studies and the association with MBL has been published (Dahl *et al.* 2001), however important details such as functionality, tissue distribution, glycosylation profile, MBL association/dissociation profile still needed to be clarified. Recombinant MASP-3 expressed by HEK293 EBNA cells was secreted in a proenzyme form, i.e. the non-activated form. Upon storage at 4°C and during purification the protein became activated. A detailed description of the degradation of MASP-1 was part of the PhD thesis by Steen Vang Petersen (University of Aarhus, August 2001) and his results were evaluated along with observations made on recombinant MASP-3.

Tunicamycin inhibits the formation of N-acetyl-glucosamine-containing lipid intermediates involved in the generation of the N-linked oligosaccharide chains (Elbein 1987). The influence of N-linked glycosylation on MASP-3 was investigated by de-glycosylation.

Structural analysis was conducted in an attempt to characterise MASP-3 but also to deduce potential substrates. A role of MASP-3 as part of the complement system was further investigated since it seemed to be the most likely system for MASP-3 to carry out an enzymatic function.

Several results lead to a mounting interest in the haemostatic system and in particular the serine protease thrombin. The effect of MASP-3 on thrombin and its substrates were investigated in collaboration with Shaun Coughlin, University of California San Francisco (UCSF), USA. Investigation of potential functional properties of MASP-3 was intensified. Proteins encoded by genes in the vicinity of the MASP-1/3 gene on chromosome 3q27 were evaluated as potential substrates.

The genes encoding adiponectin and kininogen were found near the MASP-1/3 locus by data mining (data not shown). Adiponectin has been characterised as a hormone secreted by adipocytes for regulation of energy homeostasis as well as glucose and lipid metabolism. Adipocytes also produce and secrete proteins such as leptin, factor D, properdin, C3 and tumor necrosis factor, suggesting a possible link to the immune system. The protein structure of adiponectin resembles that of soluble defence collagens (MBL, SP-A, SP-D and C1q) by

forming homotrimeric subunits with a collagen-like triple-helical structure and multimers hereof. This protein has also been named: APM1, Gelatin-binding protein of 28-kDa (GBP28) and ACRP30 (Yokota *et al.* 2000). Adiponectin has anti-inflammatory effects on the cellular components of the vascular wall (Ouchi *et al.* 1999 and 2000).

The gene encoding kininogen or high molecular weight kininogen (HMWK) was also found in close proximity to the MASP-1/3 locus on chromosome 3q27. Kininogen has been characterised as being involved in blood coagulation as described in chapter 1. Alternative splicing of the gene leads to synthesis of two isoforms of the protein, known as low and high molecular weight kininogen. Surface bound kininogen functions as a receptor for prekallikrein and FXI. The active peptide bradykinin is released from HMWK upon cleavage by proteases (Pierce 1968).

Orthologous genes encoding MASP-3 has been identified in a variety of animals from late invertebrates to human (Endo *et al.* 2003; Stover *et al.* 2003). A gene cluster of 50 genes on human chromosome 3q27 (including MASP-1/-3), were found to be similar on mouse chromosome 16 and rat chromosome 11, thus having evolved together as a cluster in these species. This suggests a possible link between proteins of the cluster and was an argument for further investigation.

4.2 Material and methods

SDS-PAGE and Western blotting was in general performed as described in chapter 2.2.5-7. Homemade ECL reagent was made using the protocol in chapter 2.2.12.

4.2.1 MASP-3 sandwich TRIFMA

Maxisorp 96 wells microtiter plates were coated with 100 µl/well monoclonal anti MASP-3 antibody 1E2 (1µg/ml of PBS, from Fujita T, Tokyo, Japan) raised against the A chain of MASP-1, and incubated in the wells overnight at 4°C. The plate was blocked with 200 µl/well TBS (defined chapter 2.2.12) containing 1 mg HSA/ml, incubated 30 min. at RT and washed three times in TBS/Tween 20 before storage at 4°C. Samples were diluted in buffer and 100 µl/well were incubated overnight at 4°C. The wells were washed three times in TBS/Tween 20 and the bound MASP-3 was detected by incubation with 100 µl biotinylated rabbit 7932 anti MASP-3 antibody (see table 2.1 for details) diluted 1/3000 in TBS/Tween 20 with 0.5 M NaCl, 1mM EDTA, 1 mg HSA/ml and 100 µg heat aggregated normal human IgG (ΔnIgG, State Serum Institute. Cat. No. 418293)/ml and 100 µg heat aggregated HSA (ΔHSA)/ml. After 4h incubation, the plate was washed three times in TBS/Tween 20 and incubated 1h with 100 µl Eu^{3+}-streptavidin diluted 1/2000 in TBS/Tween 20 with 25 µM EDTA. After a final wash the amount of bound europium could be measured by adding 200 µl enhancement buffer (Wallac Oy, Turku, Finland) to each well followed by incubation for 5 min. on a shaker and measured on a fluorimeter (Wallac). The assay was evaluated in respect to matrix influence and potential false positive signalling and found useful for quantitative determination of MASP-3 concentrations in human samples as well as for the concentration of recombinant human MASP-3 samples.

4.2.2 Activation of recombinant MASP-3

Supernatant from the stable cell line clone 3.3 (Chapter 2.3.3) expressing recombinant MASP-3 was incubated 12h at 37°C in eppendorf tubes. To samples of 200 µl supernatant were added 10 mM Ca^{2+}, 10 mM EDTA or an equal volume of water. The samples were separated by 6 - 18% SDS-PAGE and analysed by Western blotting. The Western blot was blocked in TBS with 1 mg HSA/ml and developed for 2h with rat 97 anti-MASP-3 B chain antibody (see table 2.1 for details) diluted 1/3000 as primary antibody and after a wash incubated for 2h with HRP-labelled rabbit anti-rat antibody diluted 1/4000 as secondary antibody. After a final wash the blot was incubated with 20 ml homemade ECL and developed on film. The antibodies were diluted in TBS/Tween 20 with 1 mM EDTA, 1 mg HSA/ml and 100 µg ΔHSA and ΔnIgG /ml.

4.2.3 Glycosylation of MASP-3

The characteristic sequences of potential N-linked glycosylation sites are: -Asn-Xaa-Ser- or -Asn-Xaa-Thr-. The Xaa residue can vary, but is never a Pro, and Pro cannot immediately follow the Ser or Thr residue (Bause E. 1983). There are seven potential N-linked glycosylation sites in the primary sequence of MASP-3, four in the A chain and three in the B chain.

Recombinant MASP-3 was analysed for glycosylation using the Emerald 300 glycoprotein gel staining kit (Molecular Probes, Leiden, The Netherlands Cat. No. P-21855). The kit included material to stain glycoprotein and total protein after separation by SDS-PAGE. Samples could be analysed under reduced or non-reducing conditions. The protocol supplied by the manufacture (Molecular Probes) was used for the experiment as below.

After electrophoresis, the proteins separated in the gel were fixed by incubating the gel for 30 min. in 50 ml of 50% (v/v) methanol diluted in distilled water. The fixation step was repeated to ensure all of the SDS was washed out of the gel. After a wash with 3% (v/v) glacial acetic acid in water the gel was incubated with oxidisation solution (component C from kit) for 30 min. at RT with agitation. The gel was washed three times in 3% (v/v) glacial acetic acid in

water before incubation with the staining solution (component A and B from kit) for 2h at RT with agitation. Finally the gel was washed three times in 3% (v/v) glacial acetic acid in water. The glycosylated proteins and peptides could be visualised under UV light and photographed. Subsequently the gel could be stained for total protein content by incubation in 15 ml undiluted Emerald 300 staining solution for 4h at RT. To reduce background fluorescence the gel was washed in 10% (v/v) ethanol, 7% (v/v) acetic acid diluted in water for 30 min. at RT and then rinsed with water. The total protein content in the gel could be visualised under UV light and photographed.

4.2.4 Deglycosylation of recombinant MASP-3

Purified recombinant MASP-3 from the stable cell line clone 3.3 (chapter 2.3.3) was de-glycosylated by incubation of three aliquots of 5 µl (~1.25 µg) recombinant MASP-3 with 0.5, 0.1 or 0 Units of N-glycosidase F (PNGase F from Roche, Basel, Switzerland Cat. No. 903-337) diluted in TBS/Tween 20 to a total volume of 50 µl. The samples were incubated at 37°C for 3h. The samples were analysed non-reduced in duplicate on SDS-PAGE and by Western blotting.

The membrane was blocked in TBS with 1 mg HSA/ml and cut in two pieces. One piece was developed for 2h with rat 95 anti-MASP-3 link region antibody (see table 2.1 for details) diluted 1/3000 as primary antibody and after a wash incubated 2h with HRP-labelled rabbit anti-rat antibody diluted 1/3000 as secondary antibody. The second part of the membrane was developed with rabbit 7931 anti-MASP-3 C-terminal antibody (see table 2.1 for details) diluted 1/2000 for 2h and after a wash with secondary antibody HRP-labelled goat anti-rabbit diluted 1/3000. After a final wash the blots were incubated and developed on film. The antibodies were diluted in TBS/Tween 20 with 1 mM EDTA, 1 mg HSA/ml and 100 µg ΔHSA and ΔnIgG /ml and all incubations were done at RT.

4.2.5 MASP-3 and protease activated receptors (PARs)

Protease-activated receptors (PARs) are G protein-coupled receptors that are activated by proteolytic cleavage of their N terminus (Macfarlane *et al.* 2001). PAR-1, PAR-2 and PAR-4 expressing stable cell lines from Shaun Coughlin's laboratory at UCSF were analysed by calcium flux and phosphoinositide (PI) hydrolysis for activation by different complement components including recombinant MASP-3 (Gilberto *et al.* 1999). The experiments were performed with the appreciated help of Matt Ludeman, UCSF.

For the Ca^{2+}-flux experiments, cells expressing PAR were cultured and split one day prior to the experiment. Cells were washed in 10 ml PBS (Phosphate Buffered Saline, pH 7.4, 140 mM NaCl (Merck Cat. No. 6404), 2.7 mM KCl (Merck, Cat. No. 116101), 1.5 mM KH_2PO_4 (Merck, Cat. No. 104873) and 8.1 mM Na_2HPO_4 (Merck Cat. No. 106559) and incubated in PBS containing 5 mM EDTA for 20 min. at 37°C. Cells were washed in RPMI 1640 medium and re-suspended in medium containing 0.1 % (v/v) bovine serum albumin (BSA), 5 mM EDTA and 25 mM Hepes. Fura 2AM (Molecular Probes, Leiden, The Netherlands) was added to each cell type in a concentration of 4 µg/ml medium and incubated in darkness at 37°C for 30 min. with agitation every 5 min. After the cells had been washed twice in medium containing 0.1 % (v/v) BSA, 5 mM EDTA and 25 mM Hepes, they were counted and re-suspended in medium containing 0.1 % (v/v) BSA and 25 mM Hepes to 2 x 10^6 cells/ml. The cell preparations were then kept on ice until analysed. In each Ca^{2+}-flux experiment 1 ml of the cell preparation was loaded in plastic cuvettes that were placed in a fluorimeter. The fluorescence was measured over time and the effect of additives monitored. Triton X-100 was added for lysis of the cells for maximum calcium release and by addition of EDTA, calcium was cheleated giving minimum fluorescent reading (Covic *et al.* 2000).

For the phosphoinositide hydrolysis experiments, cell lines expressing PAR were cultured and subsequently labelled with ^{3}H-inositol. Cells were washed in serum free medium, counted and re-suspended to a concentration of 10^6 cells/ml. Radioactively labelled ^{3}H-inositol was added to a final concentration of 2 µCi/ml and cells were incubated overnight at 37°C. Cells were washed 3 times in serum free media and radioactive waste was appropriately disposed off. The different agonists were added to 0.5 ml cells diluted in serum free medium containing 20 mM LiCl and samples were made in duplicate. The final concentration of the different

agonists were 1 µg/ml. Samples were incubated at 37°C for 2h then aspirated and 0.5 ml of 20 mM ice-cold formic acid was added, followed by incubation for 1h at 4°C. Each sample were loaded on a 1 ml resin column and washed with several volume of 40 mM NH_4COOH and eluted with 4 ml 2 M NH_4COOH containing 0.1 M formic acid followed by 5 ml water. The columns were eluted into labelled individual scintillation tubes (10 ml Aquasol Z). The agonist/receptor activity was reflected in the amount of radioactive material bound to the column and subsequently eluted.

4.2.6 In situ hybridisation on frosen sections from mice

To examine the expression of MASP-1 and MASP-3 mRNA in mice, 35S-labelled sense and antisense RNA was transcribed from rat MASP-1 and MASP-3 B chain cDNA and probed on cryo sections of new born mice. Pre-hybridisation was performed on sections at RT by incubation with 4% (v/v) paraformaldehyd in PBS for 60 min. After washing three times for 10 min. in PBS the slides was incubated in 0.4% (v/v) Triton-X-100 for 10 min. followed by a rinse in PBS and subsequently in water. The sections were acetylated in TEA buffer (0.1 M triethanolamine in water, pH 8.0 with addition of 0.25% (v/v) acetic anhydride immediately prior of use) for 10 min. The slides were rinsed in PBS and water and finally incubated in first 50% (v/v) for 30 sec. and then 70% (v/v) ethanol for 30 sec. The slides were left to dry and were stored at -20°C. Radioactive probes were synthesized by in vitro transcription with T3, T7 or Sp6 RNA polymerase on vectors containing cDNA encoding either mouse MASP-1 or mouse MASP-3. The MASP-3 specific probe was an 800 bp anti-sense PCR product made using SP6 RNA polymerase on vector plasmid with cDNA encoding the B chain of rat MASP-3 digested with Nco1. The MASP-1 was 200 bp sequences amplified with T3 RNA polymerase on vector with cDNA encoding the B chain of MASP-1, which had been digested with Xba1. A control sense probe was amplified on the same construct digested with Cla1 using a T7 RNA polymerase resulting in a probe of 200 bp. In vitro transcription of the linearized template and labelling of the cRNA using S_{35}-dUTP (NEN, Boston, MA) was performed by mixing 1 µl of 100 mM DDT, 1 µl buffer, 15 µl S_{35}-dUTP, 4.5 µl dNTP (des-dUTP, 3.3 mM), 0.5 µl RNAse-inhibitor, 1 µl polymerase with 5 µl template (1 µg) per slide. The three mixtures were incubated 90 min. at 37°C and additional 15 min. with 0.5 µl DNAse (10 units/µl) at the same temperature. The mixtures were incubated 5 min. at 60°C followed

by addition of 20 µl of 0.2 M Na_2CO_3, pH 10.2. The probes were purified by loading the mixture on NucTrash-columns from Stratagene (La Jolla, CA) and eluting with STE buffer (10 mM NaCl, 1 mM EDTA and 2 mM Tris in water, pH 7.5). Appropriate laboratory practise and precautions was exercised with respect to the radioactive material and wastes.
Each frozen section was incubated overnight with 50 µl probe diluted 1/10 in hybridisation buffer with 20 mM DDT and protected by a cover slide in a humidified container.
Hybridisation buffer contained 0.6 M NaCl, 10 mM Tris, 1 mM EDTA, 0.05% (w/v) t-RNA (20 mg/ml), 1x Denhardt's, 5% (w/v) dextransulfate, 100 µg sonicated salmon sperm DNA/ml in formamide diluted 1-1 in water. After hybridisation the slides were placed in a trough with a stir bar and washed in 2 x SSC (diluted from 20 x SSC containing 3 M NaCl and 0.3 M Na_3-citrate) at RT for 20 minutes and washed in 1 x SSC at RT for 20 minutes.
Sections were treated with 0.2 µg/ml RNAse A in 2 x SSC at 37°C for 40 min. and then washed in 1 x SSC for 20 min., 0.5 x SSC for 20 min. and 0.2 x SSC for 20 min. at RT. Next the slides were washed twice in 0.2 x SSC at 60°C for 30 minutes each time followed by two times in 0.2 x SSC at RT for 15 min. The slides were washed in ddH_2O at RT for 15 min. and then sequential exposure to 50% (v/v), 70% (v/v), 80 (v/v) and 98% (v/v) (Schäfer *et al.* 1995). The slides could then be developed by placing X-ray film on top.

4.2.7 Immunohistochemistry on sections of human tissues

Paraffin treated slides with sections of human tissues multi-organ slides were a gift from Ole Nielsen, University Hospital of Odense, Denmark. The slides were de-paraffinised by incubation in 70°C vegetable oil for 15 min. followed by three incubations in 99% (v/v) ethanol, one incubation in 96% (v/v) ethanol and one incubation in 70% (v/v) ethanol. Each incubation was for 3 min. Slides were then incubated for 30 min. at RT in TBS/Tween 20 with 100 µg ΔHSA and ΔnIgG/ml in a humidified chamber. The slides were rinsed in PBS/Tween 20 and incubated 15 min. with 3% (v/v) H_2O_2 in methanol followed by three times wash in PBS/Tween 20.
Primary antibodies (biotinylated) were diluted in TBS/Tween 20 with 100 µg ΔHSA and ΔnIgG/ml then added to the individual slides and covered with parafilm. The slides were incubated 1h at RT in a humidified container. After three washes in PBS/Tween 20 the slides

were developed using the Vectastain ABC kit (Vector, Burlingame, CA, Cat. No. PK-6200) and the protocol supplied by the manufacturer was used.

4.2.8 Gel filtration chromatography (GPC)

Serum proteins were separated by gel filtration on a Superose 6 HR 10/30 column (Amersham Cat. No. 17-0673-01) in TBS/Tween 20 with 1 M NaCl and 10 mM EDTA or 5 mM Ca^{2+}. To compare recombinant MASP-3 with native MASP-3, 1 µg recombinant was added to serum prior to GPC. The experiment was performed on MBL sufficient and MBL deficient serum. Serum samples of 80 µl were added to 20 µl TBS/Tween 20 with 4.25 M NaCl, 50 mM EDTA or 25 mM Ca^{2+} and +/- 5 µg recombinant MASP-3/ml. mixtures were incubated 1h at RT before fractionation was started. Fractions of 250 µl were collected in 96 well plates, which had been blocked by incubation with 300 µl TBS/Tween 20 buffer for 30 min. Native serum or EDTA plasma MASP-3 from the same donor was analysed by GPC on a Superose 12 column. Samples of 240 µl were diluted in 60 µl TBS/Tween 20 with 50 mM EDTA and loaded on the column. Fractions of 250 µl were collected in pre-blocked 96 well plates and analysed using the MASP-3 sandwich TRIFMA. The running buffer used was TBS/Tween 20 with 10 mM EDTA. Both columns were used at RT with a flow rate of 0.5 ml buffer/min.

4.2.9 Chromogenic substrates

Chromogenic substrates are small peptides often designed to resemble specific natural substrates. The peptides are made synthetically with a chemical group attached, which upon cleavage changes colour. The colour reaction is detectable at a given wavelength (depending on the chromogene) and proportional to the proteolytic activity. Inhibition studies can be conducted in systems where a specific enzyme cleaves a chromogenic substrate.
Recombinant MASP-3 was analysed using chromogenic substrates (CS. from Chromogenix, Milano, Italy) resembling potential substrates from the coagulation system. The dry chromogenic substrates was dissolved in distilled water to a concentration of 1 mM. Enzymes were diluted in TBS/Tween 20 with 5 mM Ca^{2+} in wells pre-incubated in the same buffer. The chromogenic substrates was added to a final concentration of 0.1 mM. The plate was incubated at different temperatures and colour reaction (enzymatic activity) detected

continuously by reading the OD at 405 nm or stopped at a given time by addition of acetic acid (5% v/v). Four CS compounds were initially tested with respect to MASP-3 cleavage, using thrombin as positive control enzyme. The four compounds were: S2222 (FXa substrate), S2238 (thrombin substrate), S2251 (plasmin substrate) and S2586 (chymotrypsin substrate). The chromogenic substrate S2302 (kallikrein/FXII-a substrate) was tested to evaluate the potential role of MASP-3 in the activation of the coagulation system through the contact system. The chromogenic substrates S2302 and S2222 were tested with different dilutions of different protease inhibitors to evaluate inhibition of MASP-3 (see table 4.1 for details).

Name	Formula	Substrate for
S2222	Bz-Ile-Glu(γ-OR*)-Gly-Arg-pNA•HCl (N-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-Larginine-p-nitroaniline hydrochloride and its methyl ester)	Factor Xa
S2238	HD-Phe-Pip-Arg-pNA•2HCl (HD-Phenylalanyl-L-pipecolyl-Larginine-p-nitroaniline dihydrochloride)	Thrombin
S2251	HD-Val-Leu-Lys-pNA•2HCl (HD-Valyl-L-leucyl-L-lysinep-Nitroaniline dihydrochloride)	Plasmin
S2302	HD-Pro-Phe-Arg-pNA•2HCl (HD-Prolyl-L-phenylalanyl-Larginine-p-nitroaniline dihydrochloride)	Kallikrein and FXIIa
S2586	MeO-Suc-Arg-Pro-Tyr-pNA•HCl (3-Carbomethoxypropionyl-Larginyl-L-prolyl-L-tyrosinep-nitroaniline hydrochloride)	Chymotrypsin

Table 4.1. The formula of the five chromogenic substrates S2222, S2238, S2251, S2302 and S2586 are shown together with the plasma enzymes capable of cleaving it. The OR* in S2222 indicates the 50/50 mixture of –H and $-CH_3$ groups in the chromogenic substrate.

The chromogenic substrates can be cleaved by its natural enzyme and the difference in OD_{405} between the p-nitroaniline (pNA) formed and the original substrate correlates to the enzymatic activity of the protease analysed.

4.2.10 Association and dissociation of the MBL-MASP-3 complex

The association and dissociation of the MBL/MASP-3 complex was investigated on 96 well maxisorp (Nunc, Denmark) plates coated directly with 100 µl/well of recombinant MBL (1 µg/ml) for 4h at RT. The plates were blocked by incubation with 200 µl/well TBS with 1 mg HSA/ml for 30 min. at RT. The plates were washed 3 times in TBS/Tween 20/Ca^{2+} before use or storage at 4°C.

The dissociation of the complex was investigated by incubating the MBL coated wells with 100 µl/well recombinant MASP-3 (250 ng/ml) in TBS/Tween 20 buffer containing different concentrations of EDTA and NaCl. The wells were washed three times in TBS/Tween 20/Ca^{2+} buffer before being incubated with 100 µl/well buffer containing dilutions of EDTA in TBS/Tween 20 for 30 min. at RT to establish the dissociation of the complex. The experiment was furthermore made using buffers containing a high salt concentration in combination with different EDTA concentrations. The plate was developed by incubation with 100 µl/well biotinylated rabbit 7932 anti-MASP-3 antibody (see table 2.1 for details) diluted 1/1500 in TBS/Tween 20/Ca^{2+} buffer for 2h at RT. After a wash in TBS/Tween 20/Ca^{2+} buffer the plates were incubated with 100 µl/well Eu^{3+}-labelled streptavidin diluted 1/1000 in TBS/Tween 20 with 25 µM EDTA for 1h at RT. After a final wash in TBS/Tween 20/Ca^{2+} buffer each well were added 200 µl enhancement buffer (Wallac Oy, Turku, Finland) followed by incubation for 5 min. on a shaker before the plate was measured on a fluorimeter (Wallac).

The dissociation characteristics of the MBL/MASP-3 complex was further investigated by incubating 100 µl/well recombinant MASP-3 (250 ng/ml) in TBS/Tween 20/Ca^{2+} in MBL coated plate for 2h at RT. After a wash the wells were incubated with buffers containing dilutions of EDTA for different periods of time (except the reference curve of recombinant MASP-3 dilutions). The plates were developed as described above.

The association of the MBL/MASP-3 complex was investigated by incubating the MBL coated wells with 100 µl/well recombinant MASP-3 (250 ng/ml) in TBS/Tween 20 buffer containing different concentrations of EDTA or Ca^{2+} overnight at 4°C. The wells were washed three times in TBS/Tween 20/Ca^{2+} buffer before being incubated with 100 µl/well biotinylated rabbit 7932 anti-MASP-3 diluted 1/1500 in TBS/Tween 20/Ca^{2+} buffer for 2h at

RT. After wash in TBS/Tween 20/Ca^{2+} buffer the plates were incubated with 100 µl/well Eu^{3+}-labelled streptavidin diluted 1/1000 in TBS/Tween 20 with 25 µM EDTA for 1h at RT. After a final wash in TBS/Tween 20/Ca^{2+} buffer each well was added 200 µl enhancement buffer (Wallac Oy, Turku, Finland) followed by incubation for 5 min. on a shaker before the plate was measured on a flourimeter (Wallac).

4.2.11 MASP-3 structural modelling

On the basis of published secondary and tertiary structural data on other serine proteases and domains hereof, modulation of potential MASP-3 structural features was made. Computer programs available on the web were used to predict folding and alignments. Computer modelling of MASP-3 and MBL/MASP complex was performed using Rasmol v.2.6 and RasWin v.2.6 obtained on the internet (http://www.bernstein-plus-sons.com/software/rasmol/). The molecules used were protein data bank (PDB) files for MASP-2 (Feinberg *et al.* 2003, PDB: 1NT0), MBL (Weis *et al.* 1994, PDB: 1RTM), C1r fragment (Budayova-Spano *et al.* 2002, PDB: 1GPZ) and collagen structure (Kramer *et al.* 1999, PDB: 1BKV).
Quaternary structures of MASP-3 in complex with MBL were made on the basis of the homology to MASP-2 (Chen and Wallis 2001).

4.3 Results

4.3.1 Activation of recombinant MASP-3

Recombinant MASP-3 from the stable cell lines was found to be non-activated in the culture supernatant (Figure 2.4). When the supernatants were subjected to purification on columns or stored, recombinant MASP-3 became activated. After 40 days at 4°C, purified recombinant MASP-3 (in 2 mM Tris with 1 mM EDTA and 0.4 M NaCl) was estimated to be 50% activated (Figure 4.1 lane 4). As shown in Figure 4.1, activation of MASP-3 was faster in buffer containing calcium.

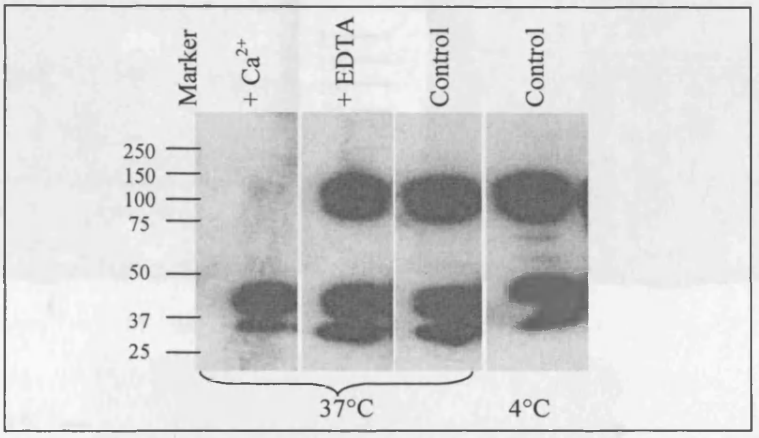


Figure 4.1. Four lanes from a Western blot produced for analysing the activation conditions of recombinant MASP-3. Lane M was loaded with 12 µl prestained protein marker and the protein lanes are indicated with lines. Equal amounts of recombinant MASP-3 were loaded in lane 1-4. The sample in lane 1 was added 10 mM Ca^{2+}, Sample in lane 2 was added 10 mM EDTA and the sample in lane 3 was added an equal volume of water. All three samples were incubated 12h at 37°C. A sample of recombinant MASP-3 diluted with water was incubated at 4°C and included as reference control in lane 4. The four samples were analysed under reducing conditions. The Western blot was developed using the polyclonal rabbit 7932 anti MASP-3 B chain antibody (chapter 2.2.7).

The intensity of the MASP-3 B chain in lane 1 of the Western blot Figure 4.1 was not found to be stronger compared with lane 2-4. It was anticipated that the band corresponding to the B chain of 100% activated MASP-3 (lane 1), would be more prominent compared with less activated protein (lane 2-4). One explanation could be further degradation of the protein in the presence of calcium.

4.3.2 Glycosylation

The potential N-linked glycosylation of MASP-3 was investigated. With an observed molecular weight of 105 kDa compared to a calculated weight of 82 kDa (as described in chapter 1.14), heavy glycosylation was likely due to seven potential N-linked glycosylation sites. Two 6-18% SDS-PAGE were loaded identically and electrophoresis conducted. One gel was Western blotted and the other stained for glycoproteins. The results are shown in Figure 4.2.

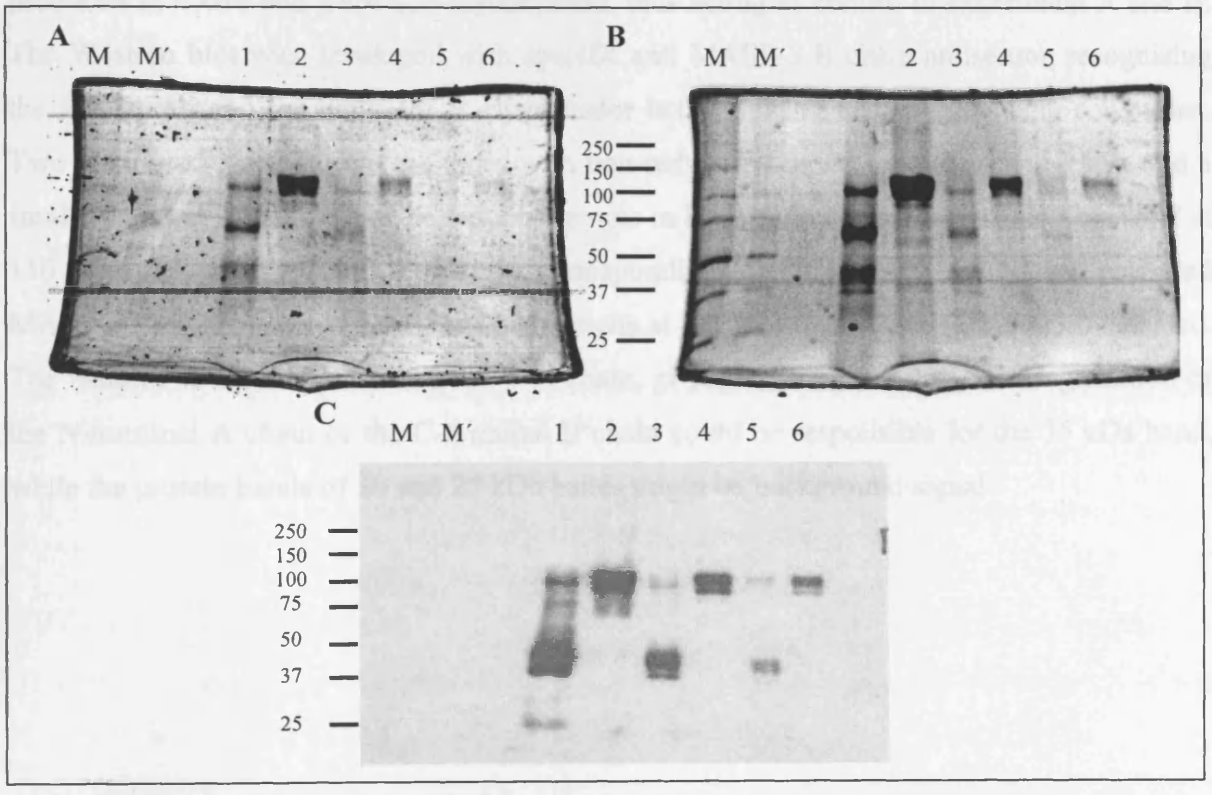


Figure 4.2. The gel (A and B) and the Western blot (C) were loaded with 5 µl unstained protein marker (BioRad) in lane M and 1 µl of the same marker in lane M'. Lane 1, 3 and 5 were loaded with reduced recombinant MASP-3, 10, 2 and 0.4 µl respectively. Lane 2, 4 and 6 were loaded with non-reduced recombinant MASP-3, 10, 2 and 0.4 µl respectively. The gel A was stained for glycoproteins and the same gel was subsequently stained for total protein content in B. The Western blot was developed using rat 97 anti-MASP-3 B chain antiserum and detected with a secondary HRP-labelled rabbit anti-rat antibody.

SDS-PAGE gels stained for glycoproteins (A) were developed for 10 min. and after subsequent staining for total protein content (B) for 15 min. under UV light. The pictures were recorded by a digital camera (FlouroS, BioRad, Denmark). The gel would dry and twist when developed for longer periods of time. The result of the staining for glycoproteins was

three clear bands at approximately 110, 70 and 40 kDa in lane 1 which contained a reduced sample of recombinant MASP-3 and corresponded to MASP-3 full-length, activated A chain and B chain, respectively. In the non-reduced lane 2 one broad band of approximately 110 kDa was detected corresponding to full-length MASP-3. The band in lane 2 could be the result of two bands with a small difference in molecular weight. The same bands could be identified in lane 3 and 4 but were barely visible in lane 5 and 6 after 10 min. of exposure with UV light. Staining for total protein content enhanced the intensity of the glycoprotein and the bands in lane 5 and 6 could be detected. The molecular marker proteins were produced in E.coli and were non-glycosylated, thus acting as control in experiment A and B. The Western blot was developed with specific anti MASP-3 B chain antiserum, recognising the full-length and the activated B chain under both reducing and non-reducing conditions. Two bands were detected in the lanes with non-reduced samples, one at 105-110 kDa and a smaller band at 95-105 kDa. The reduced sample in lane 1 showed several bands. One band at 110 kDa and a broad band at 40 kDa corresponding to full length MASP-3 and activated MASP-3 B chain, respectively. Four other bands at 75, 35, 30 and 25 kDa were also detected. The band at 75 kDa could be MASP-1 A chain, giving a background signal. Degradation of the N-terminal A chain or the C-terminal B chain could be responsible for the 35 kDa band, while the protein bands of 30 and 25 kDa bands might be background signal.

4.3.3 Deglycosylation of recombinant MASP-3

Recombinant MASP-3 could be de-glycosylated using N-glycosidase F. Figure 4.3 below shows the result of the deglycosylation analysed by Western blotting.

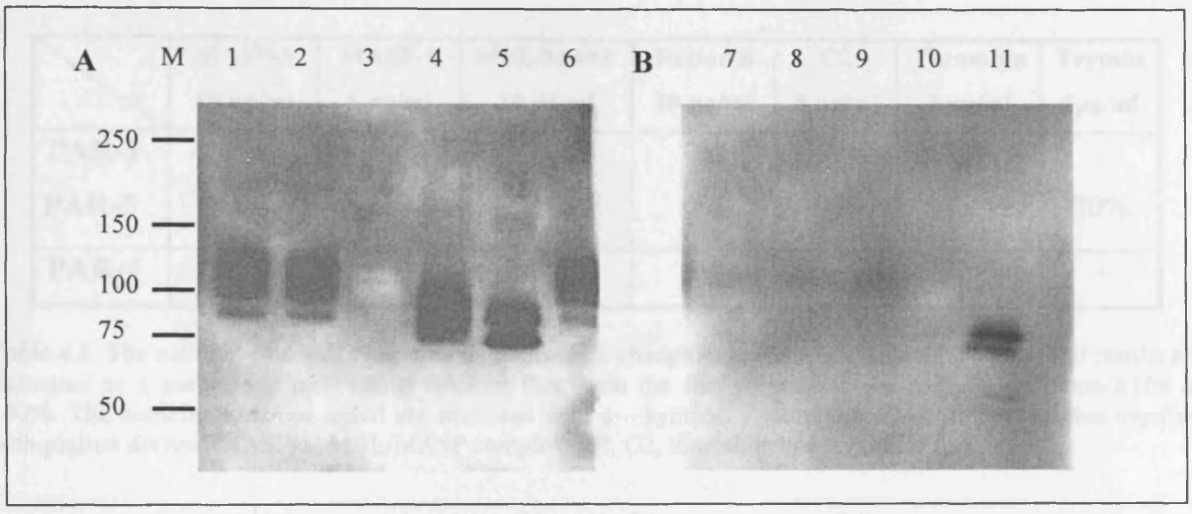


Figure 4.3. The Western blot A was developed using anti MASP-3 C-terminal B chain specific antibody (see table 2.1 for details) and blot B was developed with anti-MASP-3 link region (A chain) specific antiserum. Lane M was loaded with 12 µl pre-stained protein marker (BioRad). Lane 1, 8 and 2, 9 were loaded with 30 µl recombinant MASP-3 pre-incubated 2h with 0.5 and 1.0 µg C1-inhibitor, respectively. Lane 3 and 10 represented 1 µg C1-inhibitor diluted in water. Recombinant MASP-3 (30 µl) pre-incubated with 0.1 U PNGase was loaded in lane 4 and recombinant MASP-3 incubated with 0.5 U PNGase was loaded in lane 5 and 11. Untreated recombinant MASP-3 was loaded in lane 6. All samples were non-reduced.

By comparing recombinant MASP-3 in lane 6 with PNGase treated protein, a clear difference in molecular weight was observed. Untreated MASP-3 was observed as two bands of 110 and 90 kDa, while the deglycosylated MASP-3 had a molecular weight of 80 and 70 kDa.

Only a faint band of MASP-3 could be obtained by development with anti MASP-3 A chain specific antiserum (Figure 4.3 B lane 7, 8 and 9), while a clear signal was obtained in lane 11 containing de-glycosylated MASP-3. The band was determined to consist of two overlaying bands of 80 and 75 kDa, representing full length and possibly partial degraded recombinant MASP-3.

4.3.4 MASP-3 and protease activated receptors (PARs)

The calcium flux experiments were conducted on fibroblasts expressing either PAR-1, -2 or -4. A range of complement proteases and complexes, apart from recombinant MASP-3, were tested and the result summarised in the table 4.1 below.

	MASP-3 10 µg/ml	MASP-1 5 µg/ml	MBL/MASP 10 µg/ml	Factor B 10 µg/ml	C2 5 µg/ml	Thrombin 2 µg/ml	Trypsin 5µg/ml
PAR-1	0%	-	-	0%	0%	30%	-
PAR-2	0%	0%	0%	0%	0%	-	20%
PAR-4	0%	-	-	-	-	25%	-

Table 4.1. The calcium flux was measured as fluorescent change over time. In this table the graphical results are estimated as a percentage increase of calcium flux, with the flux respond to the addition of Triton-X100 as 100%. The combinations not tested are indicated with a – symbol. Recombinant MASP-3 was tested together with plasma derived MASP-1, MBL/MASP complex, FB, C2, thrombin, and trypsin.

The conclusion of the result from calcium flux on the PAR-1, 2 and 4 proteins, was that recombinant MASP-3 had no influence on the activation of these receptors. The other complement proteins tested were also found to have no activating effect on the receptors in the experiment.

Thrombin was used as positive controls towards PAR-1/-4 and trypsin as positive control towards PAR-2.

The influence of MASP-3 on the receptors was subsequently tested in a phosphoinositide (PI) hydrolysis experiment and the result are summarised in Figure 4.7 below.

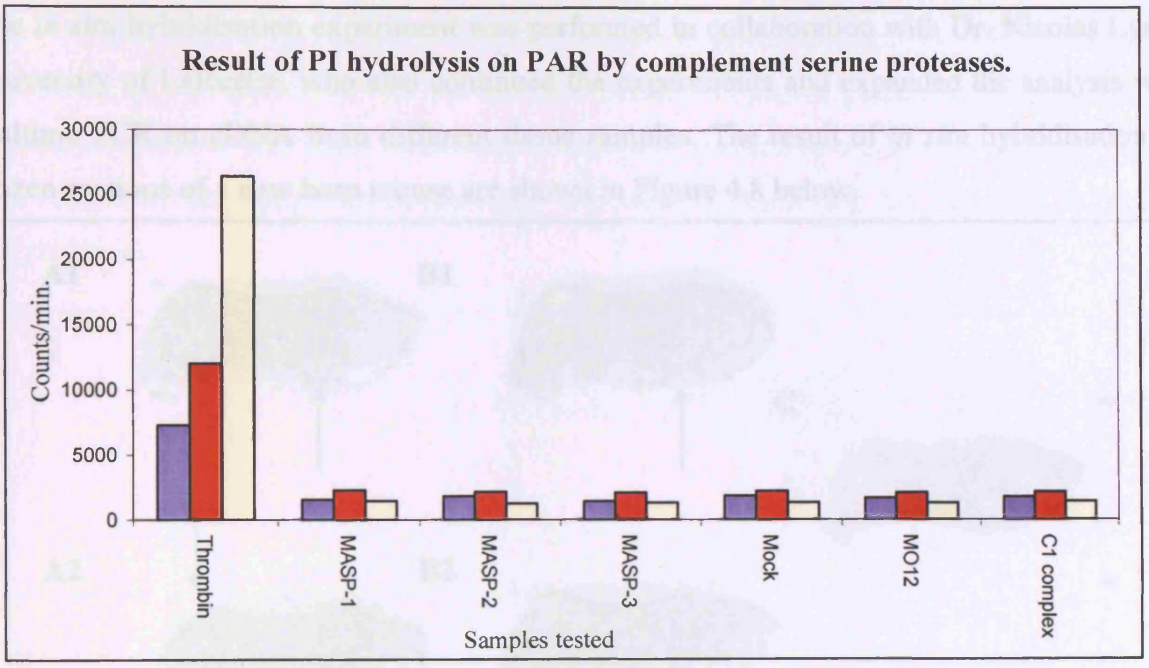


Figure 4.7. Cells expressing either PAR-1 (blue), PAR-2 (red) and PAR-4 (Yellow) were tested for activation by Thrombin, MASP-1, MASP-2, MASP-3, mock supernatant, MO12 (State serum institute, Denmark) or C1 complex (A kind gift from Sjoholm A, Lund, Sweden). Thrombin was selected as positive control and the mock supernatant as negative control.

The complement proteins tested in the PI hydrolysis experiment did not have any activating function on the PAR proteins. The result was validated by the observed activity of thrombin and supernatant from mock transfected cells.

4.3.5 *In situ* hybridisation on frozen sections from mice

The *in situ* hybridisation experiment was performed in collaboration with Dr. Nicolas Lynch University of Leicester, who also continued the experiments and expanded the analysis with realtime PCR on cDNA from different tissue samples. The result of *in situ* hybridisation on frozen sections of a new born mouse are shown in Figure 4.8 below.

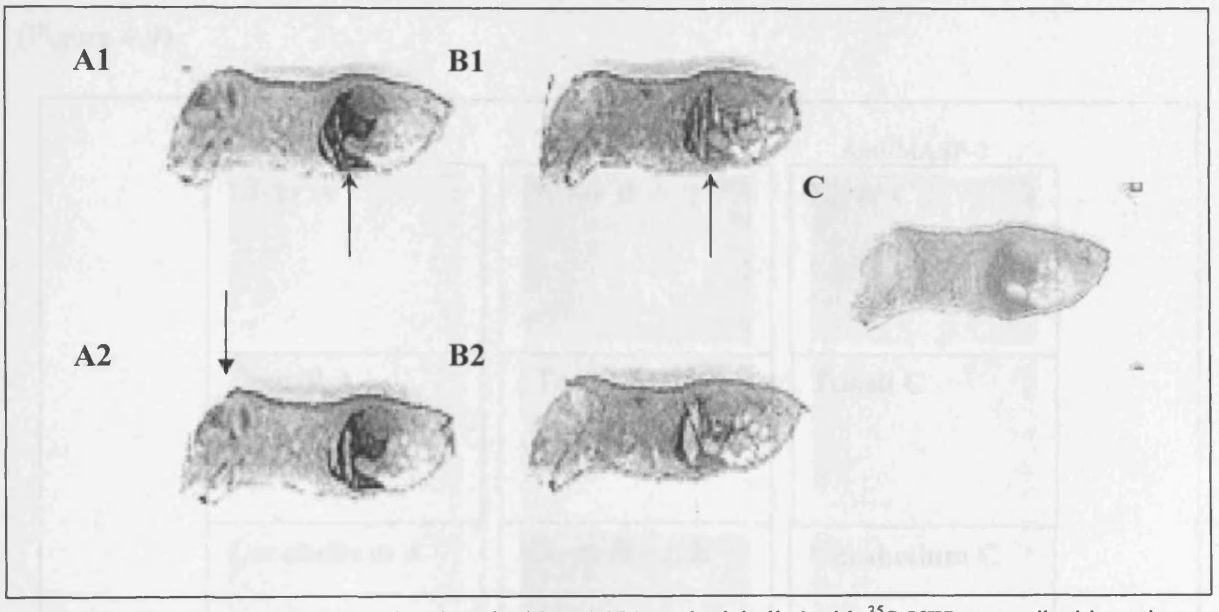


Figure 4.8. Slides A1 and A2 were incubated with a cRNA probe labelled with ^{35}S-UTP transcribed in anti-sense orientation from a c-DNA template representing the coding sequence for the B chain of MASP-3. Slides B1 and B2 were hybridised with an anti-sense c-RNA probe specific for the coding sequence for the B chain of MASP-1. As a control for the specificity of the hybridisation, the slide shown in C was hybridised with the MASP-1 specific probe transcribed in sense orientation. The slides were exposed for 2½ days on an X-ray film to obtain the pictures. The arrows on figure A and B1 indicate MASP mRNA production.

The predominant organ in which MASP-1 and MASP-3 mRNA is detected is the mouse liver (arrows on A1 and B1). A significant difference in the presence of MASP-1 or MASP-3 mRNA was observed in the mouse brain. The MASP-3 specific cRNA probe shows an abundant expression of mRNA in the brain of the mouse (arrow on slide A2). This observation triggered experiments towards identifying MASP-3 in the human brain while MASP-1 mRNA is not detectable in the same areas. The results of these experiments are shown in the next section (immunohistochemistry on sections from human tissue and in chapter 5).

4.3.6 Immunohistochemistry on sections of human tissue

The Vectastain ABC kit made use of avidin/biotin enzyme complex (ABC) formation, which upon addition of a substrate for the enzyme produced a brown colouring at the location of bound biotinylated antibody. Four tissue samples were selected out of forty one, to illustrate the results of the immunohistochemistry experiments conducted for MASP-3 localisation (Figure 4.9).

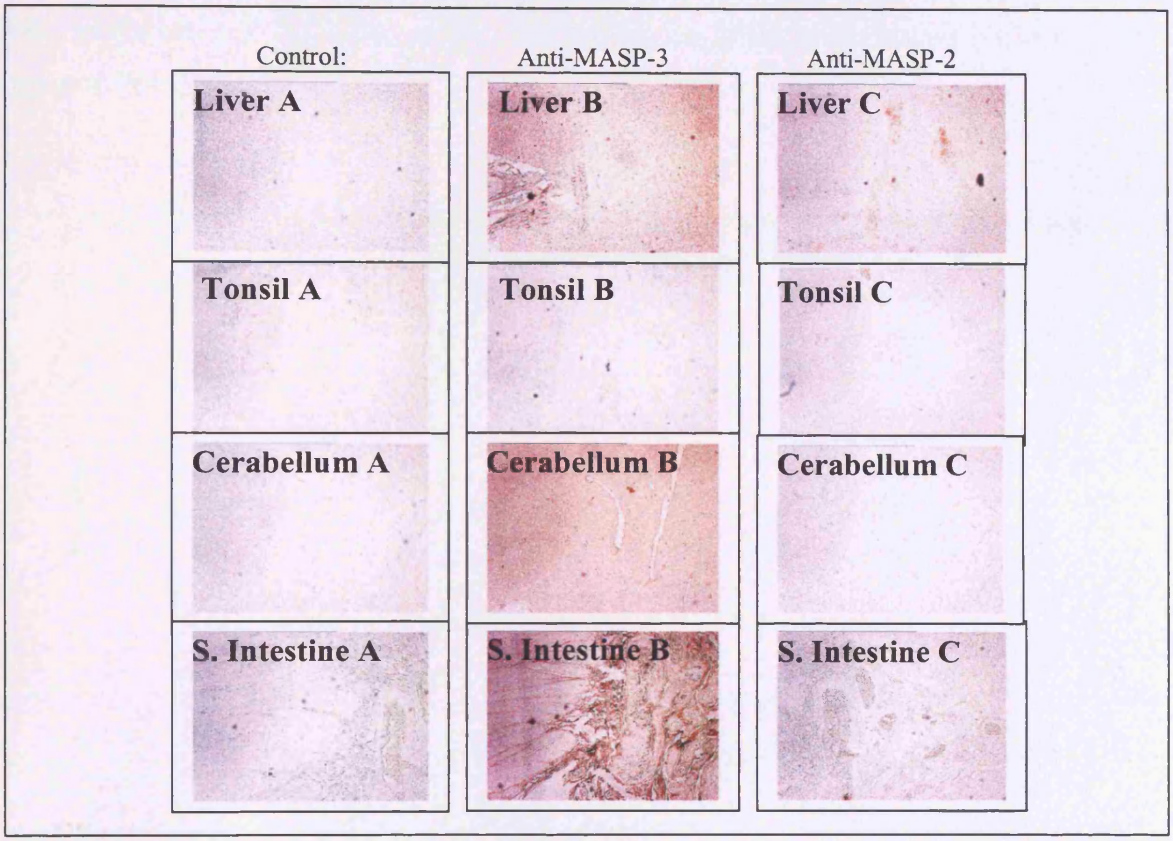


Figure 4.9. The pictures were taken using a digital camera connected to a microscope and they are all 200x magnifications of the original image. Liver, tonsil, cerebellum and small intestine (S. Intestine) were de-masked and incubated with control antibody (biotinylated rabbit anti-bovine conglutinin) (A), biotinylated Rabbit 7932 anti-MASP-3 B chain antiserum (B) and biotinylated monoclonal 6G12 anti-MASP-2 antibody (C) as primary antibody. The slides were subsequently developed using the Vectastain ABC kit. The pictures have been equally modified on the computer.

The images shown in Figure 4.9 are representative for MASP-3 immunohistochemical staining in the tissues shown. A strong brown colouring of especially cerebellum and small intestine were distinct for the slides incubated with anti-MASP-3 antiserum. Among the tissues, which were not stained by the anti-MASP-3 antiserum, were tonsil sections. This

4.3.7 Gel filtration chromatography result (GPC)

As part of the general characterisation, recombinant MASP-3 was compared to native MASP-3 from serum by GPC on a Superose 6 column. The recombinant MASP-3 was previously determined to be 105 kDa by SDS-PAGE and Western blotting (Dahl *et al.* 2001). Fractions were diluted 1-1 in TBS/Tween 20 with 10 mM EDTA, 1 M NaCl and 100 µg/ml ΔnIgG and 100 µg/ml ΔHSA/ml on the microtitre plates. The GPC experiment was performed on both MBL sufficient and deficient serum. The results on MBL sufficient serum are merged in Figure 4.10 below.

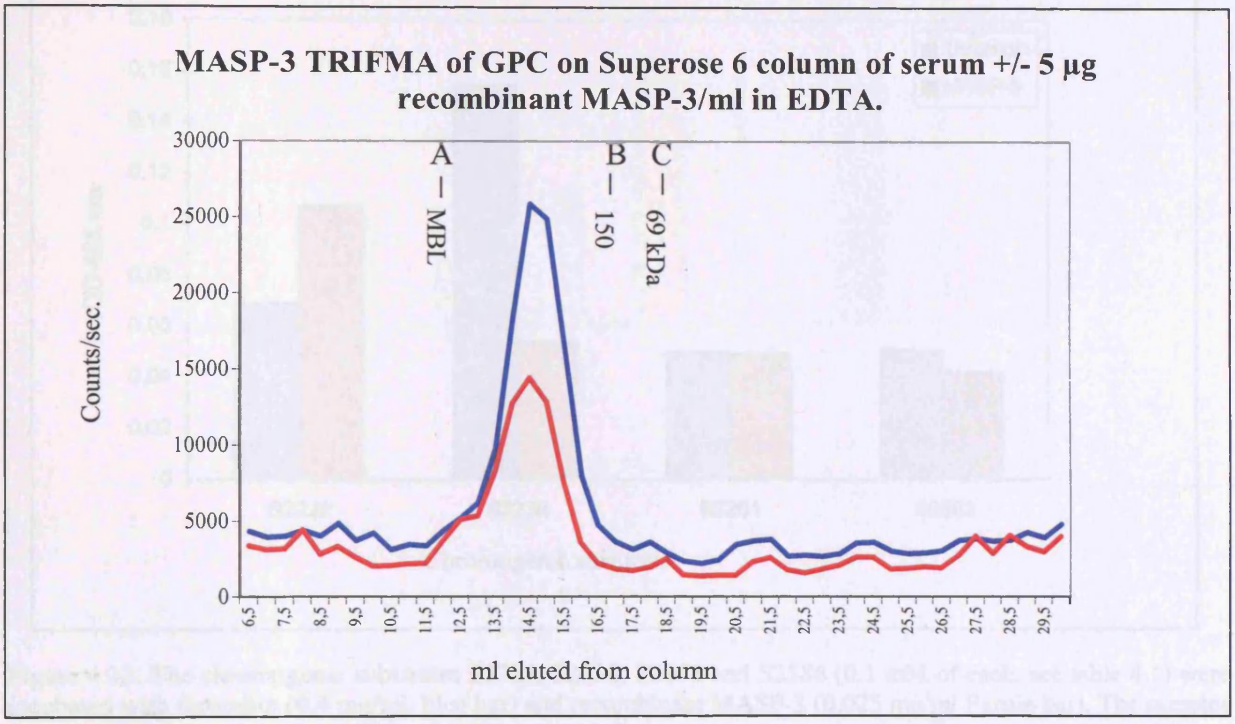


Figure 4.10. GPC fractions from two experiments were analysed using the sandwich MASP-3 TRIFMA. The red curve represents MASP-3 levels from 80 µl serum diluted in 20 µl TBS/Tween 20 with 4.25 M NaCl and 50 mM EDTA. The blue curve represents MASP-3 levels from 80 µl serum diluted in 20 µl TBS/Tween 20 with 4.25 M NaCl, 50 mM EDTA and 5µg recombinant MASP-3/ml. MBL was eluted after 12.0 ml, IgG after 17.5 ml and HSA after 19.0 ml indicated by the letters A,B and C, respectively. MBL levels were determined by MBL TRIFMA (not shown), IgG and HSA could be detected directly from the OD_{280}.

MASP-3 was eluted in a single peak after 14.5 ml at a position indicating a dimeric form when compared to C1r and C1s (Thiel *et al.* 2000). The recombinant MASP-3 was eluted at the same position as the native protein, suggesting recombinant and native MASP-3 form the

same major oligomer. Results on MBL deficient serum were similar and there was no indication that MBL levels influencing the results (data not shown).

4.3.8 Chromogenic substrates and inhibition

Recombinant MASP-3 was tested for enzymatic activity on four chromogenic substrates. The sample combinations were made in wells and OD measurements could be obtained at different time points or stopped at a given time (Figure 4.12).

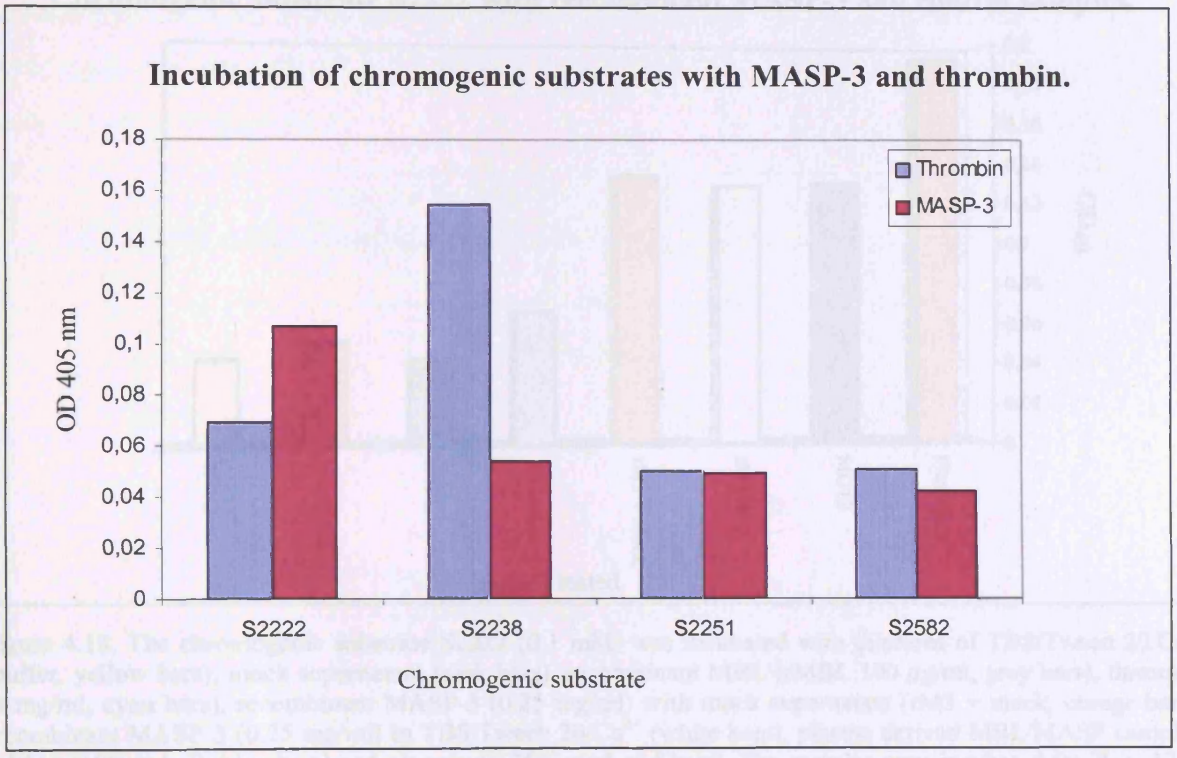


Figure 4.12. The chromogenic substrates S2222, S2238, S2251 and S2586 (0.1 mM of each, see table 4.1) were incubated with thrombin (0.4 mg/ml, blue bar) and recombinant MASP-3 (0.025 mg/ml Purple bar). The samples were incubated for 30 min. at 37°C and OD_{405} measured.

Recombinant MASP-3 was found to cleave the chromogenic substrate S2222 (see table 4.1 for details). The compound was designed as a substrate specific for FXa. The difference in concentration between MASP-3 and thrombin was 16 fold and MASP-3 cleaved the chromogenic substrate S2222 approximately twice as efficiently as thrombin, thus MASP-3 was estimated to be 30 times more efficient cleaving S2222 than thrombin. MASP-3 was found to cleave the other three substrates with a much lower efficiency and efforts were concentrated on the FXa substrate (Figure 4.13).

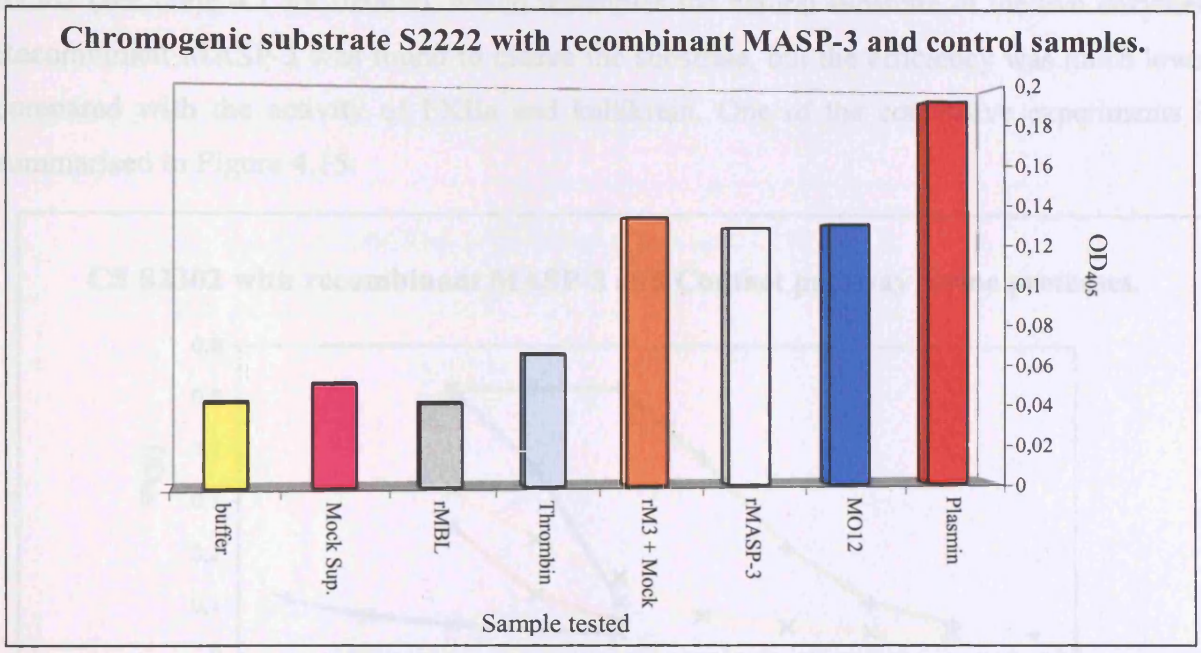


Figure 4.13. The chromogenic substrate S2222 (0.1 mM) was incubated with dilutions of TBS/Tween 20/Ca^{2+} (buffer, yellow bars), mock supernatant (pink bars), recombinant MBL (rMBL 100 µg/ml, grey bars), thrombin (4 mg/ml, cyan bars), recombinant MASP-3 (0.25 mg/ml) with mock supernatant (rM3 + mock, orange bars), recombinant MASP-3 (0.25 mg/ml) in TBS/Tween 20/Ca^{2+} (white bars), plasma derived MBL/MASP complex (MO12 (100 µg/ml), blue bars) and plasmin (1.25 mg/ml, red bars). The samples were incubated for 2h at 37°C and OD measured at 405 nm.

Dilutions of mock supernatant and recombinant MBL were found to have no enzymatic activity on S2222, while recombinant MASP-3 and MO12 were found to have a dose dependent activity towards the substrate. Plasmin was found to cleave S2222 approximately twice as efficiently as recombinant MASP-3, taking the difference in stock concentration into account. The enzymatic activity of thrombin on S2222 was confirmed to be low. The substrate was not tested using FXa due to the high cost of the protein. The natural substrate of FXa, prothrombin, was tested. Recombinant MASP-3 was incubated with prothrombin (Hyphen BioMed Cat. No. PP006B) at 37°C for 2, 6 and 16h and samples were analysed on

silver stained SDS-PAGE under both reducing and non-reducing conditions. No enzymatic activity of recombinant MASP-3 on the conversion of prothrombin into thrombin in any of the incubation experiments conducted was found (data not shown).

Focus was now on the initiation of the contact system (part of the haemostatic system) and the proteins involved in activation of coagulation through this pathway. The proteins analysed were High Molecular Weight Kininogen (HMWK), FXII and prekallikrein.

FXII and prekallikrein were initially investigated with the use of the chromogenic substrate S2302 (see table 4.1 for details), which resembles the natural substrate of the two enzymes. Recombinant MASP-3 was found to cleave the substrate, but the efficiency was much lower compared with the activity of FXIIa and kallikrein. One of the conclusive experiments is summarised in Figure 4.15.

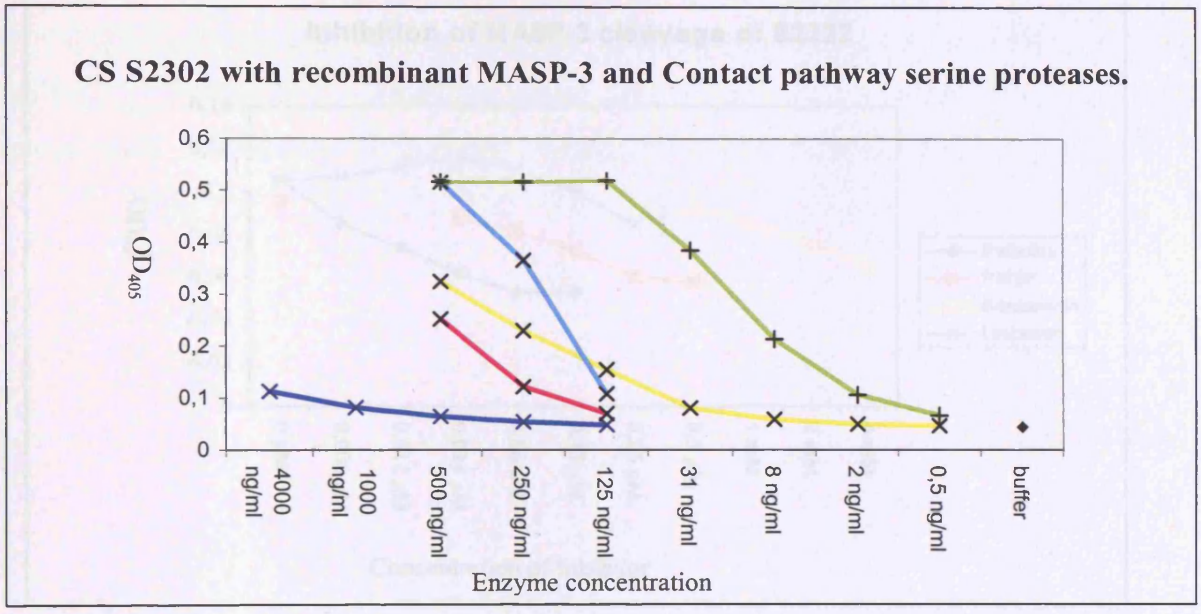


Figure 4.15. Incubation of S2302 with dilutions of recombinant MASP-3 (blue line), FXII (pink line), FXII-a (yellow line), prekallikrein (cyan line) and kallikrein (green line). A buffer control was included and indicated with a black diamond. The samples were incubated at 37°C and OD measured at various time points. The time for the result shown was 95 min.

It was evident that recombinant MASP-3 was much less effective in cleaving S2302 than FXII-a and kallikrein, respectively. Pre-incubations of recombinant MASP-3 with FXII or prekallikrein before addition of S2302 indicated that no activation of either the proteins was performed by MASP-3. Analysis of incubations on silver stained SDS-PAGE confirmed the result (data not shown). Potential cleavage of HMWK by MASP-3 was investigated by

incubating recombinant HMWK (Enzyme Research Laboratories Cat. No. 2HK1301) with dilutions of recombinant MASP-3 overnight at 37°C and analyses on silver stained SDS-PAGE under reducing and non-reducing conditions. Dilutions of FXII-a, kallikrein and HMWK stored at 4°C were used as controls. It was found that recombinant MASP-3 did not cleave HMWK (data not shown).

Cleavage of chromogenic substrate S2222 and S2302, mediated by recombinant MASP-3, was used in inhibition studies. This made the substrates cleaved by recombinant MASP-3 optimal for determining the effect of different inhibitors. Several inhibitors were tested and four, found to inhibit the enzymatic activity of MASP-3, are summarised in Figure 4.16.

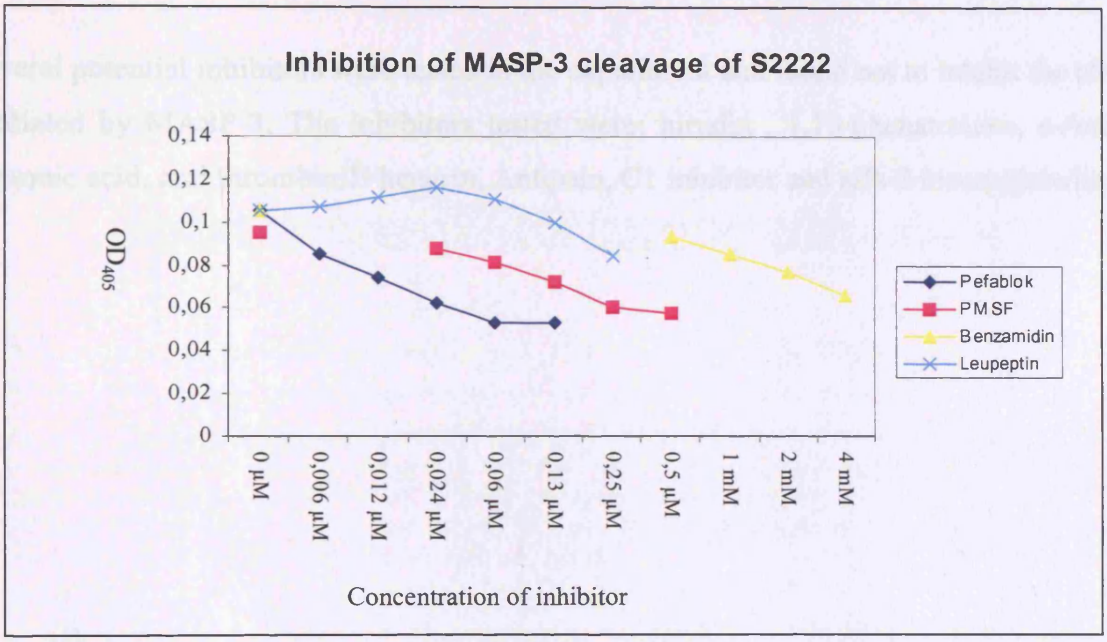


Figure 4.16. Inhibition of S2222 cleavage by recombinant MASP-3 was summarised by comparing MASP-3 of cleaved substrate at a given concentration of inhibitor. The cleavage effect of S2222 was estimated by comparing recombinant MASP-3 without any inhibitor added and cleavage as the maximum inhibition by each individual inhibitor. The concentration of recombinant MASP-3 was in all incubations fixed on 1,25 µg/ml and the CS concentration was 0.5 mM. The four curves represent the dose dependent inhibitory effect of Pefablok (SC plus, Roche Diagnostics, UK) (blue), Leupeptin (Ac-Leu-Leu-Argininal, red), PMSF (cyan) and benzamidin (yellow). The black line represents 50% inhibition of substrate cleavage.

The result using chromogenic substrate S2302 in the inhibition experiment was identical to the results shown in Figure 4.15.

The concentration of each inhibitor necessary for 50% inhibition (estimated as the inhibitor concentration needed to obtain half the maximum inhibition for the individual inhibitors) was estimated and is listed in table 4.2 together with the other inhibitors tested.

	Pefablok SC plus	PMSF	Benzamidin	Leupeptin	IAA	PPACK
Conc. 50% inhibition	20 μM	130 μM	1 mM	60 μM	3 mM	60 μM

Table 4.2. The inhibitor concentration necessary for inhibiting recombinant MASP-3 mediated cleavage of S2222 or S2302 by 50% were calculated for each inhibitor separately. Iodoacetamide (IAA) and PPACK (Dihydrochloride, Calbiochem, San Diego, CA) were tested in two separate experiments and were not included in Figure 4.16.

Several potential inhibitors were tested in the experiment and found not to inhibit the cleavage mediated by MASP-3. The inhibitors tested were: hirudin , 1,10-phenatrolene, ϵ-Amino-n-capronic acid, anti thrombinIII/heparin, Antipain, C1 inhibitor and alfa-2 macroglobulin.

4.3.9 Dissociation and association of the MBL-MASP-3 complex

Dissociation and association of the MBL/MASP-3 complex was investigated on microtiter plates coated with recombinant MBL and recombinant MASP-3 as described in chapter 4.2.11. Figure 4.17 shows a dilution curve of recombinant MASP-3 used as reference curve and wells with a fixed amount of recombinant MASP-3 incubated with different buffers.

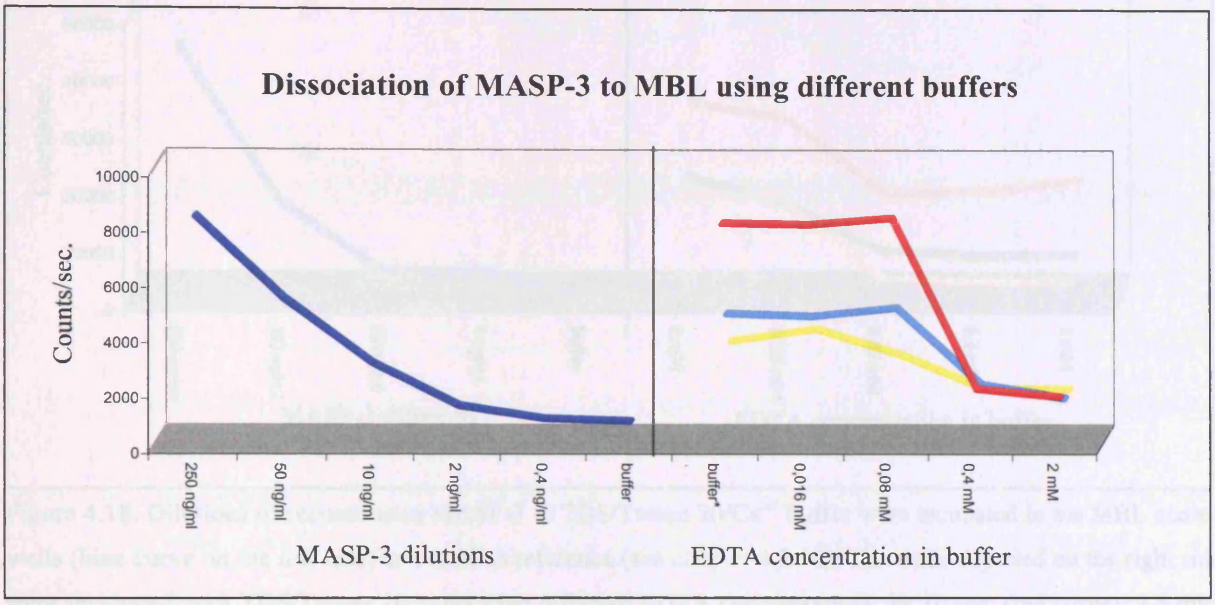


Figure 4.17. Dilutions of recombinant MASP-3 in TBS/Tween 20 buffer were incubated in the MBL coated wells (blue curve on the left side) and used as reference (see chapter 4.2.10). Samples of 250 ng/ml recombinant MASP-3 were incubated in TBS/Tween 20 buffer (red curve), TBS/Tween 20 with 0.25 M NaCl (cyan curve) or TBS/Tween 20 with 0.5 M NaCl (yellow curve). The three buffers were made +/- EDTA to a final concentration of 0 M (buffer*), 0,016 mM, 0,08 mM, 0.4 mM and 2 mM.

In buffer with physiological salt concentration (TBS/Tween 20) an EDTA concentration of 0.4 mM was necessary to dissociate the MBL/MASP-3 complex. Addition of 0.25 M NaCl increased the dissociation by a factor 10 independent of the EDTA concentration. The additional 0.25 M NaCl in the TBS/Tween 20 buffer reduces the EDTA concentration necessary for dissociation of the MBL/MASP complex slightly but 0.4 mM EDTA was still required for dissociation. Increase of the ionic strength of the buffer by addition of 0.5 M NaCl, further de-stabilised the complex.

The time of dissociation was investigated by incubating recombinant MASP-3 in wells coated with recombinant MBL and subsequently incubating in buffers containing different concentration of EDTA for a given period of time (Figure 4.18).

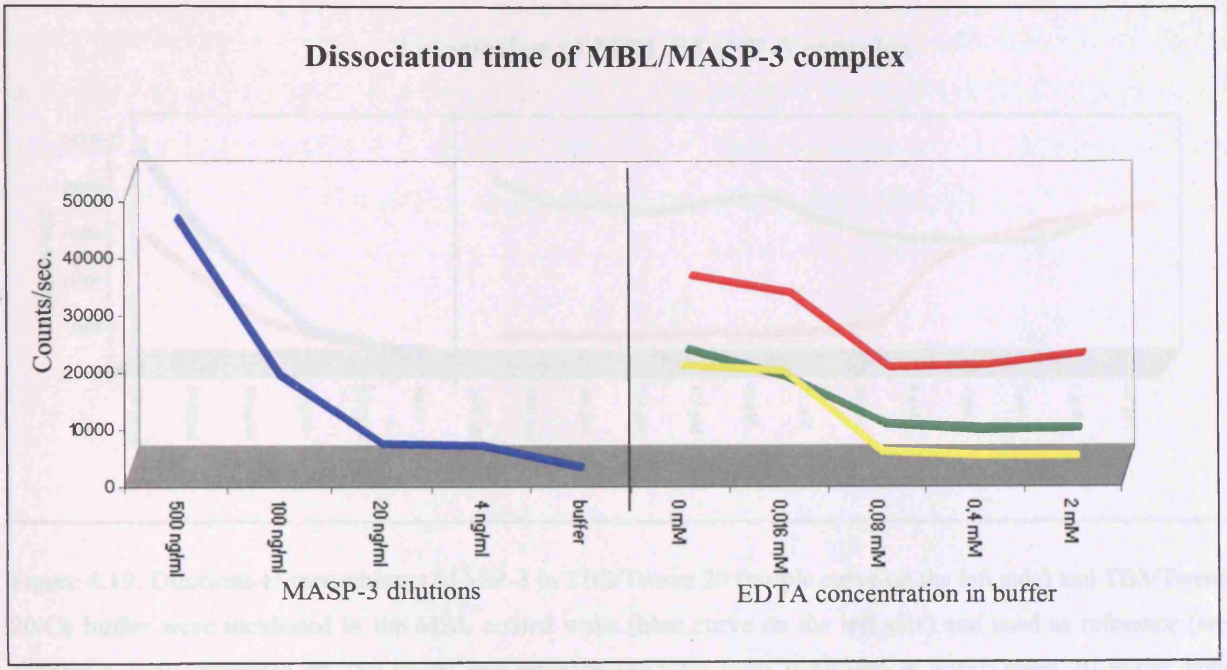


Figure 4.18. Dilutions of recombinant MASP-3 in TBS/Tween 20/Ca^{2+} buffer were incubated in the MBL coated wells (blue curve on the left side) and used as reference (see chapter 4.2.10). The wells depicted on the right side were incubated with TBS/Tween 20 containing different EDTA concentrations for 10 sec. (red curve), 2.5 min. (green curve) or 60 min. (Yellow curve).

By comparing the short rinse with TBS/Tween 20 (orange curve at 0 µM EDTA) with the 2.5 min. incubation (green curve at 0 µM EDTA) it could be seen that even a wash without Ca^{2+} had a marked influence on the stability of the complex. It could be seen that a wash or incubation with 80 µM EDTA would almost fully dissociate the MBL/MASP-3 complex.

Association conditions of the MBL/MASP-3 protein complex were investigated with a similar experiment, with the difference that recombinant MASP-3 was incubated directly on the MBL coated surface in different buffers (Figure 4.19 below).

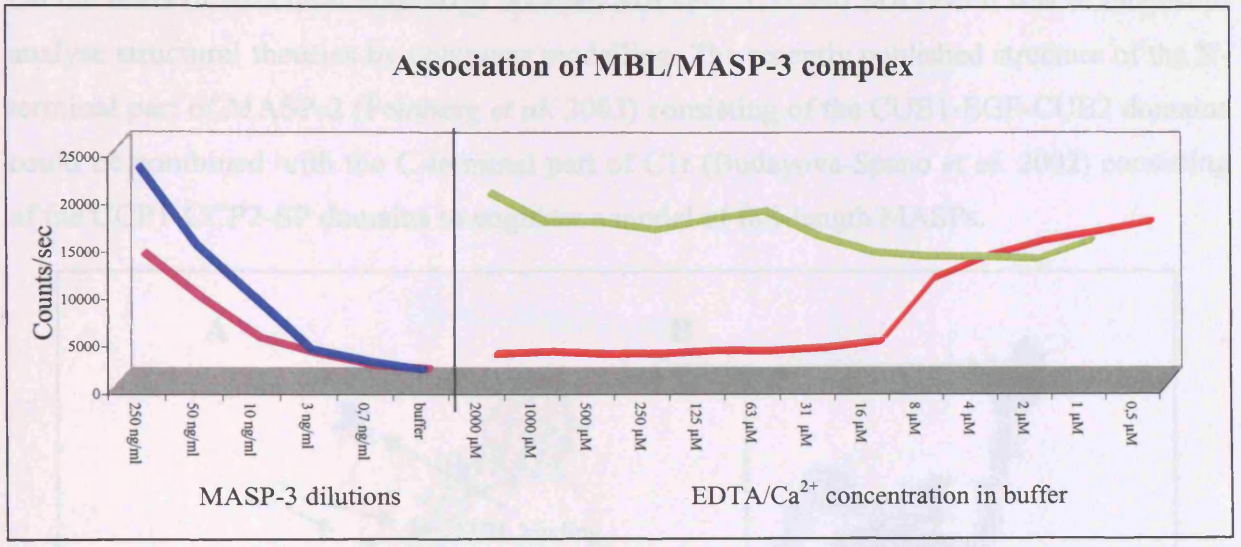


Figure 4.19. Dilutions of recombinant MASP-3 in TBS/Tween 20 (purple curve on the left side) and TBS/Tween 20/Ca buffer were incubated in the MBL coated wells (blue curve on the left side) and used as reference (see chapter 4.2.10). Samples of 250 ng/ml recombinant MASP-3 were incubated in TBS/Tween 20 buffer with different concentrations of EDTA (red curve) and Ca^{2+} (green curve) ranging from 0,5µM to 2000 µM in 2 fold dilutions.

It was found that recombinant MASP-3 could associate with MBL in buffer containing an EDTA concentration of 8 µM or less. Compared with the concentration of EDTA needed for dissociation, the difference was 10 fold. It could be concluded, that MASP-3 and MBL was capable of forming a protein complex in low concentrations of EDTA.

4.3.10 MASP-3 structural modelling

On the basis of structural homology between MASP-2, C1r and MASP-3 it was of interest to analyse structural theories by computer modelling. The recently published structure of the N-terminal part of MASP-2 (Feinberg *et al.* 2003) consisting of the CUB1-EGF-CUB2 domains could be combined with the C-terminal part of C1r (Budayova-Spano *et al.* 2002) consisting of the CCP1-CCP2-SP domains to engineer a model of full-length MASPs.

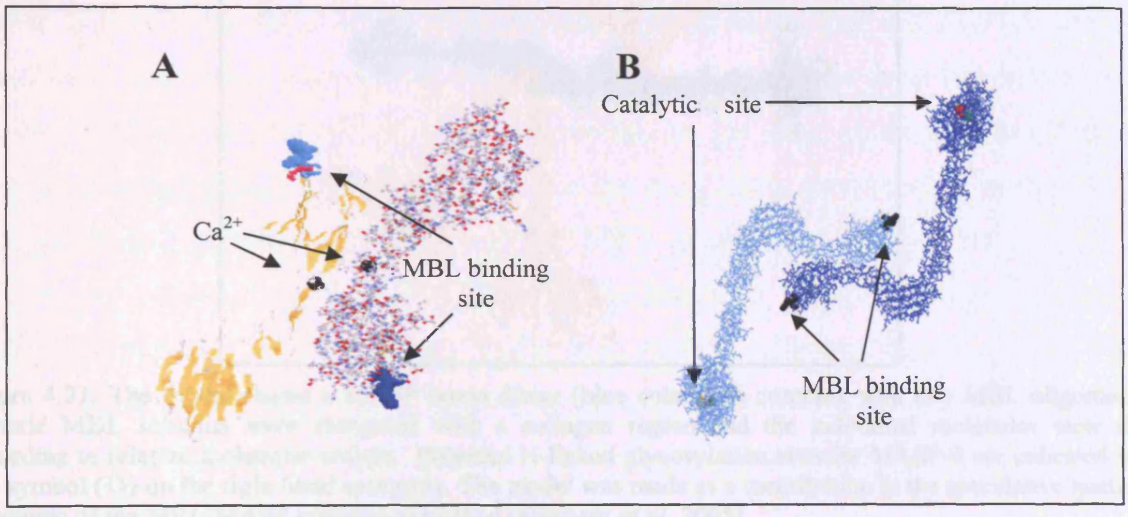


Figure 4.20. Computer model of the dimeric structure CUB1-EGF-CUB2 domains of MASP-2 (A) with the EGF calcium binding site indicated with an arrow. The monomers are drawn with secondary structure (left) and balls/pins (right). Indicated by blue space-filling, are the potential MBL binding site of the CUB-1 domain. Each monomer has a curving shape away from one-another. The combined structure of C1r (CCP1-CCP2-SP) and MASP-2 (CUB1-EGF-CUB2) was made to illustrate a plausible dimeric structure of the full length protein (B). The C-terminal catalytic site are indicated by space filling and pointed out with arrows.

The dimeric MASP-2 structure in Figure 4.20-A, promoted by the CUB1 and the EGF domains was well described by Feinberg *et al.* (2003). The calcium binding site was situated at a central position of the dimeric structure thereby being essential for the stability of the structure. The two MBL binding sites were well separated (more then 100Å) and could potentially bind two different MBL oligomers. Modelling of the full length protein in Figure 4.20-B indicated a profound separation (more then 250Å) of the serine protease domains. The model suggested that activation of MASP-3 in a dimeric form by one another would depend on extensive structural change, due to the distance between the active site of one to the activation site of the other subunit.

An engineered MBL/MASP-3 structure could be constructed by combining the MASP model in Figure 4.20 with the proposed structure of MBL (Weis *et al.* 1994), which was combined with a collagen structure (Kramer *et al.* 1999), (Figure 4.21).

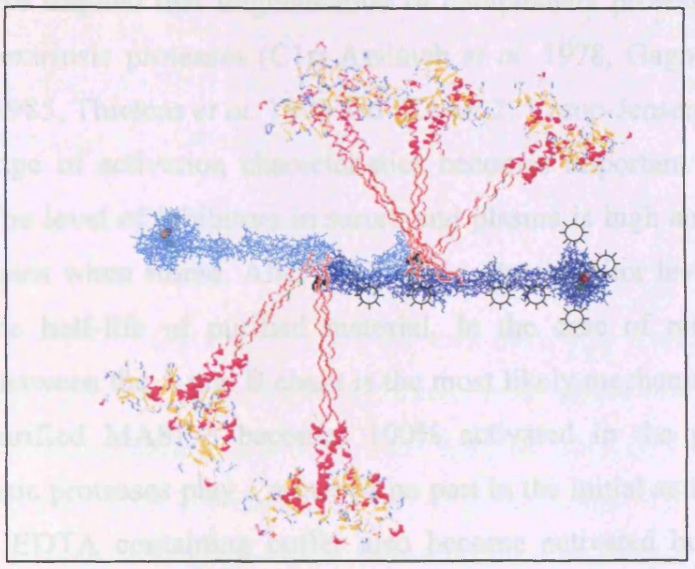


Figure 4.21. The model shows a MASP homo dimer (blue colour) in complex with two MBL oligomer. The trimeric MBL subunits were elongated with a collagen region and the individual molecules were drawn according to relative molecular weight. Potential N-linked glycosylation sites for MASP-3 are indicated with a sun symbol (☼) on the right hand monomer. The model was made as a contribution to the speculative quaternary structures of the MBL/MASP complex published (Feinberg *et al.* 2003).

The MBL/MASP complex could have a structure as indicated in Figure 4.21 with two MBL molecules and a MASP dimer. The orientation of MBL was selected due to the anti-parallel orientation of the MASP-3 in the dimer. The structure indicated a slight possibility of the serine protease domain having an influence on MBL binding. The actual size of the carbohydrate groups were unknown and therefore only indicated with a symbol.

4.4 Discussion

4.4.1 Activation of recombinant MASP-3

Previous studies have implied that fragmentation of complement proteases may occur either by autolysis or by extrinsic proteases (C1r: Assimeh *et al.* 1978, Gagnon and Arlaud 1985 C1s: Villiers *et al.* 1985, Thielens *et al.* 1990 and MASP-2: Vorup-Jensen *et al.* 2000).

A detailed knowledge of activation characteristics becomes important when working with purified material. The level of inhibitors in serum and plasma is high and may prevent rapid degradation of proteins when stored. After purification, the inhibitor level has decreased and this may reduce the half-life of purified material. In the case of recombinant MASP-3, autolytic cleavage between the A and B chain is the most likely mechanism of activation. The observation that purified MASP-3 becomes 100% activated in the presence of calcium indicates that extrinsic proteases play a minor or no part in the initial activation. Recombinant MASP-3 stored in EDTA containing buffer also become activated but in a much slower tempo suggesting that calcium mediate activation. Native MASP-3 was also found to be strongly influenced by calcium. This was seen in the difference in MASP-3 levels between serum and EDTA plasma.

The influence of calcium in controlling activation of MASP-3 could be due to calcium dependent stabilisation of the catalytic triad by the A chain of MASP-3, or structural changes. The CUB1 and the EGF domain were shown to be responsible for the calcium influence of activation of C1r (Cseh *et al.* 1996).

The recombinant MASP-3 preparations were in general stored at 4°C in TBS/Tween 20 with 5 mM Ca^{2+} and 1M $MgCl_2$ without added inhibitors and MASP-3 became activated over time. It was noted that future recombinant MASP-3 preparations should be stored in TBS/ Tween 20/ 5 mM EDTA.

Recombinant MASP-3 was determined to be N-glycosylated in both the A and B chain by staining for glycoproteins and de-glycosylation. Glycosylation of MASP-3 may play a role in the stability and half-life of the protein.

4.4.2 MASP-3 and protease activated receptors (PARs)

The investigation of potential activation of the PARs by MASP-3 could be concluded as negative. There was no evidence of MASP-3 having any activity on cells expressing PAR-1, PAR-2 and PAR-4 and no further experiments were made in this field. The results from the laboratory of Kevan Hartshorn, who included recombinant MASP-3 in his HI assay, were also negative in the sense that MASP-3 had no effect on the assay. The idea of MASP-3 being involved in direct cleavage of a viral substrate was abandoned.

4.4.3 *In situ* and immunohistochemistry discussion

The result of the *in situ* hybridisation on frozen sections from new born mice was interesting, since it showed a difference in the mRNA expression level of the brain (data supported by real time PCR reactions conducted on mouse tissue specific RNA by Nick Lynch, Leicester, UK.) This suggested a possible function of MASP-3 in the brain. With the results from the MASP-1/-3 knockout mice in mind (the mice were smaller in size) it could be speculated that MASP-3 had an influence on development, possibly in the form of NO signalling and cell communication. The novel research area of cell communication through NO signalling in the brain seems quite distant from the complement system. The finding of cell bound MASP-3 in kidney tissue, being S-nitrosylated is difficult to comprehend in terms of protease activity but it could be a functional inactivation of MASP-3 by nitrosylation (Kuncewicz *et al.* 2003). Further investigation on this subject would have to be conducted in collaboration with another specialised laboratory.

Immunohistochemistry on sections from human tissue were evaluated by Ole Nielsen (Odense University, Denmark) and he described a high level of unspecific binding in the majority of the 41 tissue samples tested. The unspecific binding could be the result of using the polyclonal rabbit anti-MASP-3 C-terminal antibody instead of a monoclonal antibody. The interpretation of the selected tissues in Figure 4.9 should therefore be viewed as influenced by the results of the in situ hybridisation. Nevertheless it was my immediate impression that the MASP-3 protein detection by immunohistochemistry correlated with the MASP-3 mRNA detection by the in situ hybridisation experiments.

4.4.4 Gel filtration chromatography discussion (GPC)

Gel filtration chromatography was used to evaluate the similarity between native MASP-3 and recombinant MASP-3. The results indicated no difference in the mobility of MASP-3 from the two sources, thus the overall structure would be the same. The EDTA and the high salt concentration in the first GPC experiment (Figure 4.10) dissociated the MBL/MASP-3 protein complex. It was anticipated that the conditions would also dissociate MASP-3 from any other complexes e.g. the ficolins and dimeric structure.

The sandwich TRIFMA used for detection of MASP-3 in the fractions, would not detect all the observed degradation fragment of recombinant MASP-3 due the assay design. Recombinant MASP-3 cleaved at the activation site would be detected, while degradation of MASP-3 in the A chain and C-terminal B chain could be undetectable in the assay leading to a decrease in the level detected.

4.4.5 Chromogenic substrates and inhibition discussion

The detection of less MASP-3 in EDTA plasma compared with serum from the same individual let to the extensive examination of a possible involvement of MASP-3 in the coagulation system. MASP-1 was reported to cleave fibrinogen and FXIII (Hajela *et al.* 2002), thus further strengthening the arguments for investigating potential MASP-3 involvement in activation of the contact system.

The experiments on chromogenic substrates were the first strong evidence of MASP-3 having an enzymatic function. The cleavage of the chromogenic substrate S2222 was interpreted as very strong and as an indication of a potential function of MASP-3 The similarity in cleavage of S2222 between recombinant MASP-3 and plasmin inspired the experiment in a very different part of the coagulation system; fibrinolysis.

The chromogenic substrate S2302 was used for experiments resembling the activation of the contact system pathway of the coagulation system. Prekallikrein, kallikrein, FXII and FXII-a were used in combinations with recombinant MASP-3 to evaluate the potential role of MASP-3 in this context. It was concluded that MASP-3 did not participate or influence the function of these two proteins in neither activated or proenzyme form.

Experiments were done to find inhibitors of recombinant MASP-3 mediated cleavage of S2222 and S2302. A range of different inhibitors were tested and several were found to inhibit MASP-3 efficiently. The broad spectrum inhibitor compounds such as PPACK SC plus, Pefablok (Dihydrochloride) and PMSF are irreversible and can therefore not be used in preparations intended for enzymatic analysis. The identification of leupeptin (reversible inhibitor) as a potent MASP-3 inhibitor could be used in future production of recombinant MASP-3 and clarify the questions regarding auto activation and degradation.
The initiation of the coagulation by the proteins of the contact system: HMWK, FXII and kallikrein were analysed using the best material available on the market. The experiments were carefully controlled and repeated several times to ensure a clear conclusion, which was that recombinant MASP-3 had no enzymatic activity on the three proteins.

4.4.6 Association and dissociation of the MBL-MASP-3 complex

Association and dissociation experiments could not be conducted on the mannan/MBL surface since the buffers containing EDTA would elute MBL off the mannan. The experiments were important in the evaluation of the complex stability and interpretation of assay results. Dissociation of the complex was a consequence of Ca^{2+} from the MASP-3 A chain being chelated by EDTA, or disruption of interacting residues between MBL and MASP-3. Dissociation of the MBL/MASP-3 complex was found to be sensitive to high salt concentration in the buffer with a 10 fold decrease in associated MASP-3 in TBS buffer containing an additional salt concentration of 0.25 M. The relatively low amount of MBL bound MASP-3 (reflected in a low amount of counts/sec.) in the first experiment (Figure 4.17) could be explained by incubation buffer being TBS/Tween 20 without calcium.
Time as a factor for dissociation could be evaluated by the second experiment (Figure 4.18). By including the 10 sec. incubation it was demonstrated how sensitive the assays were in terms of buffer selection and how easily an experiment could be ruined. The similarity between the 2.5 min. and 60 min. incubation indicated a critical point for dissociation at an concentration of 80 μM EDTA. By comparing the graduated course of the curves, there were some indications of a two step dissociation profile. The rinse with ≥80 μM EDTA dissociated an equal amount of bound MASP-3 and a small proportion was dissociated by incubation with

16 μM EDTA for 1h. The result could reflect MASP-3 bound to MBL by two different sites, MASP-3 bound to different MBL oligomeric structures or dimeric MASP-3.
The recombinant MASP-3 was diluted 1/1000 (stock concentration of: 250 μg/ml MASP-3), thus the influence of the storage could be neglected. Also the incubation step in TBS/Tween 20 containing 25 μM EDTA could be concluded to have minimum influence on the stability of the complex.
Association and thereby formation of a MBL/MASP-3 complex was found to be 10 fold more sensitive to EDTA concentration compared with dissociation. The result indicated that MASP-3 would be more stable in complex with MBL than unbound.

4.4.7 MASP-3 structural modelling discussion

MASP forms homodimers through interactions of CUB1-EGF-CUB2, but there have been contradicting reports about calcium dependence of the dimer formation (Chen and Wallis 2001 and Thielens et al 2001).
Structural analysis suggested that the two calcium ions of the EGF domains in a MASP dimer were within 10 Å of each other. With a size of ~1 Å and a strong influence of the surroundings within 5-10 Å, the two calcium ions could influence the stability of the dimer due to the short distance between the ions.
The results from GPC and MASP-3 TRIFMA experiments suggested that MASP-3 was found as dimers in EDTA plasma and serum.
On the basis of the structural analysis and experimental results it was concluded that the MBL/MASP-3 complex would be dissociated by EDTA chelation of calcium. Given this conclusion, it could be speculated that a proportion of MASP would be dissociated from MBL after separation of the dimer. This could explain how MASP-2 cleaves C4 and subsequently C2 on the surface of a pathogen (Moller-Kristensen *et al.* 2003).
Due to the tertiary structure of the CUB1-EGF-CUB2 domains and the direction of individual monomers in the structure, the suggested structures in the paper (Feinberg *et al.* 2003, Figure 7) were impossible. The suggested quaternary structure would only be possible if the MASP chains were parallel.

MBL/MASP complexes could potentially consist of several different MBL oligomers connected by dimeric MASP. The proposed structure of the MBL/MASP complex opens the possibility of having a large number of unstable high molecular weight complexes with a broad functional range. This would make the MBL/MASP complex even more unorganised compared with the C1 complex.

This type of multi-oligomeric complexes could explain the experimental difficulties of oligomeric separation. The natural function of MAp19 could then be size restriction of the complexes.

5.1 Determination of MASP-3 levels by TRIFMA

The MASP-3 TRIFMA was considered a tool for estimation of the MASP-3 level in samples and capable of identifying potential MASP-3 deficient individuals. The subsequent sample collections were analysed for levels of MASP-3. The samples were selected on the basis of information from literature (see chapter 1) and MASP-3 characteristics (see chapter 4). Furthermore samples from healthy individuals were of interest as reference data.

Cerebrospinal fluid (CSF) and sera from seventeen patients diagnosed with Alzheimer disease was part of the material from the Oxford project to investigate memory and ageing (OPTIMA). The samples were used in a study evaluating correlation between MBL levels and Alzheimer disease (Lanzrein *et al.* 1998).

The 398 serum samples from a study of individuals with and without diabetes 1 were used in a study to evaluate correlation with MBL levels and MBL genotypes. In patients with type 1 diabetic the frequencies of high- and low-expression MBL genotypes were similar to healthy control subjects. (Hansen *et al.* 2003).

Sperm plasma and serum samples were collected by B. Bundgaard University of Aarhus and samples of urine, lung-, pleural-, ascites- and amnion fluids were collected by J. Haurum, University of Aarhus.

Plasma samples from healthy individuals were collected during a one-year period and from patients with cancer over a 30 day period.

5.2 Material and methods

5.2.1 MASP-3 TRIFMA

Maxisorp 96 wells microtiter plates were coated with monoclonal anti MASP-3 antibody 1E2 (a kind gift from Dr. Teizo Fujita, Japan) against the A chain common to both MASP-1 and MASP-3. The concentration of 1E2 was 1 µg/ml diluted in PBS (Phosphate Buffered Saline, pH 7.4, 140 mM NaCl (Merck Cat. No. 6404), 2.7 mM KCl (Merck, Cat. No. 116101), 1.5 mM KH_2PO_4 (Merck, Cat. No. 104873) and 8.1 mM Na_2HPO_4 (Merck Cat. No. 106559) and 100 µl was incubated in the wells overnight at 4°C. The plate was blocked with 200 µl/well TBS (defined in chapter 2.2.12) containing 1 mg HSA/ml, incubated for 30 min. at RT and washed three times in TBS/Tween 20 before storage at 4°C. Samples were diluted in buffer and 100 µl /well were incubated overnight at 4°C. The wells were washed three times in TBS/Tween 20 and the bound MASP-3 was detected by incubation with 100 µl biotinylated rabbit 7932 anti MASP-3 antibody (see table 2.1 for details) diluted 1/3000 (~1 µg/ml). After incubation for 4h, the plate was washed three times in TBS/Tween 20 and incubated 1h with 100 µl Eu^{3+}-streptavidin (Perkin Elmer Cat.No.1244-360 DELFIA Eu-N1, 250 µg) diluted 1/2000 in TBS/Tween 20 with 25 µM EDTA. After a final wash the amount of bound europium could be measured by adding 200 µl enhancement buffer (Wallac Oy, Turku, Finland) to each well followed by incubation for 5 min. on a shaker and measured on a flourimeter.

5.2.2 Stability of MASP-3 over time and after freezing/thawing

A pool of EDTA plasma was incubated in 50 µl aliquots at -20, 4, RT and 37°C for different periods of time up to 30 days. After incubation, the samples were stored at -80°C until analysed by MASP-3 TRIFMA. To evaluate the stability of MASP-3, which had been tested in other assays and stored at -80°C, nine aliquots of 10 µl purified recombinant MASP-3 was frozen at -80°C and thawed at RT up to eight times. Analysis of the samples was performed by MASP-3 sandwich TRIFMA as described above.

5.2.3 MASP-3 levels in serum/plasma samples

Serum and plasma samples were initially analysed without discrimination. Only after the discovery of a two fold difference in MASP-3 concentration between EDTA plasma on one side and heparinised, citrated plasma and serum on the other side, could the appropriate precautions be taken. The problem was temporarily solved by introducing two standards, one for EDTA plasma and one for other plasma and serum.

MASP-3 levels in citrate-, heparin-, EDTA plasma and serum were analysed using the sandwich MASP-3 TRIFMA assay. Samples were diluted 1/50 in TBS/Tween 20 buffer containing 0.35 M NaCl, 5 mM EDTA and 100 µg heat aggregated HSA (ΔHAS)/ml and heat aggregated normal human IgG (ΔnIgG, State Serum Institute. Cat. No. 418293)/ml and incubated overnight in 96 wells plates coated with monoclonal 1 µg 1E2 anti MASP-1/3 A chain antibody/ml. Dilutions of recombinant MASP-3 or standardised samples were used to determine the MASP-3 level in the samples.

5.2.4 MASP-3 levels in EDTA plasma with added calcium or EDTA

A pool of EDTA plasma from three individuals was diluted 1-1 in TBS containing 10 mM Ca^{2+} or 5mM EDTA and incubated in 37°C water bath. Aliquots were taken out at the beginning of the experiment and at time intervals. The aliquots were diluted 1/25 in TBS/Tween 20 buffer containing 0.35 M NaCl, 5 mM EDTA and 100 µg ΔHSA and ΔnIgG/ml (see chapter 5.2.4). After 60 min. of incubation the EDTA plasma in which Ca^{2+} was added were split in two, one half for continuous incubation as before and the other half was diluted 1-1 in TBS with 40 mM EDTA. Aliquots were taken at different time points and diluted in TBS/Tween 20 buffer described above. The second dilution with TBS/EDTA was accounted for before all samples were incubated overnight in 96 well plates coated with monoclonal 1 µg 1E2 anti MASP-1/3 A chain antibody/ml. Development of the experiment was conducted as described for the MASP-3 TRIFMA.

5.2.5 MASP-3 levels in normal human serum/plasma

The average level of MASP-3 in human serum and plasma was determined using the MASP-3 TRIFMA, which was standardised on the basis of recombinant MASP-3 characterisation. EDTA plasma samples from 100 healthy individuals had been previously used for determination of the average MBL level (Steffensen *et al.* 2000). The serum samples used were introduced in chapter 5.1.

5.2.6 Specific lectin pathway complement activation

Microtitre wells were coated with 100 µl of 10 µg mannan/ml in coating buffer (0.1 M sodiumcarbonate/bicarbonate buffer pH 9.6) for 2h at RT. The plates were blocked as described in MASP-3 TRIFMA. After a wash the wells received dilutions of serum samples, standard control serum and a buffer control. The dilutions were made using buffer containing 20 mM Tris, 1 M NaCl, 10 mM Ca^{2+}, 0.05% (v/v) Triton X-100 and 1 mg HSA/ml and adjusted to pH 7.4. Samples were incubated overnight at 4°C followed by wash in TBS/Tween 20/Ca buffer. Complement C4 was purified as described by Alister Dodds (Dodds 1993) and was diluted to 7 µg C4/ml barbital buffer. The barbital buffer contained 4 mM barbital, 145 mM NaCl, 2 mM Ca^{2+}, 1 mM Mg^{2+} adjusted to pH 7.4. 100 µl of C4 dilutions were added to each well and incubated 1h at 37°C. After wash in TBS/Tween 20/Ca^{2+} buffer, the C4b deposition was determined by development using monoclonal Eu^{3+}-labelled anti-C4b antibody (hybridoma 162-2, State Serum Institute, Denmark) diluted to 500 ng/ml in TBS/Tween 20 buffer with 25 µM EDTA. After a final wash the amount of bound europium was measured as described in MASP-3 TRIFMA (chapter 5.2.1).

5.3 Results

5.3.1 Stability of MASP-3 over time and after freezing/thawing

MASP-3 levels in different types of samples and sample collections were analysed using the sandwich MASP-3 TRIFMA. The sample collections had been stored for as long as 10 years at -80°C and been analysed in several studies. The stability of MASP-3 upon storage over a period of 30 days was analysed by MASP-3 TRIFMA to evaluate the effect of storage (Figure 5.1).

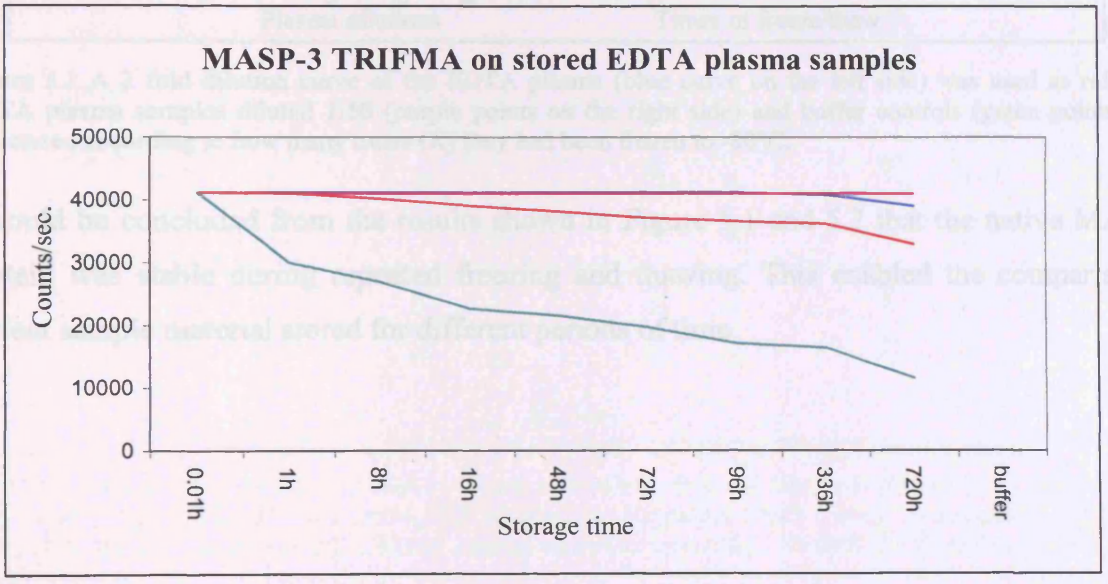


Figure 5.1 Storage effects of EDTA plasma on MASP-3 was analysed by MASP-3 TRIFMA after incubation at -20°C (blue curve), 4°C (purple curve), RT (red curve) and 37°C (green curve). A buffer control (orange) indicated the background level in the experiment. Control samples were included in the experiment to validate the result.

The stability of MASP-3 was found to be high for samples stored under normal conditions at -20°C or 4°C. A MASP-3 level decrease was found in samples stored at RT after 30 days, while most MASP-3 was lost when stored at 37°C for the same period of time. Approximately half of the protein was undetectable within the first 24h of incubation at this temperature. The samples were not analysed by Western blotting, due to previous experience with full serum analysis using this method.

It was suspected that the decrease in detectable MASP-3 was due to protein degradation.

The stability of MASP-3 upon repeated freeze/thaw from storage at -80°C to RT was analysed by MASP-3 TRIFMA (Figure 5.1).

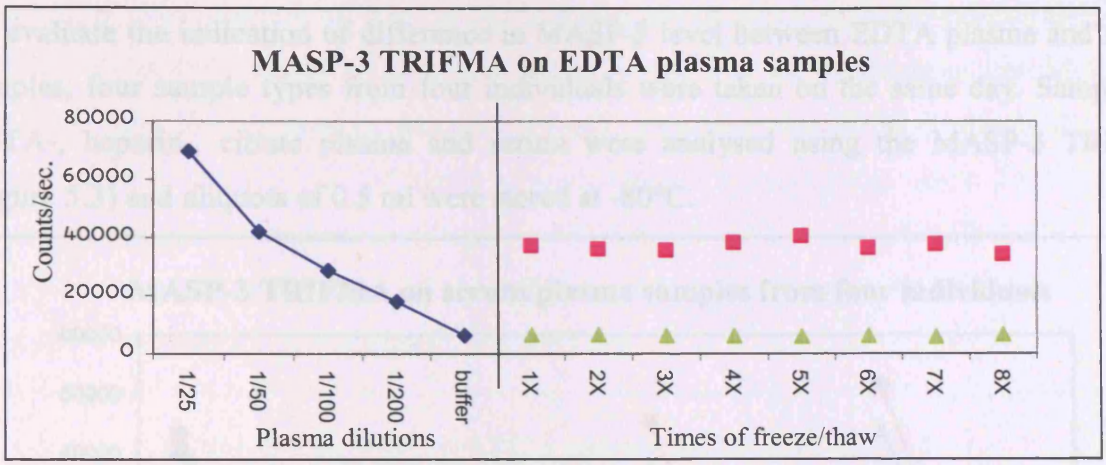


Figure 5.2 A 2 fold dilution curve of the EDTA plasma (blue curve on the left side) was used as reference. EDTA plasma samples diluted 1/50 (purple points on the right side) and buffer controls (green points) were depictured according to how many times (X) they had been frozen to -80°C.

It could be concluded from the results shown in Figure 5.1 and 5.2 that the native MASP-3 protein was stable during repeated freezing and thawing. This enabled the comparison of patient sample material stored for different periods of time.

5.3.2 MASP-3 levels in serum/plasma

To evaluate the indication of difference in MASP-3 level between EDTA plasma and serum samples, four sample types from four individuals were taken on the same day. Samples of EDTA-, heparin-, citrate plasma and serum were analysed using the MASP-3 TRIFMA (Figure 5.3) and aliquots of 0.5 ml were stored at -80°C.

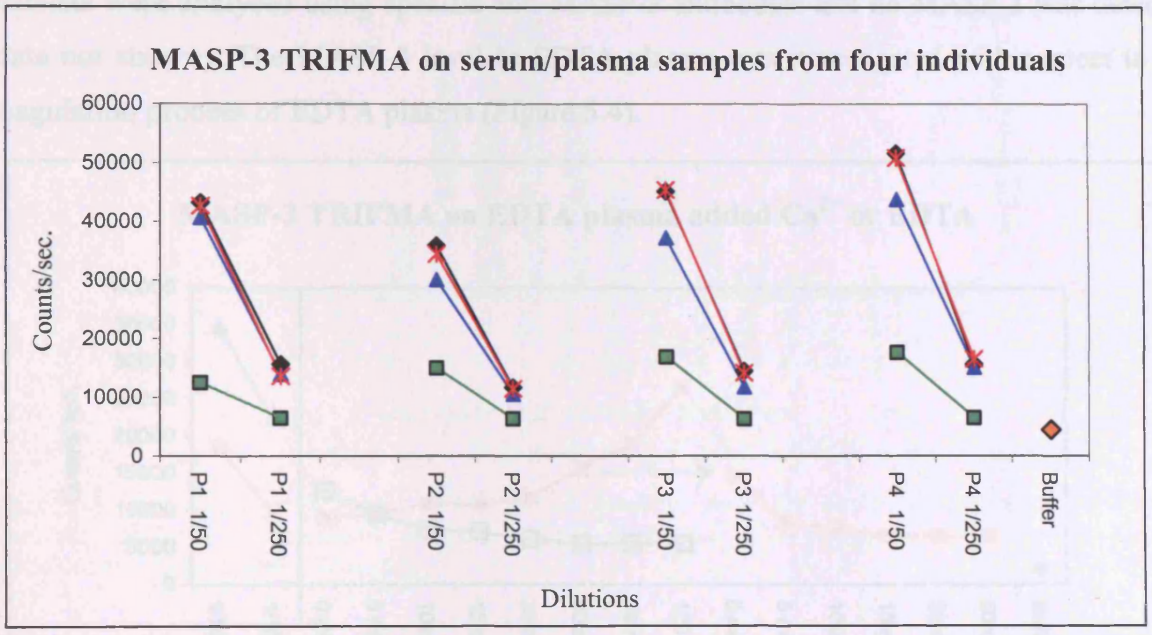


Figure 5.3 shows two dilutions (1/50 and 1/250) of each sample from the four individuals P1, P2, P3 and P4. The four sample types analysed were: serum (red curve), heparin plasma (black curve), citrate plasma (blue curve) and EDTA plasma (green curve). A buffer control was used included to indicate the background level (orange point).

The result in Figure 5.3 suggested an equal amount of MASP-3 detectable in serum and heparin plasma, while the MASP-3 level in citrate plasma was measured as ~15% lower in P3 and P4 diluted 1/50. The level of MASP-3 detected in EDTA plasma was significantly lower in all four individuals compared to the other three sample types. To determine the concentration of MASP-3 in samples it was essential to distinguish between EDTA plasma and the other three types of blood samples.

The experiment was repeated several times with variation of buffers and pre-treatment of samples. The result remained the same, except when EDTA plasma was re-calcified and left to coagulate. Serum made from EDTA plasma had approximately the same detectable level of MASP-3 obtained in serum samples. Experiments were conducted to investigate and clarify

the findings. MASP-3 levels in EDTA plasma enriched with thrombocytes were compared with cell free EDTA plasma to test for potential release of intracellular protein in relation to coagulation. The coagulation time of EDTA plasma with added calcium was compared with EDTA plasma with added recombinant MASP-3 and calcium to evaluate potential direct influence. Both experiments showed no difference with regard to MASP-3 compared to inter-experimental controls (data not shown). Furthermore, Western blots made from lysate of platelets were analysed using specific anti-MASP-3 antibodies and no MASP-3 was detected (data not shown). The MASP-3 level in EDTA plasma was investigated with respect to the coagulation process of EDTA plasma (Figure 5.4).

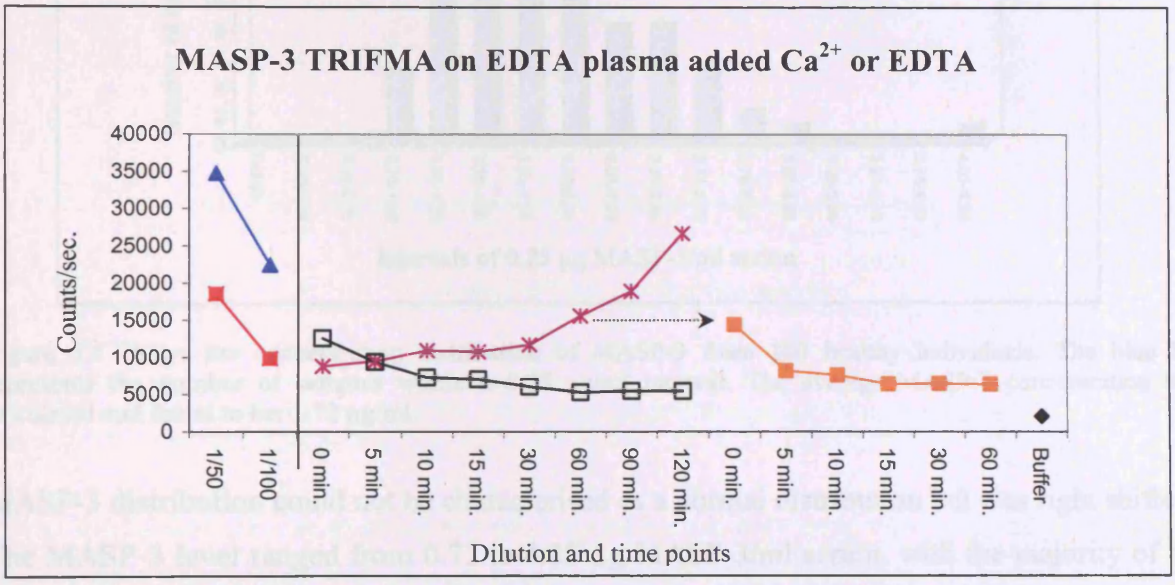


Figure 5.4 Two dilutions of serum (blue) and EDTA plasma (red) from one individual were used as control samples (left side) and a buffer control was used as negative control (black diamond, far right side). EDTA plasma with added calcium (5 mM, purple curve) or EDTA (1 mM, black curve) were analysed immediately after addition and after 5, 10, 15, 30, 60, 90 and 120 min. After 60 min. of incubation (indicated with black arrow) an aliquot of the EDTA plasma with added calcium were further added 5 mM EDTA and analysed immediately after addition and after 5, 10, 15, 30 and 60 min (orange curve).

Addition of calcium to EDTA plasma had a profound effect on the amount of MASP-3 detected in the MASP-3 TRIFMA. The effect of addition of EDTA after 60 min. suggested a reversible process of complex formation. The complex in question could not be MBL/MASP-3 since the EDTA concentration was too high for association of MASP-3 to MBL (Figure 4.19) and was probably due to MASP-3 complex formation (see discussion chapter 4, Figure 4.21).

5.3.3 MASP-3 levels in 100 EDTA plasma samples from healthy individuals

To evaluate the concentration of MASP-3 in EDTA plasma from healthy individuals, 100 samples were submitted to the MASP-3 TRIFMA (Figure 5.5).

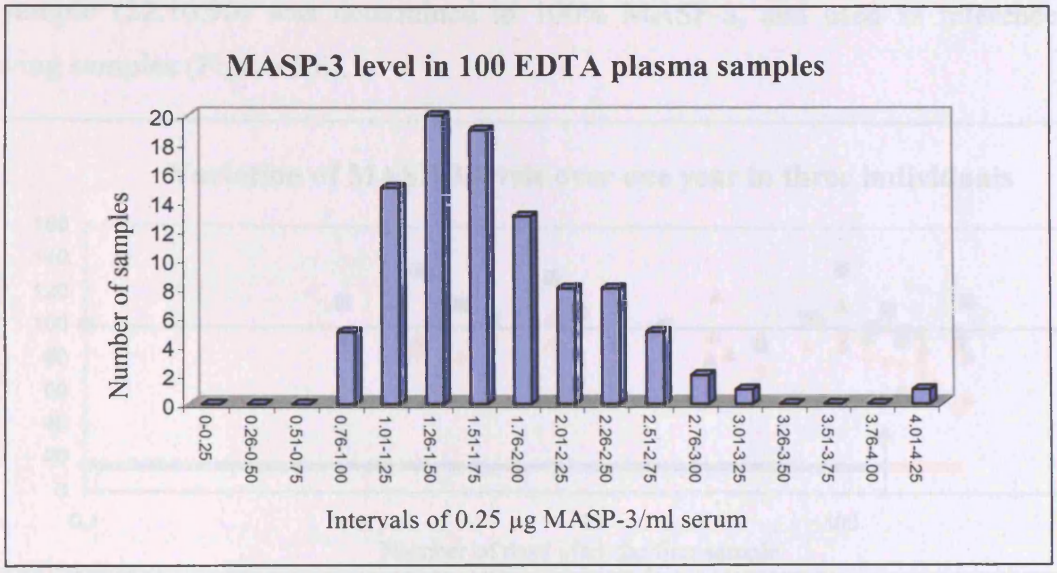


Figure 5.5 shows the concentration distribution of MASP-3 from 100 healthy individuals. The blue bar represents the number of samples within a 0.25 µg/ml interval. The average MASP-3 concentration was calculated and found to be: 1.72 µg/ml.

MASP-3 distribution could not be characterised as a normal distribution but was right shifted. The MASP-3 level ranged from 0.73 to 4.25 µg MASP-3/ml serum, with the majority of the population having between 1 and 2.5 µg MASP-3/ml serum. A single serum contained more than 3.25 µg/ml and no MASP-3 deficient individuals were identified.

5.3.4 MASP-3 levels in samples over one year

Samples collected form three healthy individuals in the period form 22.10.1990 to 23.9.1991 was analysed using the MASP-3 TRIFMA (Figure 5.6). The concentration of MASP-3 in the first sample (22.10.90) was determined to 100% MASP-3, and used as reference of the following samples (Figure 5.6).

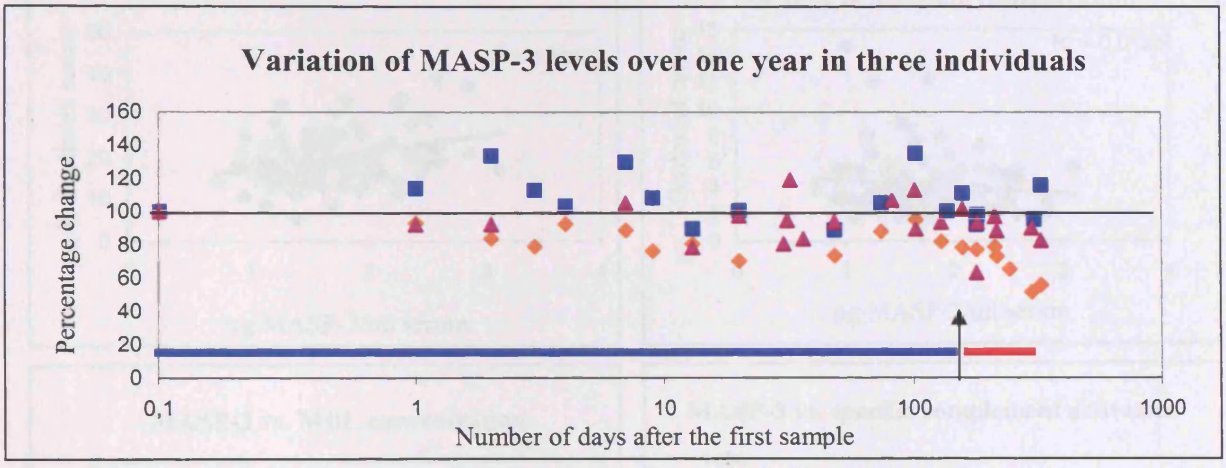


Figure 5.6 shows the percentage change in MASP-3 levels in sera from three individuals (blue, purple and orange points) over a period of one year. The first samples at day 0.1 were set to represent a 100% level (black line). The arrow indicates the date 2.4.1991, which was selected to separate the summer (red line) period of the year and the winter period (blue line).

It was found that the variation of MASP-3 could be +/- 40% from the initial level and could change 20% within 24h. The average level of MASP-3 was in general lower in the summer half of the year (mean percentage change of 90%) compared with the winter half (mean percentage change of 96%).

The level of MBL in the EDTA plasma from 100 normal healthy individuals was determined by the MBL TRIFMA described in chapter 4 and levels of L- and H-ficolin were estimated by Anders Krarup, University of Aarhus. The specific activation of complement mediated by the lectin pathway was determined using the C4 assay described above. The results were combined and plotted against the result of MASP-3 level determination (Figure 5.7 A-D).

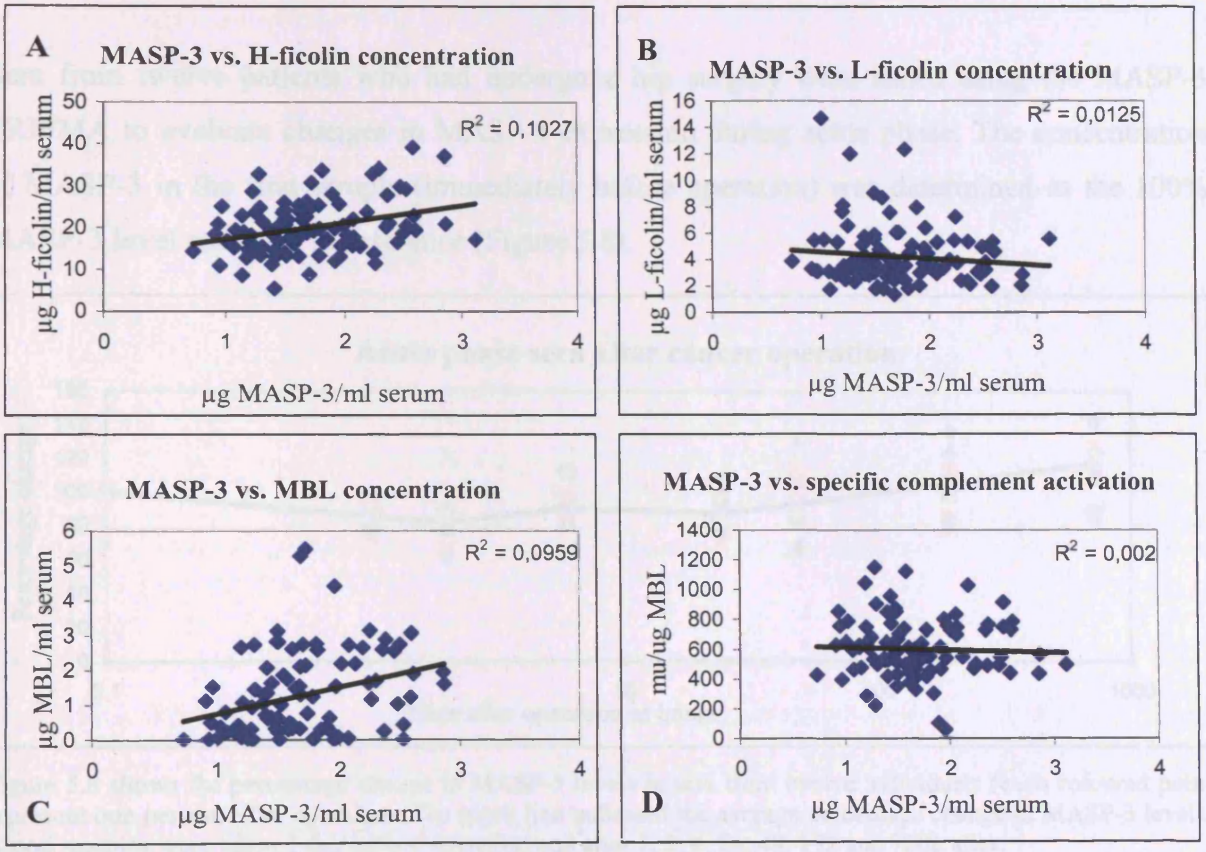


Figure 5.7 shows four plots where the MASP-3 concentration of 100 normal sera are compared with the corresponding concentration of H-ficolin (A), L-ficolin (B), MBL (C) and the specific complement activation mediated by the lectin pathway. Linearly tendencies are indicated with a black line and the R^2 value of correlation noted.

A positive dependence was observed between the concentrations of MASP-3/H-ficolin and MASP-3/MBL, thus low level of MASP-3 could be associated with lower MBL and H-ficolin levels or vice-versa. A slight negative correlation was observed between MASP-3 and L-ficolin. There was found no correlation between specific MBL mediated lectin pathway activation of complement and the concentration of MASP-3.

The observations could only be regarded as guiding and suggested dependence between MASP-3, H-ficolin and MBL. Other factors such as the serum level of MASP-1, MASP-2, MAp19 and MBL genotypes could influence the result.

5.3.5 MASP-3 levels in acute phase

Sera from twelve patients who had undergone hip surgery were tested using the MASP-3 TRIFMA to evaluate changes in MASP-3 expression during acute phase. The concentration of MASP-3 in the first sample (immediately before operation) was determined as the 100% MASP-3 level and used as reference (Figure 5.8).

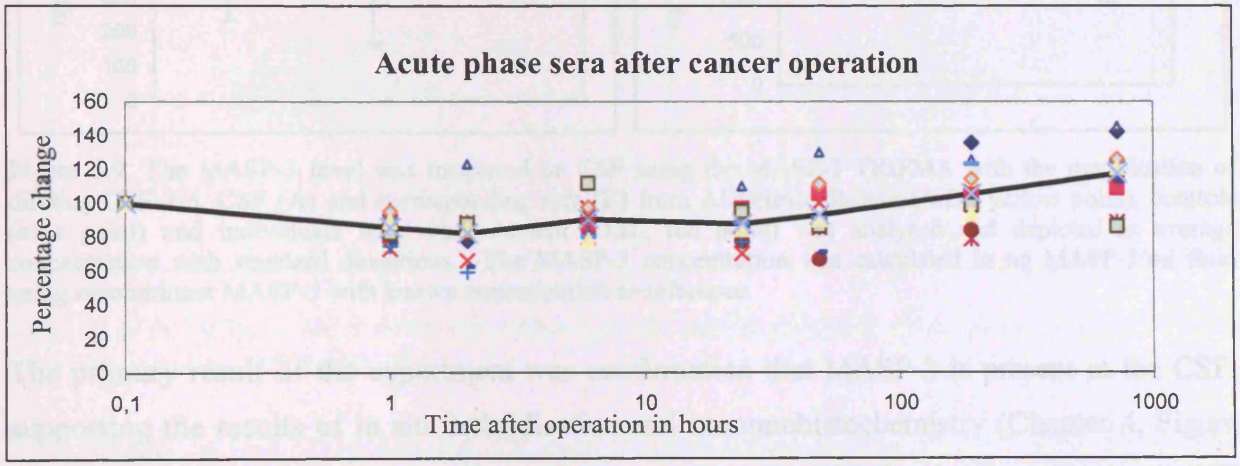


Figure 5.8 shows the percentage change in MASP-3 levels in sera from twelve individuals (each coloured point represent one person) after operation. The black line indicated the average percentage change in MASP-3 levels. Blood samples were taken 1 day before operation and after 1, 2, 6, 24, 48, 192 and 720h after.

The average level of MASP-3 was stable in the first 48h and approximately 20% higher 720h after operation. By comparing this variation with the variation seen over one year (Figure 5.6), there was no indication of MASP-3 being an acute phase protein.

5.3.6 MASP-3 levels in samples from patients with Alzheimer and other dementias

Cerebrospinal fluid (CSF) and sera from seventeen patients diagnosed with Alzheimer disease was analysed using the MASP-3 TRIFMA. The result was compared with CSF and sera from four healthy individuals and seven patients with other dementias (Figure 5.9-A and -B).

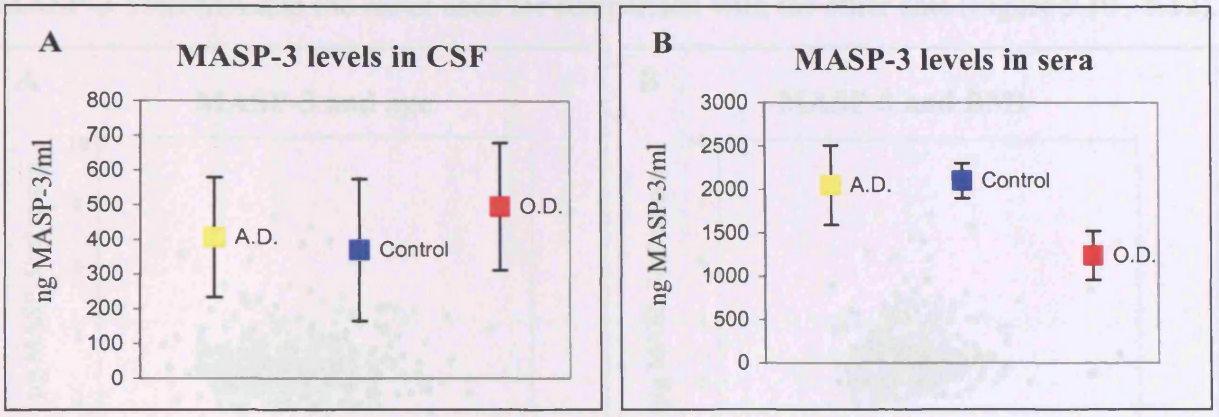


Figure 5.9. The MASP-3 level was measured on CSF using the MASP-3 TRIFMA with the modification of diluting CSF 1/5. CSF (A) and corresponding sera (B) from Alzheimer disease (A.D., yellow point), controls (blue point) and individuals with other dement (O.D., red point) was analysed and depicted as average concentration with standard deviations. The MASP-3 concentration was calculated in ng MASP-3/ml fluid using recombinant MASP-3 with known concentration as reference.

The primary result of the experiment was confirmation that MASP-3 is present in the CSF, supporting the results of in situ hybridisation and immunohistochemistry (Chapter 4, Figure 4.8 and 4.9). There was no significant difference between CSF from AD patients and CSF from healthy individuals, both groups had an average MASP-3 level of approximately 400 ng/ml (AD: 407 +/- 173 ng/ml, control: 370 +/- 205 ng/ml). A slightly higher average MASP-3 concentration was detected in CSF from patients suffering from other types of dementias (OD: 496 +/-184 ng/ml). The MASP-3 level in the corresponding sera was; AD: 2049 +/-458 ng/ml, normal: 2099 +/-200 ng/ml and OD: 1239 +/-282 ng/ml. A significantly lower average MASP-3 concentration was detected in sera from patients suffering from other types of dementias, while the values for AD and healthy individuals were found to be similar. The result indicated a local production of MASP-3 in the brain, since the average MASP-3 level in patients suffering from other dementias was highest in CSF and lowest in the sera samples. It was concluded that there was no correlation between MASP-3 levels and Alzheimer's disease.

5.3.7 MASP-3 levels in samples from patients with type 1 diabetes

A collection of 398 serum samples from patients suffering from diabetes was well characterised in terms of physical data, MBL level and genotypes by Troels K Hansen, University hospital of Aarhus, Denmark. MASP-3 concentration was determined using the MASP-3 TRIFMA and the result used for comparison with the other data (Figure 5.10 - 5.12).

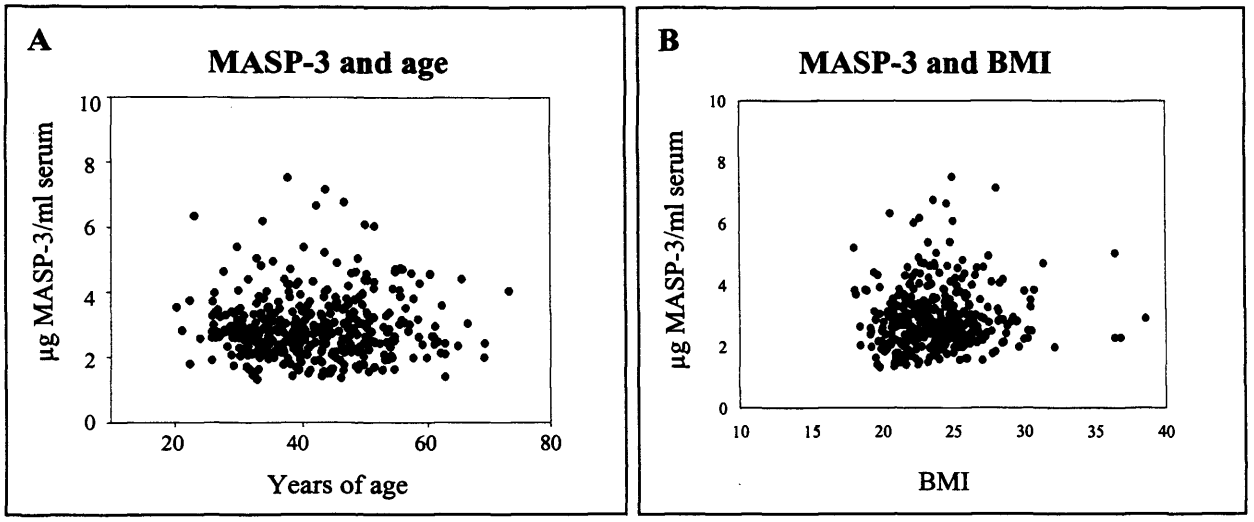


Figure 5.10 shows the MASP-3 serum concentration of 398 individuals plotted against the age (A) and the body mass index (BMI) of each person (B).

The average MASP-3 level in the 398 samples was determined to 2.92 +/- 0.97 µg MASP-3/ml and did not differ from the MASP-3 level in the controls.

No correlation was found between serum MASP-3 level and age or BMI of individuals. The data was further used to compare serum MASP-3 levels with: sex, duration of diabetes,"glycosylated haemoglobin" (Hba1c), serum creatinine, and blood sugar levels (data not shown). No correlation was detected in any of the analysis and it was concluded that serum MASP-3 levels had no influence on the diabetes.

The 398 individuals, genotyped in terms of MBL, were grouped and compared in terms of MASP-3 levels (Figure 5.11).

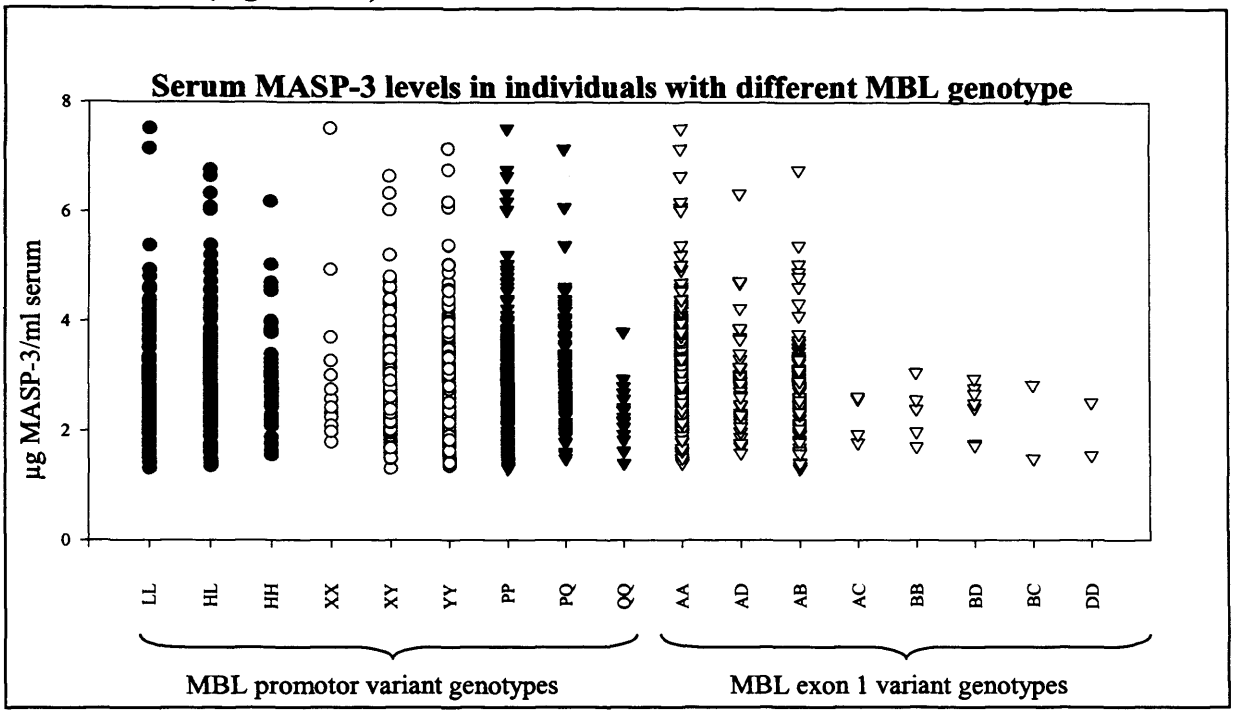


Figure 5.11 shows the 398 samples grouped by MBL genotypes and compared with the corresponding serum MASP-3 concentration. Variants LL, HL and HH (black diamond). Variants XX, XY and XX (white circle). Variants PP, PQ and QQ (black triangle). Variants AA (wildtype), AD, AB, AC, BB, BD, BC and DD (white triangle).

No sample contained less then 1.3 µg MASP-3/ml and the highest concentration detected was 7.5 µg MASP-3/ml. The plot showed some variation and potential dependence between MASP-3 concentration and MBL genotypes. The MBL promoter variant QQ and the exon 1 variants AC, BB, BD, BC and DD seemed to have a slightly lower concentration of MASP-3. The average MASP-3 concentrations of each genotype were calculated and are shown in table 5.1 below.

	LL	HL	HH	XX	XY	YY	PP	PQ	QQ	AA	AD	AB	AC	BB	BD	BC	DD
Mean	3.03	2.96	2.84	3.12	2.94	2.9	2.89	3.05	2.34	3.08	2.81	2.77	2.22	2.34	2.41	2.16	2.03
Std.Dev	1.02	0.97	0.95	1.58	0.9	0.97	0.99	0.94	0.59	1.01	0.89	0.92	0.43	0.52	0.45	0.95	0.68
n.	49	185	155	13	142	234	246	128	15	221	49	98	4	5	8	2	2

Table 5.1 shows the average (mean) MASP-3 concentration in µg/ml with standard deviation (std.) and the number of individuals (n) of each genotype. The genotype nomenclature corresponds to the one given in figure 5.1.

Some differences between MBL genotypes and MASP-3 levels was observed. The MASP-3 level in individuals with MBL genotype variant QQ, AC, BB, BD, BC and DD was substantialy lower compared with the average MASP-3 level of the population. The most interesting genotypes were the exon 1 mutation variants B, C and D. The three mutations which cause amino acid changes in the collagen region of MBL all corresponding with a lower MASP-3 level. Due to the low incidence of homozygote and heterozygote mutated individuals; the population was regrouped into three groups (Figure 5.12).

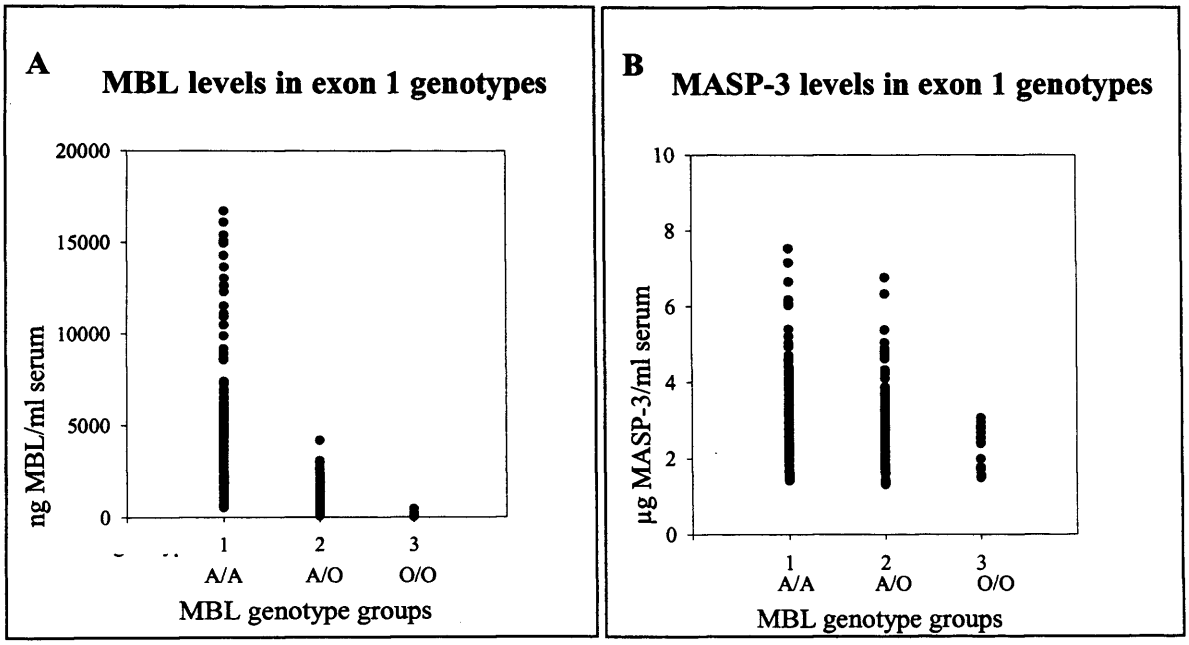


Figure 5.12. The population of 398 individuals was grouped with respect to the genotypes of MBL exon 1 mutations. Group 1 (A/A) included the wildtype (A/A) variant, group 2 (A/O) included heterozygote wildtype variants (A/B, A/C and A/D) and group 3 (O/O) included the homozygote variants (B/B, B/C, B/D and D/D). The MBL level of individuals from each group is depicted on the left side (A) and corresponding MASP-3 levels to the right in the Figure (B).

The MBL concentration was strongly influenced by the exon 1 mutations (Figure 5.12-A) and the homozygote variants B/B, B/C, B/D and D/D were almost all functionally deficient with respect to the detected MBL concentration (defined as below 50 ng MBL/ml serum). A similar tendency could be observed for the MASP-3 concentration in Figure 5.12-B although not as pronounced as for MBL. No MASP-3 deficiency was observed.

The average MBL and MASP-3 concentrations were calculated with standard deviations (Table 5.2).

	A/A: MBL	A/A: MASP-3	A/O: MBL	A/O: MASP-3	O/O: MBL	O/O: MASP-3
Mean	4.21	3.08	0.82	2.76	0.10	2.32
Std.Dev.	3.13	1.01	0.71	0.9	0.10	0.51
No.	221	221	151	151	17	17

Table 5.2. Show the average (mean) concentration in µg/ml with standard deviation (std.) of MBL and MASP-3 in the three groups of MBL genotypes (A/A, A/O and O/O) as described in Figure 5.12. The numbers of individuals in each group are indicated (No.).

The result in table 5.2 indicated a tendency of the MASP-3 concentration being influenced by the MBL genotype. The MASP-3 concentration of MBL genotype A/A was determined to 3.08 +/- 1.01 and MBL genotype O/O to 2.32 +/- 0.51.

5.3.8 Levels in different body fluids

Serum and sperm plasma from 27 individuals were submitted to the MASP-3 TRIFMA. Sperm plasma was diluted 1/10 and 1/50 to enhance detection (see chapter 5.2.1).
The average serum level was 3.6 µg MASP-3/ml and less then 5 ng MASP-3/ml sperm plasma was detected when sperm plasma was diluted 1/10. The MASP-3 content in sperm plasma was detectable, but was considered to low to be of any major functional significance in terms of reproduction.
MASP-3 levels were in addition to sperm plasma quantified in urine, lung-, pleural-, ascites- and amnion fluids summarised in table 5.3.

	Lung fluid	Ascites fluid	Pleura fluid	Urine	Amnion fluid
ng MASP-3/ml	UD	775	500	UD	25
No. Of samples	2	1	2	3	4

Table 5.3 shows the estimated MASP-3 concentration in ng/ml of five body fluids. MASP-3 was undetectable (UD) in lung fluid diluted 1/10. Due to the low number of samples, standard deviations were omitted.

A relatively high level of MASP-3 could be detected in ascites- and pleura fluids, while the amount in urine, lung- and amnion fluids was very low. The results could only be regarded as preliminary, due to the low number of samples included which does not allow to make a more general statement.

5.4 Discussion

Native MASP-3 was found to be stable under normal storage conditions and substantial degradation was only observed when incubated at 37°C. The results regarding MASP-3 stability could be used as a validation argument of results obtained on sample collections stored for several years at -80°C and the fact that they had been analysed (thawed) before would not affect the measurement of MASP-3.

MASP-3 levels did not change during acute phase and a variation of MASP-3 concentration of +/- 40% was observed in healthy individuals over a one-year period. The theoretical possibility of MASP-3 being involved in Alzheimer's disease was not supported by experimental data, nor was there any correlation with type 1 diabetes found.

Differences in the amount of MASP-3 detectable in EDTA plasma and serum were concluded to be a consequence of the MASP-3 TRIFMA design. The assay protocol was probably not capable of separate MASP-3 in a dimeric form and despite of several attempts to standardise the assay for all sample types using high salt buffers with detergents and EDTA the result remained the same. Pre-treatment of samples with calcium and/or heating did have some positive effect but the process was too laborious and theoretical.

The result suggested that the MASP-3 TRIFMA used would have to be adjusted according to the type of samples analysed. This was a limitation of the assay and further development of the MASP-3 assay would be needed. It seemed logical to make this effort at the time of the introduction of the monoclonal anti-MASP-3 antibodies derived against the C-terminal of the MASP-3 B chain. The assay buffer could contain high salt (0.7 M - 1 M of NaCl), 1 mM calcium and additives to decrease the background.

It was decided to evaluate EDTA plasma samples with a separate set of standards. To approximate the corresponding serum MASP-3 level, multiplication by a factor 1.7 was needed.

The MASP-3 concentration in healthy individuals was found to be 1.73 +/- 0.56 µg MASP-3/ml EDTA plasma. The concentration could be compared with other human serum/plasma serine proteases to the average MBL concentration of 1.28 +/- 1.20 µg MBL/ml EDTA plasma in the same material.

The average serum level of MASP-3 from 398 individuals with type 1 diabetes was calculated to 2.92 +/- 0.97 µg MASP-3/ml serum. As expected from previous observations, the measured

level was significantly higher compared with the level estimated from the EDTA plasma samples. There was no indication of any relation between MASP-3 levels and diabetes, thus the average MASP-3 level was concluded to be representative of the general serum concentration.

The previous estimation of MASP-1 serum level (Teria *et al.* 1997) was done when the excistence of MASP-3 was not known. The sandwich assay was designed using the two anti-MASP-1/3 A chain antibodies: 1E2 and 2B11, both recognising recombinant MASP-3 (data not shown for 2B11). The published concentration was 6.27 +/- 1.85 µg MASP-1 (and MASP-3)/ml serum in a material counting 1063 healthy individuals. The average MBL concentration in the material was 1.71 +/- 1.13 µg MBL/ml serum.

Comparing the two results from serum, regardless of differences in design and methods, it could be argued that the MASP-1 concentration in serum would be in the range of: 3.35 +/- 1 µg/ml. The higher MASP-1 concentration could explain why the authors did not notice MASP-3 on their Western blots (Teria *et al.* 1997, Figure 1).

The result presented in Figure 5.7-A and -B indicating a slight correlation between the concentrations of MASP-3, MBL and H-ficolin in EDTA plasma. The correlation could be due to a mutual enhancement of protein stability upon complex formation. There was no indication of MBL/MASP-3 concentration dependence in the 398 serum samples. This strongly suggested the difference in average MASP-3 levels between EDTA plasma and serum to be the consequence of MBL/MASP-3 complex structures. The finding could be of physiological relevance, if changes in calcium level at a given site could cause an activation of MASP-3 and potentially MASP-1/-2. In the case of MASP-2 one effect could be complement activation in concert with activation of the contact system.

With a concentration of 1.3 µg MASP-3/ml serum as a minimum level detected, deficiency due to availability (plasma level) was concluded to be rare. It would therefore be sensible to screen samples for MASP-3 functional deficiency in addition to determining the actual concentration. The mannan/MBL coated surface based TRIFMA already in use could be used for screening of potential MASP-3 variants unable to associate with MBL in line with the MASP-2 mutant variant (Stengaard-Pedersen *et al.* 2003).

The potential concentration dependence between MBL and MASP could be part of the explanation of considerable variation between individuals with the same MBL genotype. A 6 fold variation in MBL levels has been seen between humans with the same genotypes

(Steffensen *et al.* 2000) and a 2.5 fold variation in MBL levels has been detected in mice with identical genotypes (Liu *et al.* 2001).

The relatively high level of H-ficolin in EDTA plasma (Yae *et al.* 1991) compared to MBL made H-ficolin an important component to consider when analysing MASP-3 complexes in EDTA plasma. The finding of a slight negative correlation between MASP-3 and L-ficolin concentrations could question the existence of such a complex, indicate an enzymatic effect of MASP-3 on L-ficolin, or indicate an influence on the oligorimerisation of L-ficolin by MASP-3. The size influence would only be realistic if the L-ficolin assay could not distinguish between different L-ficolin oligomers.

Based on previous results (Dahl et al 2001) a correlation between MASP-3 concentration and lectin pathway activation of complement was expected. Recombinant MASP-3 was found to compete with recombinant MASP-2 for binding to plasma derived MBL bound to a mannan coated surface. The result in Figure 5.7-D indicates that a more physiological experimental approach would be required to conclude MASP-2/-3 competition, due to the many components involved in the MBL/MASP complex formation.

The finding of relatively high levels of MASP-3 in pleural and ascites fluids suggested a potential function of MASP-3 to be relevant after penetration of a pathogen into the lumen or injury of the mucosa. The MASP-3 levels in pleural and ascites fluids could be explained by pathological serum leakages.

6. Future perspectives

The MASP proteins were named because of their association with MBL. However this was before the discovery of the ficolins. The nomenclature is not appropiate and should be revised so that it reflects the reality. The name MASP could be changed to LASP (lectin associated serine protease), or all the serine proteases of the complement system should be given a more systematic name like CSP1 (complement serine protease 1 for MASP-1). A problem with the last suggestion would be if MASP-3 (as example) were to have an enzymatic function outside the complement system.

The fundamental understanding of the lectin pathway of complement activation is still a matter for investigation. MBL/MASP and ficolin/MASP complexes has proven to be numerous and difficult to simplify, due to the dynamics and diversity of the complexes. Finding the actual function of MASP-1, MAp19 and MASP-3 would greatly improve the understanding of the lectin pathway.

The theoretical and physical material produced during the course of this thesis facilitates the possibility of determining the function of MASP-3 in the future. The MASP-3 sandwich TRIFMA makes it possible to identify individuals with low levels of MASP-3 and the mannan/MBL TRIFMA could identify individuals with mutated MASP-3 (functional deficient) in line with MASP-2 deficiency (Stengaard-Pedersen *et al.* 2003).

The recombinant MASP-3 can be used for the production of monoclonal antibodies, in order to obtain a range of anti-MASP-1/-3 and anti-MASP-3 antibodies capable of recognising native MASP-3. The antibodies could be coupled on beads and used for purification of native MASP-3, MASP-3 in complex with MBL, H-ficolin and possibly other ligand complexes. The monoclonal antibodies could also be used for further development of the MASP-3 TRIFMA. It would not be anticipated that introduction of novel monoclonal antibodies raised against the same epitope as the rabbits 7931/32 in the assay, would change the difference in MASP-3 levels detected in serum and EDTA plasma. The difference could only be equalised by ensuring separation of dimers and MASP-3 in complexes. Development of a TRIFMA independent of the sample type demands further buffer optimisation. The large scale production of recombinant MASP-3 and subsequent affinity purification to produce mg amounts of 95% pure MASP-3, should be exploited in attempts of crystallisation. The

production and purification could be performed in the presence of specific MASP-3 inhibitor such as leupeptin to produce non-activated protein.

Production of recombinant MASP-3 mutated in the codon encoding the Asp_{106} of MASP-3 would potentially produce a protein unable to associate with MBL and thereby reflecting the characteristic of the MASP-2 mutant (Stengaard-Pedersen *et al.* 2003). The mutant could be used for investigating the binding site of MASP-3 on MBL.

Production of recombinant MASP-3 mutated in the codon encoding the active site serine residue could clarify the activation/degradation profile of MASP-3 and be used for functional and structural studies. The mutated protein could be covalently bound to beads and used for catching a potential MASP-3 substrate without having the enzymatic function of MASP-3.

MASP-3 was found to be present in blood, cerebrospinal-, ascites-, pleura- and amnion fluid but absent in urine and lung fluid. It would be of interest to expand these studies with a larger material and include lymph fluid. The MASP-3 TRIFMA would be the preferred method in such a study.

One could also consider the possibility of MASP-3 having a more general function and not one triggered by external factors such as an infection or a tissue damage. C1q and MBL have suggested involvement in the clearance of apoptotic cells (Ogden *et al.* 2001) and this could also involve MASP-3 and potentially MASP-1. The binding of MBL to apoptotic cells, which could release intracellular calcium, could activate MASP-3. The activated MASP-3 could then cleave its substrate, which in theory could be an intracellular protein. The theory would explain the remarkably high level of homology between species throughout the evolution (Schwaeble *et al.* 2002), not allowing much diversity in the substrate selectivity of MASP-3.

Nitric-oxide synthase isozymes (Kone and Baylis 1997) responsible for the synthesis of NO from L-arginine have restricted tissue distribution and are partly controlled by calcium transients. Among the prominent roles of NO are regulation of cellular functions, energy metabolism and apoptosis (Kuncewicz *et al.* 2003).

Calpains are a family of calcium dependent proteases, which has a regulatory function in relation to mast cell adhesion during inflammation (Forsythe and Befus 2003). Extracellular NO-donor proteins has been found to regulated cell adhesion of mesangial cells (Yao *et al.* 1998) and inhibition of nitric-oxide synthase isozymes causes neutrophil adhesion to endothelium via a mast cell (Niu *et al.* 1996). S-Nitrosylation of MASP-3 suggests possible

function in relation to self-cell maintenance and cellular immunity. Collaboration with experts in this area should be established to evaluate these theories.

CL-P1, a membrane bound trimeric glycoprotein of about 300 kDa, was identified in human umbilical vein or arterial endothelial cells and placental membrane extracts. The protein has a CRD, collagen-like structure and membrane spanning region (Ohtani *et al.* 2001). Due to the collagen-like region, CL-P1 could be a candidate for MASP association. Cell surface bound MASP-3 should be analysed by incubation of endothelial cells with e.g. FITC- or texas red-labelled anti-MASP-3 antibody and evaluated by confocal microscopy. Analysis of CL-P1/MASP-3 complexes could be investigated by simultaneous incubation using fluorescently labelled anti CL-P1 antibody or by solid phase CL-P1 incubated with recombinant MASP-3.

Long term use of alcohol leads to disability of sialyltransferase and subsequently to carbohydrate-deficient proteins of both N- and O-linked glycosylation (Lakshman *et al.* 1999). It would be interesting and informative to evaluate the level of lectin pathway proteins in blood samples from alcoholics, who have higher risk of recurrent infections compared with non-alcoholics (Di Luzio *et al.* 1980 and Kawasaki *et al.* 1978).

The study could alternatively be performed using samples from new born suffering from alcohol syndrome with the perspective of treatment (Johnson *et al.* 1981).

6. Future perspectives

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7. Publications and presentations

Papers during my PhD

Schwaeble W, **Dahl MR**, Thiel S, Stover C and Jensenius JC. (2002). The Mannan-Binding Lectin-Associated Serine Proteases (MASPs) and MAp19: Four Components of the Lectin Pathway Activation Complex Encoded by Two Genes. *Immunobiol.* **205**:455-466.

Stover CM, Lynch NJ, **Dahl MR**, Hanson S, Takahashi M, Frankenberger M, Ziegler-Heitbrock L, Eperon I, Thiel S and Schwaeble WJ. (2003) Murine serine proteases MASP-1 and MASP-3, components of the lectin pathway activation complex of complement, are encoded by a single structural gene. *Genes Immun.* **4**(5):374-84.

Zundel S, Cseh S, Lacroix M, **Dahl MR**, Matsushita M, Andrieu JP, Schwaeble WJ, Jensenius JC, Fujita T, Arlaud GJ, Thielens NM. (2004). Characterization of recombinant mannan-binding lectin-associated serine protease (MASP)-3 suggests an activation mechanism different from that of MASP-1 and MASP-2 *J. Immunol* **172**(7): 4342-4350

Presentations during my PhD

Seminar at Leicester University, England Aug.2001.

Fifth International workshop on C1, Mainz, Germany Oct.2001.

Seminar at Aarhus University, Denmark Nov.2001.

Interlec, Copenhagen, Denmark 20 May 2002

Seminar at Aarhus University, Denmark Mar.2003.

Danish Society of Immunology, Annual meeting 24/3-2003

11th International congress of Immunology, Stockholm, Sweden July 26 2001

XIXth Int. Complement Workshop, Palermo, Italy Sep. 2002

References

Ambrus G, Gal P, Kojima M, Szilagyi K, Balczer J, Antal J, Graf L, Laich A, Moffatt BE, Schwaeble W, Sim RB, Zavodszky P. (2003). Natural substrates and inhibitors of mannan-binding lectin-associated serine protease-1 and -2: a study on recombinant catalytic fragments. *J Immunol.* **170**(3):1374-82.

Arif S and Mufti A. (1998). Immune, Blood, and Lymphatic systems. Mosby International Ltd. pp.77-81.

Arlaud GJ, Gagnon J. (1985). Identification of the peptide bond cleaved during activation of human C1r. *FEBS Lett.* **180**(2):234-8.

Arlaud GJ, Colomb MG and Gagnon J. (1987). A functional model of the human C1 complex Emergence of a functional model. *Immunol. Today* **8**:106-111.

Arlaud GJ, Gaboriaud C, Thielens NM, Rossi V, Bersch B, Hernandez JF, Fontecilla-Camps JC. (2001). Structural biology of C1: dissection of a complex molecular machinery. *Immunol Rev.* **180**:136-45.

Assimeh SN, Chapuis RM, Isliker H. (1978). Studies on the precursor form of the first component of complement. II. Proteolytic fragmentation of C1r. *Immunochemistry* **15**(1):13-7.

Aude CA, Lacroix MB, Arlaud GJ, Gagnon J, Colomb MG. (1988). Differential accessibility of the carbohydrate moieties of C1s-C1r-C1r-C1s, the catalytic subunit of human C1. *Biochemistry.* **27**(23):8641-8.

Barrett AJ. (1994). Classification of peptidases. *Methods Enzymol.* **244**:1-15.

Bause E. (1983). Structural requirements of N-glycosylation of proteins. Studies with proline peptides as conformational probes. *J Biochem.* **209**:331-336.

Blake MS, Johnston KH, Russell-Jones GJ, Gotschlich EC. (1984). A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. *Anal Biochem.* **136**(1):175-9.

Bond JS and Butler PE. (1987). Intracellular proteases. *Ann. Rew. Biochem.* **56**:333-64.

Bordet J and Streng O. (1906). Les phénomènes d'absorption de la conglutinin du serum de boeuf. *Ann. Inst. Pasteur.* **49**:260-276.

Bork P, Beckmann G. (1993). The CUB domain. A widespread module in developmentally regulated proteins. *J Mol Biol.* **231**(2):539-45.

Budayova-Spano M, Lacroix M, Thielens N, Arlaud G, Fontecilla-Camps J, Gaboriaud C. (2002). The Crystal Structure of the Zymogen Catalytic Domain of Complement Protease C1R Reveals that a Disruptive Mechanical Stress is Required to Trigger Activation of the C1 Complex. *Embo J.* **21** *pp.* 231.

Butenas S. and Mann KG. (2002). Blood coagulation. *Biochemistry* **67**(1):3-12.

Campbell, I.D, Bork, P. (1993). Epidermal growth factor-like modules. *Curr.Opin.Struct.Biol.* **3**:385-392.

Carroll MC. (2000). The role of complement in B cell activation and tolerance. *Adv Immunol.* **74**:61-88.

Chen CB, Wallis R. (2001). Stoichiometry of complexes between mannose-binding protein and its associated serine proteases. Defining functional units for complement activation. *J Biol Chem.* **276**(28):25894-902.

References

Colman PM. (1988). Structure of antigen-antibody complexes: Implications for recognition. *Adv Immunol.* **43**:99-132.

Cooper NR, Muller-Eberhard HJ. (1970). The reaction mechanism of human C5 in immune hemolysis. *J Exp Med.* **1**;132(4):775-93.

Covic L, Gresser AL, Kuliopulos A. (2000). Biphasic Kinetics of Activation and Signaling for PAR1 and PAR4 Thrombin Receptors in Platelets *Biochemistry,* **39**(18):5458 -67.

Cregg JM, Vedvick TS, Raschke WC. (1993). Recent advances in the expression of foreign genes in Pichia pastoris. *Biotechnology* **11**(8):905-10.

Cseh S, Gal P, Sarvari M, Dobo J, Lorincz Z, Schumaker VN, Zavodszky P. (1996). Functional effects of domain deletions in a multidomain serine protease, C1r. *Mol Immunol.* **33**(4-5):351-9.

Dahl MR, Thiel S, Matsushita M, Fujita T, Willis AC, Christensen T, Vorup-Jensen T, Jensenius JC. (2001). MASP-3 and its association with distinct complexes of the mannan-binding lectin complement activation pathway. *Immunity* **15**(1):127-35.

Day AJ, Barlow PN, Steinkasserer A, Campbell ID, Sim RB. (1993). Progress in determining module structures in C1r and C1s. *Behring Inst Mitt.* **93**:31-40.

Delves PJ, Roitt IM. 2000. The immune system (2/2). *N Engl J Med.* **343**(2):108-17.

Delves PJ, Roitt IM. 2000. The immune system (1/2). *N Engl J Med.* **343**(1):37-49.

Di Luzio NR, Williams DL. (1980). Enhancement of host susceptibility to Staphylococcus aureus infection by chronic ethanol ingestion--modification by glucan immunostimulation. *Alcohol Clin Exp Res.* **4**(3):254-60.

Doolittle RF. (1985). The genealogy of some recently evolved vertebrate proteins. *Trend Biochem Sci.* **10**:233-237.

Drickamer K. (1988). Two distinct classes of carbohydrate-recognition domains in animal lectins. *J.Biol.Chem.* **263**:9557-60.

Drickamer K. (1993). Evolution of Ca^{2+}-dependent animal lectins. Proc. Nucl. Acid Res. Mol. Biol. **45**:207-32.

Elbein AD. (1987). Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. *Annu Rev Biochem.* **56**:497-534.

Endo Y, Takahashi M, Nakao M, Saiga H, Sekine H, Matsushita M, Nonaka M, Fujita T. (1998). Two lineages of mannose-binding lectin-associated serine protease (MASP) in vertebrates. *J Immunol.* **161**(9):4924-30.

Endo Y, Nonaka M, Saiga H, Kakinuma Y, Matsushita A, Takahashi M, Matsushita M, Fujita T. (2003). Origin of mannose-binding lectin-associated serine protease (MASP)-1 and MASP-3 involved in the lectin complement pathway traced back to the invertebrate, amphioxus. *J Immunol.* **170**(9):4701-7.

Ezekowitz RA, Day LE, Herman GA. (1988). A human mannose-binding protein is an acute-phase reactant that shares sequence homology with other vertebrate lectins. *J Exp Med.* **167**(3):1034-46.

Fearon DT. (1979). Regulation of the amplification C3 convertase of human complement by an inhibitory protein isolated from human erythrocyte membrane. *Proc Natl Acad Sci U S A.* **76**(11):5867-71.

References

Fearon DT, Locksley RM. (1996). The instructive role of innate immunity in the acquired immune response. *Science* **272**:50-53

Feinberg H, Uitdehaag JC, Davies JM, Wallis R, Drickamer K, Weis WI. (2003). Crystal structure of the CUB1-EGF-CUB2 region of mannose-binding protein associated serine protease-2. *EMBO J.* **22**(10):2348-59.

Fingeroth JD, Weis JJ, Tedder TF, Strominger JL, Biro PA, Fearon DT. (1984). Epstein-Barr virus receptor of human B lymphocytes is the C3d receptor CR2. *Proc Natl Acad Sci.* **81**(14): 4510-4. Fleischman JB, Pain RH, Porter RR. (1962). Reduction of gamma-globulins. *Arch Biochem Biophys.* **1**:174-80.

Forsythe P, Befus AD. (2003). Inhibition of calpain is a component of nitric oxide-induced down-regulation of human mast cell adhesion. *J Immunol.* **170**(1):287-93.

Freer ST, Kraut J, Robertus JD, Wright HT, Xuong NH. (1970). hymotrypsinogen: 2.5-angstrom crystal structure, comparison with alpha-chymotrypsin, and implications for zymogen activation. *Biochemistry* **9**(9):1997-2009.

Gaboriaud C, Rossi V, Fontecilla-Camps JC, Arlaud GJ. (1998). Evolutionary conserved rigid module-domain interactions can be detected at the sequence level: the examples of complement and blood coagulation proteases. *J Mol Biol.* **282**(2):459-70.

Gaboriaud C, Rossi V, Bally I, Arlaud GJ, Fontecilla-Camps JC. (2000). Crystal structure of the catalytic domain of human complement c1s: a serine protease with a handle. *EMBO J.* **19**(8):1755-65.

Garred P, Madsen HO, Hofmann B, Svejgaard A. (1995). Increased frequency of homozygosity of abnormal mannan-binding-protein alleles in patients with suspected immunodeficiency. *Lancet* **346**(8980):941-3.

Georgio G. (1996). Expression of proteins in bacteria. In Protein Engineering, Wiley-Liss, NY 101-127.

Gilberto R. Sambrano and Shaun R. Coughlin. (1999). The Carboxyl Tail of Protease-activated Receptor-1 Is Required for Chemotaxis *J Biol Chem* 274(29):20178-20184.

Guesdon JL, Ternynck T, Avrameas S. (1979). The use of avidin-biotin interaction in immunoenzymatic techniques. *J Histochem Cytochem.* **27**(8):1131-9.

Guo N, Mogues T, Weremowicz S, Morton CC, Sastry KN. (1998). The human ortholog of rhesus mannose-binding protein-A gene is an expressed pseudogene that localizes to chromosome 10. *Mammalian Genome* **9**:246-249.

Hajela K, Kojima M, Ambrus G, Wong KH, Moffatt BE, Ferluga J, Hajela S, Gal P, Sim RB. (2002). The biological functions of MBL-associated serine proteases (MASPs). *Immunobiology* **205**(4-5):467-75.

Hansen S, Holm D, Moeller V, Vitved L, Bendixen C, Reid KB, Skjoedt K, Holmskov U. (2002) CL-46, a novel collectin highly expressed in bovine thymus and liver. *J Immunol.* **169**(10):5726-34.

Hansen TK, Thiel S, Knudsen ST, Gravholt CH, Christiansen JS, Mogensen CE, Poulsen PL. (2003). Elevated levels of mannan-binding lectin in patients with type 1 diabetes. *J Clin Endocrinol Metab.* **88**(10):4857-61.

References

Hartshorn KL, Crouch EC, White MR, Eggleton P, Tauber AI, Chang D, Sastry K. (1994). Evidence for a protective role of pulmonary surfactant protein D (SP-D) against influenza A viruses. *J. Clin. Invest.* **94**: 311-319).

Heimark RL, Kurachi K, Fujikawa K, Davie EW. (1980). Surface activation of blood coagulation, fibrinolysis and kinin formation. *Nature.* **286**(5772):456-60.

Hemmila I, Dakubu S, Mukkala VM, Siitari H, Lovgren T. (1984) Europium as a label in time-resolved immunofluorometric assays. *Anal Biochem.* **137**(2):335-43.

Hess D, Schaller J, Rickli EE. 1991 Identification of the disulfide bonds of human complement C1s. *Biochemistry* **30**(11):2827-33.

Holmskov U, Thiel S, Jensenius JC. (2003). Collectins and ficolins: humoral lectins of the innate immune defense. *Annu. Rev. Immunol.* **21**:547-78.

Horstmann RD, Pangburn MK, Muller-Eberhard HJ. (1985). Species specificity of recognition by the alternative pathway of complement. *J Immunol.* **134**:1101-1104.

Hoover RR and Floros J. (1998). Organization of human SP-A and SP-D at 10q22-q23: physical and radiation hybrid mapping reveal gene order and orientation. *Am. J. Respr. Cell Mol. Biol.* **18**:353-362.

Illy C, Thielens NM, Gagnon J, Arlaud GJ. (1991). Effect of lactoperoxidase-catalyzed iodination on the Ca(2+)-dependent interactions of human C1s. Location of the iodination sites. *Biochemistry* **30**(29):7135-41.

Ikeda K, Sannoh T, Kawasaki N, Kawasaki T, Yamashina I. (1987). Serum lectin with known structure activates complement through the classical pathway. *J. Biol. Chem.* **262**:7451-54.

Inai S, Akagaki Y, Moriyama T, Fukumori Y, Yoshimura K, Ohnoki S, Yamaguchi H. (1989). Inherited deficiencies of the late-acting complement components other than C9 found among healthy blood donors. *Int Arch Allergy Appl Immunol.* **90**(3):274-9.

Janeway CA. and Travers P. 1997, Immunobiology, 3rd ed. Current Biology Ltd., Garland Publishing Inc., NY-London.

Jentoft N. (1990). Why are proteins O-glycosylated? *Trends Biochem Sci.* **15**:291-294.

Johnson S, Knight R, Marmer DJ, Steele RW. (1981). Immune deficiency in fetal alcohol syndrome. *Pediatr Res.* **15**(6):908-11.

Kan C, Solomon E, Belt K T, Chain AC, Hiorns LR, Fey G. (1985). Nucleotide sequence of cDNA encoding human alpha-2-macroglobulin and assignment of the chromosomal locus. *Proc. Nat. Acad. Sci.* **82**: 2282-2286.

Katyal SL, Singh G, Locker J. (1992). Characterization of a second human pulmonary surfactant-associated protein SP-A gene. *Am J Respir Cell Mol Biol.* **6**(4):446-52.

Kawakami M, Meyer AA, Johnson MC, deSerres S, Peterson HD. (1990). Chronic ethanol exposure before injury produces greater immune dysfunction after thermal injury in rats. *J Trauma.* **30**(1):27-31.

Kawasaki T, Etoh R, Yamashina I. (1978). Isolation and characterization of a mannan-binding protein from rabbit liver. *Biochem Biophys Res Commun.* **81**(3):1018-24.

References

Kawasaki N, Kawasaki T, Yamashina I. (1983). Isolation and characterization of a mannan-binding protein from human serum. *J. Biochem.* (Tokyo). **94** (3):937-47.

Kawasaki N, Kawasaki T, Yamashina I. (1985). Mannan-binding protein and conglutinin in bovine serum. *J Biochem* (Tokyo). **98**(5):1309-20.

Kilpatrick DC. (2002). Mannan-binding lectin: clinical significance and applications. *Biochim Biophys Acta.* **1572**(2-3):401-13.

Klickstein LB, Barbashov SF, Liu T, Jack RM, Nicholson-Weller A. (1997). Complement receptor type 1 (CR1, CD35) is a receptor for C1q. *Immunity* 7(3): 345-55.

Kramer RZ, Bella J, Mayville P, Brodsky B, Berman HM. (1999). Sequence dependent conformational variations of collagen triple-helical structure. *Nat Struct Biol.* **6** *pp.* 454.

Krem MM, Rose T, Di Cera E. (1999). The C-terminal sequence encodes function in serine proteases. *J Biol Chem.* **274**(40):28063-6.

Kolble K, Lu J, Mole SE, Kaluz S, Reid KB. (1993). Assignment of the human pulmonary surfactant protein D gene (SFTP4) to 10q22-q23 close to the surfactant protein A gene cluster. *Genomics* **17**(2):294-8.

Kone BC, Baylis C. (1997). Biosynthesis and homeostatic roles of nitric oxide in the normal kidney. *Am J Physiol.* **272**(5 Pt 2):F561-78.

Kozutsumi Y, Kawasaki T, Yamashina I. (1980). Isolation and characterization of a mannan-binding protein from rabbit serum. *Biochem Biophys Res Commun* **95**(2):658-64).

Kuncewicz T, Sheta EA, Goldknopf IL, Kone BC. 2003. Proteomic analysis of s-nitrosylated proteins in mesangial cells. *Mol Cell Proteomics.* **2**(3):156-63.

Lanzrein AS, Jobst KA, Thiel S, Jensenius JC, Sim RB, Perry VH, Sim E. (1998). Mannan-binding lectin in human serum, cerebrospinal fluid and brain tissue and its role in Alzheimer's disease. *Neuroreport.* **9**(7):1491-5.

Laursen SB, Dalgaard TS, Thiel S, Lim BL, Jensen TV, Juul-Madsen HR, Takahashi A, Hamana T, Kawakami M, Jensenius JC. (1998). Cloning and sequencing of a cDNA encoding chicken mannan-binding lectin (MBL) and comparison with mammalian analogues. *Immunology* **93**(3):421-30.

Lakshman MR, Rao MN, Marmillot P. (1999). Alcohol and molecular regulation of protein glycosylation and function. *Alcohol.* **19**(3):239-47.

Law SKA, Reid KBM. (1995). Complement, 2^{ed}, IRL Press, Oxford, UK, pp88.

Lipscombe RJ, Lau YL, Levinsky RJ, Sumiya M, Summerfield JA, Turner MW. (1992). Identical point mutation leading to low levels of mannose binding protein and poor C3b mediated opsonisation in Chinese and Caucasian populations. *Immunol Lett.* **32**(3):253-7.

Lipscombe RJ, Sumiya M, Hill AV, Lau YL, Levinsky RJ, Summerfield JA, Turner MW. ((1992(b)). High frequencies in African and non-African populations of independent mutations in the mannose binding protein gene. *Hum Mol Genet.* **1**(9):709-15.

Liu H, Jensen L, Hansen S, Petersen SV, Takahashi K, Ezekowitz AB, Hansen FD, Jensenius JC, Thiel S. (2001). Characterization and quantification of mouse mannan-binding lectins (MBL-A and MBL-C) and study of acute phase responses. *Scand J Immunol.* **53**(5):489-97.

References

Lu JH, Thiel S, Wiedemann H, Timpl R, Reid KB. (1990). Binding of the pentamer/hexamer forms of mannan-binding protein to zymosan activates the proenzyme C1r2C1s2 complex, of the classical pathway of complement, without involvement of C1q. *J Immunol.* **144(6)**:2287-94.

Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R. (2001). Proteinase-activated receptors. *Pharmacol Rev.* 53(2):245-82.

Madsen HO, Garred P, Kurtzhals JA, Lamm LU, Ryder LP, Thiel S, Svejgaard A. (1994). A new frequent allele is the missing link in the structural polymorphism of the human mannan-binding protein. *Immunogenetics* **40**(1):37-44.

Madsen HO, Garred P, Thiel S, Kurtzhals JA, Lamm LU, Ryder LP, Svejgaard A. (1995). Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein. *J Immunol.* **155**(6):3013-20.

Madsen HO, Satz ML, Hogh B, Svejgaard A, Garred P. (1998). Different molecular events result in low protein levels of mannan-binding lectin in populations from southeast Africa and South America. *J Immunol.* **161**(6):3169-75.

Malhotra R, Haurum JS, Thiel S, Sim RB. (1994). Binding of human collectins (SP-A and MBP) to influenza virus. *Biochem J.* **304** (Pt 2):455-61.

Matsushita M and Fujita T. (1992). Activation of the classical complement pathway by mannose-binding protein in association with a novel C1s-like serine protease. *J. Exp. Med.* **176**:1497-502.

Matsushita M, Fujita T. (1995). Cleavage of the third component of complement (C3) by mannose-binding protein-associated serine protease (MASP) with subsequent complement activation. Immunobiology **194**:443-448.

Matsushita M, Ezekowitz RA, Fujita T. (1995). The Gly-54-->Asp allelic form of human mannose-binding protein (MBP) fails to bind MBP-associated serine protease. *Biochem J.* **311**(Pt 3):1021-3.

Matsushita M, Thiel S, Jensenius JC, Terai I, Fujita T. (2000). Proteolytic activities of two types of mannose-binding lectin-associated serine protease. *J Immunol.* **165**(5):2637-42.

Matsushita M, Endo Y, Fujita T. (2000). Cutting edge: complement-activating complex of ficolin and mannose-binding lectin-associated serine protease. *J Immunol.* **164**(5):2281-4.

Matsushita M. and Fujita T. (2001). Ficolins and the lectin complement pathway. *J. Immunol. Rev.* **180**:78-85.

Matsushita M, Kuraya M, Hamasaki N, Tsujimura M, Shiraki H, Fujita T. (2002). Activation of the lectin complement pathway by H-ficolin (Hakata antigen). *J Immunol.* **168**(7):3502-6.

Medicus RG, Gotze O, Muller-Eberhard HJ. (1976). Alternative pathway of complement: recruitment of precursor properdin by the labile C3/C5 convertase and the potentiation of the pathway. *J Exp Med.* **144**(4):1076-93.

Medof ME, Kinoshita T, Nussenzweig V. (1984). Inhibition of complement activation on the surface of cells after incorporation of decay-accelerating factor (DAF) into their membranes. *J Exp Med.* **160**(5): 1558-78.

References

Meuth JL, Morgan EL, DiSipio RG, Hugli TE. (1983). Suppression of T lymphocyte functions by human C3 fragments. I. Inhibition of human T cell proliferative responses by a kallikrein cleavage fragment of human iC3b. *J Immunol.* **130**(6):2605-11.

Miller ME, Seals J, Kaye R, Levitsky LC. (1968). A familial, plasma-associated defect of phagocytosis: a new cause of recurrent bacterial infections. *Lancet* **2**: 60-63.

Mizuno Y, Kozutsumi Y, Kawasaki T, Yamashina I. (1981). Isolation and characterization of a mannan-binding protein from rat liver. *J Biol Chem.* **256**(9):4247-52.

Moller-Kristensen M, Thiel S, Hansen AG, Jensenius JC. (2003). On the site of C4 deposition upon complement activation via the mannan-binding lectin pathway or the classical pathway. *Scand J Immunol.* **57**(6):556-61

Morgan BP. (1999). Regulation of the complement membrane attack pathway. *Crit Rev Immunol.* **19**(3):173-98.

Muller-Eberhard HJ. (1986). The membrane attack complex. *Annu Rev immunol.* **4**:503-528.

Mullertz S. (1986). Fibrinolysis, general aspects, characteristic features and perspectives. *Fibrinolysis* **1**:3-12.

Najakajima T. and Ballou CE. 1974. Characterization of the carbohydrate fragment obtained from Saccharomyces e'cerevisiae mannan by alkaline degradation. *J. Biol. Chem.* **249**:7679-84.

Nemerow GR, Wolfert R, McNaughton ME, Cooper NR. (1985). Identification and characterization of the Epstein-Barr virus receptor on human B lymphocytes and its relationship to the C3d complement receptor (CR2). *J Virol.* **55**(2): 347-51.

Nicholson-Weller A, Burge J, Fearon DT, Weller PF, Austen KF. (1982). Isolation of a human erythrocyte membrane glycoprotein with decay-accelerating activity for C3 convertases of the complement system. *J Immunol.* **129**(1): 184-9.

Niu XF, Ibbotson G, Kubes P. (1996). A balance between nitric oxide and oxidants regulates mast cell-dependent neutrophil-endothelial cell interactions. *Circ Res.* **79**(5):992-9.

Nonaka M, Miyazawa S. (2002). Evolution of the initiating enzymes of the complement system. *Genome Biol.* **3**(1):REVIEWS1001.

Norman DG, Barlow PN, Baron M, Day AJ, Sim RB, Campbell ID. (1991). Three-dimensional structure of a complement control protein module in solution. *J Mol Biol.* **219**(4):717-25.

Ogata RT, Low PJ, Kawakami M. (1995). Substrate specificities of the protease of mouse serum Ra-reactive factor. *J Immunol.* **154**(5):2351-7.

Ogden CA, deCathelineau A, Hoffmann PR, Bratton D, Ghebrehiwet B, Fadok VA, Henson PM. (2001). C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. *J Exp Med.* **194**(6):781-95.

Ohsawa K, Ebata N. (1983) Silver stain for detecting 10-femtogram quantities of protein after polyacrylamide gel electrophoresis. *Anal Biochem.* **135**(2):409-15.

Ohtani K, Suzuki Y, Eda S, Kawai T, Kase T, Keshi H, Sakai Y, Fukuoh A, Sakamoto T, Itabe H, Suzutani T, Ogasawara M, Yoshida I, Wakamiya N. (2001). The membrane-type collectin CL-P1 is a scavenger receptor on vascular endothelial cells. *J Biol Chem.* **276**(47):44222-8.

References

Ohtani K, Suzuki Y, Eda S, Kawai T, Kase T, Yamazaki H, Shimada T, Keshi H, Sakai Y, Fukuoh A, Sakamoto T, Wakamiya N. (1999). Molecular cloning of a novel human collectin from liver (CL-L1). *J Biol Chem.* **274**(19):13681-9.

Oka S, Ikeda K, Kawasaki T, Yamashina I. (1988). Isolation and characterization of two distinct mannan-binding proteins from rat serum. *Arch Biochem Biophys.* **260**(1):257-66.

Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y. (1999). Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. *Circulation* **100**: 2473-2476.

Ouchi N, Kihara S, Arita Y, Okamoto Y, Maeda K, Kuriyama H, Hotta K, Nishida M, Takahashi M, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y. (2000). Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-kappa-B signaling through a cAMP-dependent pathway. *Circulation* **102**: 1296-1301.

Pangburn MK. (2000). Host recognition and target differentiation by factor H, a regulator of the alternative pathway of complement. *Immunopharmacology* **49**(1-2): 149-57.

Patthy L. (1985). Evolution of the proteases of blood coagulation and fibrinolysis by assembly from modules. *Cell.* **41**(3):657-63.

Perona JJ and Craik CS. (1995). Structural basis of substrate specificity in the serine proteases. *Protein science* **4**:337-360.

Perona JJ, Craik CS. (1997). Evolutionary divergence of substrate specificity within the chymotrypsin-like serine protease fold. *J Biol Chem.* **272**:29987-29990.

Persson A, Rust K, Chang D, Moxley M, Longmore W, Crouch E. (1988). CP4: a pneumocyte-derived collagenous surfactant-associated protein. Evidence for heterogeneity of collagenous surfactant proteins. *Biochemistry* **27**:8576-84.

Petersen SV, Thiel S, Jensen L, Vorup-Jensen T, Koch C, Jensenius JC. (2000). Control of the classical and the MBL pathway of complement activation. *Mol Immunol.* **37**(14):803-11.

Pierce JV. (1968). Structural features of plasma kinins and kininogens *Fed. Proc.* **27**(1), 52-57.

Pikaar JC. (1995). Opsonic activities of surfactant proteins A and D in phagocytosis of gram-negative bacteria by alveolar macrophages. *J Infect Dis.* **172**(2):481-9.

Rao Z, Handford P, Mayhew M, Knott V, Brownlee GG, Stuart D. (1995). The structure of a Ca(2+)-binding epidermal growth factor-like domain: its role in protein-protein interactions. *Cell* **82**(1):131-41.

Ratnoff WD. (1996). Inherited deficiencies of complement in rheumatic diseases. *Rheum Dis Clin North Am.* **22**(1):75-94.

Rawlings ND, Barrett AJ. (1994). Families of serine peptidases. *Methods Enzymol.* **244**:19-61.

Reid KBM, Bently DR, Campbell RD, Chung LP, Sim RB, Kristensen T, Tack BF. (1986). Complement system proteins which interact with C3b or C4b. A superfamily of structurally related proteins. *Immunol Today* 7:230-234.

Rojkjaer R, Schmaier AH. (1999). Activation of the plasma kallikrein/kinin system on endothelial cells. *Proc Assoc Am Physicians.* **111**(3):220-7.

References

Romero A, Romao MJ, Varela PF, Kolln I, Dias JM, Carvalho AL, Sanz L, Topfer-Petersen E, Calvete JJ. (1997). The crystal structures of two spermadhesins reveal the CUB domain fold. *Nat Struct Biol.* **4**(10):783-8.

Ross GD, Medof ME. (1985). Membrane complement receptors specific for bound fragments of C3. *Adv Immunol.* **37**:217-267.

Rossi V, Cseh S, Bally I, Thielens NM, Jensenius JC, Arlaud GJ. (2001) Substrate specificities of recombinant mannan-binding lectin-associated serine proteases-1 and -2. *J Biol Chem.* **276**(44):40880-7.

Sastry K, Herman GA, Day L, Deignan E, Bruns G, Morton CC, Ezekowitz RA. (1989). The human mannose-binding protein gene: exon structure reveals its evolutionary relationship to a human pulmonary surfactant gene and localization to chromosome 10. *J. Exp. Med.* **170**:1175-1189.

Schapira M, de Agostini A, Schifferli JA, Colman RW. (1985). Biochemistry and pathophysiology of human C1 inhibitor. *Complement* **2**(2-3):111-26.

Schechter I, Berger A. (1967). On the size of the active site in proteases. I. Papain. *Biochem Biophys Res Commun.* **27**(2):157-62.

Schäfer, M. K.-H., R. Day. (1995). In situ hybridization techniques to map processing enzymes. *Methods Neurosci. 23:16.*

Schwaeble W, Dahl MR, Thiel S, Stover C, Jensenius JC. (2002). The mannan-binding lectin-associated serine proteases (MASPs) and MAp19: four components of the lectin pathway activation complex encoded by two genes. *Immunobiology.* **205**(4-5):455-66.

Seya T, Atkinson JP. (1989). Functional properties of membrane cofactor protein of complement. *Biochem J.* **264**(2): 581-8.

Shariat-Madar Z, Mahdi F, Schmaier AH. (2002). Assembly and activation of the plasma kallikrein/kinin system: a new interpretation. *Int Immunopharmacol.* **2**(13-14):1841-9.

Sheriff S, Chang CY, Ezekowitz RA. (1994). Human mannose-binding protein carbohydrate recognition domain trimerizes through a triple alpha-helical coiled-coil. *Nat Struct Biol.* **1**(11):789-94.

Sim RB, Arlaud GJ, Colomb MG. (1979). C1 inhibitor-dependent dissociation of human complement component C1 bound to immune complexes. *Biochem J. 179*(3):449-57.

Sim RB, Reid KB. (1991). C1: molecular interactions with activating systems. *Immunol Today.* **12**:307-311.

Sim RB, Day AJ, Moffatt BE, Fontaine M. (1993). Complement factor I and cofactors in control of complement system convertase enzymes. *Methods Enzymol.* **223**:13-35.

Steffensen R, Thiel S, Varming K, Jersild C, Jensenius JC. (2000). Detection of structural gene mutations and promoter polymorphisms in the mannan-binding lectin (MBL) gene by polymerase chain reaction with sequence-specific primers. *J Immunol Methods.* **241**(1-2):33-42.

Stengaard-Pedersen K, Thiel S, Gadjeva M, Moller-Kristensen M, Sorensen R, Jensen LT, Sjoholm AG, Fugger L, Jensenius JC. (2003). Inherited deficiency of mannan-binding lectin-associated serine protease 2. *N Engl J Med.* **349**(6):554-60.

References

Stover CM, Schwaeble WJ, Lynch NJ, Thiel S, Speicher MR. (1999). Assignment of the gene encoding mannan-binding lectin-associated serine protease 2 (MASP2) to human chromosome 1p36.3-->p36.2 by in situ hybridization and somatic cell hybrid analysis. *Cytogenet Cell Genet.* **84**(3-4):148-9.

Stover CM, Thiel S, Thelen M, Lynch NJ, Vorup-Jensen T, Jensenius JC, Schwaeble WJ. (1999b).Two constituents of the initiation complex of the mannan-binding lectin activation pathway of complement are encoded by a single structural gene. *J Immunol.* **162**:3481-3490.

Stover CM, Lynch NJ, Dahl MR, Hanson S, Takahashi M, Frankenberger M, Ziegler-Heitbrock L, Eperon I, Thiel S, Schwaeble WJ. (2003). Murine serine proteases MASP-1 and MASP-3, components of the lectin pathway activation complex of complement, are encoded by a single structural gene. *Genes Immun.* **4**(5):374-84.

Stroud RM. (1974) A family of protein-cutting proteins. *Sci Amer.* **231**:74-88.

Sumiya M, Super M, Tabona P, Levinsky RJ, Arai T, Turner MW, Summerfield JA. (1991). Molecular basis of opsonic defect in immunodeficient children. *Lancet* **337**(8757):1569-70.

Summerfield JA, Ryder S, Sumiya M, Thursz M, Gorchein A, Monteil MA, Turner MW. (1995). Mannose binding protein gene mutations associated with unusual and severe infections in adults. *Lancet* **345**(8954):886-9.

Super M, Thiel S, Lu J, Levinsky RJ, Turner MW. (1989). Association of low levels of mannan-binding protein with a common defect in opsonisation. *Lancet* **2**:1236-1239.

Takada F, Takayama Y, Hatsuse H, Kawakami M. (1993). A new member of the C1s family of complement proteins found in a bactericidal factor, Ra-reactive factor, in human serum. *Biochem Biophys Res Commun.* **196**(2):1003-9.

Takahashi M, Endo Y, Fujita T, Matsushita M. (1999). A truncated form of mannose-binding lectin-associated serine protease (MASP)-2 expressed by alternative polyadenylation is a component of the lectin complement pathway. *Int Immunol.* **11**(5):859-63.

Takahashi M. Miura S. Ishii N. Matsushita M. Endo Y. Sugamura K. Fujital T. (2000). An essential role of MASP-1 in activation of the lectin pathway *Immunopharmacology* **49**(1) 3

Takahashi M, Minura S, Ishii N, Matsushita M, Endo Y, Sugamura K, Fujita T. (2001). Activation of the complement lectin pathway in MASP-1 deficient mice. Vth International Workshop on C1, the first component of complement, and collectins. Mainz, Germany, October 26-28, 2001, Abstract III-9.

Taylor ME, Brickell PM, Craig RK, Summerfield JA. (1989). Structure and evolutionary origin of the gene encoding a human serum mannose-binding protein. *Biochem. J.* **262**:763-771.

Teh C, Le Y, Lee SH, Lu J. (2000). M-ficolin is expressed on monocytes and is a lectin binding to N-acetyl-D-glucosamine and mediates monocyte adhesion and phagocytosis of Escherichia coli. *Immunology* **101**(2):225-32.

Terai I, Kobayashi K, Matsushita M, Fujita T, Matsuno K. (1995). alpha 2-Macroglobulin binds to and inhibits mannose-binding protein-associated serine protease. *Int Immunol.* **7**(10): 1579-84.

Terai I, Kobayashi K, Matsushita M, Fujita T. (1997). Human serum mannose-binding lectin (MBL)-associated serine protease-1 (MASP-1): determination of levels in body fluids and identification of two forms in serum. *Clin Exp Immunol.* **110**(2):317-23.

References

Thiel S, Vorup-Jensen T, Stover CM, Schwaeble W, Laursen SB, Poulsen K, Willis AC, Eggleton P, Hansen S, Holmskov U, Reid KB, Jensenius JC. (1997). A second serine protease associated with mannan-binding lectin that activates complement. *Nature* **386**(6624):506-10.

Thiel S, Petersen SV, Vorup-Jensen T, Matsushita M, Fujita T, Stover CM, Schwaeble WJ, Jensenius JC. (2000). Interaction of C1q and mannan-binding lectin (MBL) with C1r, C1s, MBL-associated serine proteases 1 and 2, and the MBL-associated protein MAp19. *J Immunol.* **165**(2):878-87.

Thielens NM, Cseh S, Thiel S, Vorup-Jensen T, Rossi V, Jensenius JC, Arlaud GJ. (2001). Interaction properties of human mannan-binding lectin (MBL)-associated serine proteases-1 and -2, MBL-associated protein 19, and MBL. *J Immunol.* **166**(8):5068-77.

Thielens NM, Van Dorsselaer A, Gagnon J, Arlaud GJ. (1990). Chemical and functional characterization of a fragment of C1-s containing the epidermal growth factor homology region. *Biochemistry* **29**(14):3570-8.

Tschopp J, Villiger W, Fuchs H, Kilchherr E, Engel J. (1980). Assembly of subcomponents C1r and C1s of first component of complement: electron microscopic and ultracentrifugal studies. *Proc Natl Acad Sci USA.* 77(12):7014-8.

Turner MW, Hamvas RM. (2000). Mannose-binding lectin: structure, function, genetics and disease associations. *Rev Immunogenet.* **2**(3):305-22.

Villiers CL, Arlaud GJ, Colomb MG. (1985) Domain structure and associated functions of subcomponents C1r and C1s of the first component of human complement. *Proc Natl Acad Sci U S A.* **82**(13):4477-81.

Villiers MB, Thielens NM, Reboul A, Colomb MG. (1982). A study of a covalent-like interaction between soluble nascent C4b and C4-binding protein. *Biochim Biophys Acta.* **704**(2):197-203.

Vorup-Jensen T, Petersen SV, Hansen AG, Poulsen K, Schwaeble W, Sim RB, Reid KB, Davis SJ, Thiel S, Jensenius JC. (2000). Distinct pathways of mannan-binding lectin (MBL)- and C1-complex autoactivation revealed by reconstitution of MBL with recombinant MBL-associated serine protease-2. *J Immunol.* **165**(4):2093-100

Wallis R, Drickamer K. (1999). Molecular determinants of oligomer formation and complement fixation in mannose-binding proteins. *J Biol Chem.* **274**(6):3580-9.

Wallis R, Dodd RB. (2000). Interaction of mannose-binding protein with associated serine proteases: effects of naturally occurring mutations. *J Biol Chem.* **275**(40):30962-9.

Wallevik K, Jensenius JC. (1982). A simple and reliable method for the drying of polyacrylamide slab gels. *J Biochem Biophys Methods.* **6**(1):17-21.

Warshel A, Naray-Szabo G, Sussman F, Hwang JK. (1989). How do serine proteases really work? *Biochemistry* **28**(9):3629-37.

Weber K, Osborn M. (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J Biol Chem.* **244**(16):4406-12.

Weis WI, Drickamer K, Hendrickson WA. (1992). Structure of a C-type mannose-binding protein complexed with an oligosaccharide. *Nature* **360**(6400):127-34.

Weis WI. Drickamer K. (1994). Trimeric structure of a C-type mannose-binding protein. *Structure* **2**(12):1227-40.

References

White RT, Damm D, Miller J, Spratt K, Schilling J, Hawgood S, Benson B, Cordell B. (1985). Isolation and characterization of human pulmonary surfactant apoprotein gene. *Nature* **317**:361-63.

Wiggins RC, Giclas PC, Henson PM. (1981). Chemotactic activity generated from the fifth component of complement by plasma kallikrein of the rabbit. *J Exp Med.* **153**(6):1391-404.

Wirthmueller U, Dewald B, Thelen M, Schafer MK, Stover C, Whaley K, North J, Eggleton P, Reid KB, Schwaeble WJ. (1997). Properdin, a positive regulator of complement activation, is released from secondary granules of stimulated peripheral blood neutrophils. *J Immunol.* **158**(9): 4444-51.

Wong NK, Kojima M, Dobo J, Ambrus G, Sim RB. (1999). Activities of the MBL-associated serine proteases (MASPs) and their regulation by natural inhibitors. *Mol Immunol.* **36**(13-14):853-61.

Y. Yae, S. Inaba, H. Sato, K. Okochi, F. Tokunaga and S. Iwanaga , Isolation and characterization of a thermolabile β-2 macroglycoprotein (thermolabile substance or Hakata antigen) detected by precipitating (auto) antibody in sera of patients with systemic lupus erythematosus. *Biochim. Biophys. Acta* **1078** (1991), pp. 369–376.

Yao J, Schoecklmann HO, Prols F, Gauer S, Sterzel RB. (1998). Exogenous nitric oxide inhibits mesangial cell adhesion to extracellular matrix components. *Kidney Int.* **53**(3):598-608.

Yokota T, Oritani K, Takahashi I, Ishikawa J, Matsuyama A, Ouchi N, Kihara S, Funahashi T, Tenner AJ, Tomiyama Y, Matsuzawa Y. (2000). Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. *Blood* **96**:1723-1732.

Ziccardi RJ. (1981). Activation of the early components of the classical complement pathway under physiologic conditions. *J Immunol.* **126**(5): 1769-73.

Appendix

Appendix 1

Protein	Plasma Conc. (μg/ml)	Zymogen Mw (kDa)	Substrate	Domain structure	Accession No.	Chromosom location
Factor D	2	25	Factor B	S	M84526	19p13
C1r	34	173	C1s	CECPPS	X04701	12p13
C1s	31	80	C2 and C4	CECPPS	X06596	12p13
MASP-1	4	93	C3 and C2 ?	CECPPS	D28593	3q27
MASP-2	0,5	76	C2 and C4	CECPPS	Y09926	1q36
MASP-3	2	105	?	CECPPS	AF284421	3q27
Factor B	180	90	C3 and C5	PPPVS	L15702	6p21
C2	23	100	C3 and C5	PPPVS	X04481	6p21
Factor I	35	88	C3b and C4b	FDLLS	Y00318	4q25
Plasminogen	100	92	Fibrin	AKKKKKS	NP000292	6q26
Factor XII	30	80	FXI	N2EN1EKS	P00748	5q33
Prekallikrein	50	86	FXII	AAAAS	P03952	4q35
Prothrombin	150	72	Prothrombin	GKKS	P00734	11p11
Factor X	10	59	FXIII,FG,PAR	GES	P00742	13q34
Factor IX	5	56	FX	GES	P00740	Xq27
Factor XI	5	160	FIX	AAAAS	P03951	4q35
Factor VII	0,5	50	FIX and FX	GES	P08709	13q34
Protein C	4	62	FVa and VIIIa	GES	P04070	2q13
u-PA	0,005	53	Plasminogen	KS	NP002649	10q24
t-PA	0,005	60	Plasminogen	KKS	NP127509	8p12

Appendix 1. A list of data concerning the serine proteases of the complement system and the haemolytic system. The abbreviations for the domain structure are given as **S**: Trypsin-like serine protease domain(see chapter 1.18). **C**: CUB domain(see chapter 1.15). **E**: Calcium-binding EGF-like domain (see chapter 1.16). **P**: The complement control protein (CCP) domain (see chapter 1.17). **A**: divergent subfamily of APPLE domains; Apple-like domains present in Plasminogen. **N2**: Fibronectin type 2 domain; One of three types of internal repeat within the plasma protein, fibronectin. Also occurs in coagulation factor XII, 2 type IV collagenases, PDC-109, and cation-independent mannose-6-phosphate and secretory phospholipase A2 receptors. In fibronectin, PDC-109, and the collagenases, this domain contributes to collagen-binding function. **N1**: Fibronectin type 1 domain, approximately 40 residue long with two conserved disulfide bridges. FN1 is one of three types of internal repeats which combine to form larger domains within fibronectin. FN1 domains also found in coagulation factor XII, HGF activator, and tissue-type plasminogen activator. In tissue plasminogen activator, FN1 domains may form functional fibrin-binding units with EGF-like domains C-terminal to FN1. Data mainly obtained through the individual protein accession number and domain structure. **G**: Domain containing Gla (gamma-carboxyglutamate) residues. A hyaluronan-binding domain found in proteins associated with the extracellular matrix, cell adhesion and cell migration. **K**: Kringle domain. Kringle domains have been found in plasminogen, hepatocyte growth factors, prothrombin, and apolipoprotein A. Structure is disulfide-rich, nearly all-beta.