PROLYL OLIGOPEPTIDASE IN MULTIPLE SCLEROSIS

Anne Penttinen
University of Helsinki
Faculty of Pharmacy
Division of Pharmacology and Toxicology

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PART I: LITERATURE REVIEW

PROLYL OLIGOPEPTIDASE IN MULTIPLE SCLEROSIS

1 INTRODUCTION

Prolyl oligopeptidase (POP, E.C. 3.4.21.26) cleaves short peptides, of less than 30 amino acid long, at the C-side of an internal proline (Fülöp et al. 1998). POP is a member of the prolyl oligopeptidase family of serine proteases, and has been associated with many pathophysiological processes, such as neurodegeneration, depression and hypertension (Brant et al. 2007; Garcia-Horsman et al. 2007; Männistö et al. 2007). POP seems to have a role in inflammatory diseases, such as rheumatoid arthritis and mycobacterium tuberculosis induced delayed-type allergic inflammation, and in diseases, that have a notable inflammatory and/or neurodegenerative component (Hashimoto et al. 2001, Kagewaga et al. 2004; Brand et al. 2007; Gaggar et al. 2009). Thus, POP function may have a connection to both the inflammatory and the neurodegenerative phase of multiple sclerosis (MS). At the moment there are no studies that have been focused on POP function in MS. A preliminary study in a Spanish cohort reported altered POP activity in plasma samples of patients with relapsing-remitting multiple sclerosis (RR-MS) (Tenorio-Laranga et al. 2010). However, there is a number of studies that associates matrix metalloproteinases (MMPs) with the course of MS disease (inter alia Bar-Or et al. 2003). MMPs and POP have been shown to work in concert in a chronic neutrophilic inflammation in the airways (Gaggar et al. 2009). Some of the peptide hormones suggested to be regulated by POP have also been shown to be mediators in inflammation (Lühder et al. 2009).

POP may exhibit highest concentration among brain peptidases (Polgár 2002). POP mRNA is significantly expressed in activated microglial cells that have been associated with neurodegenerative disorders (Klegeris et al. 2008). Other studies have linked POP to neurodegeneration based on its possible role in regulation of neuronal apoptosis.
(Männistö et al. 2007). A peptidomic study of porcine brain homogenates found fragments of myelin basic protein to be POP substrates (Brandt et al. 2005). In addition, cytoplasmic POP has been shown to be a regulator of the inositol phosphate signaling pathway (Garcia-Horsman et al. 2007). Disturbances in this pathway have been implicated in mood disorders, such as depression, a common symptom of MS.

This literature review focuses on the POP functions interpreted to be implicated in inflammatory and neurodegenerative processes, since these processes are the most relevant in the course of MS disease. The aim of this review is to clarify the role of POP activity alterations in the pathology of this autoimmune disease.

2 PROLYL OLIGOPEPTIDASE

The POP family proteins seem to be of ancient origin (Venäläinen et al. 2004). POP is distributed in archaeal, bacterial and eukaryotic species, but it has not been found in fungi. The high conservation of POP family enzyme sequences in different species suggests their presence in the last universal common ancestor (LUCA). This conservation points strongly to the importance of these enzymes in physiological processes.

2.1 Structure, activity and genetics

Mammal POP is a cylinder shaped 80 kDa globular soluble protein of 710 amino acids constituted by two domains (Fülöp et al. 1998) (Fig.1). Both N- and C-termini of the polypeptide chain constitute the peptidase domain (residues 1-72 and 428-710) which presents an α/β-hydrolase fold and contains the catalytic triad (Ser554, Asp641 and His680). The peptidase domain is covalently attached to a seven-bladed β-propeller domain (residues 73-427). The active site is located in a large cavity at the interface of the two domains. POP interacts maximally with six amino acids residues of the
substrate peptide, and specificity is provided by stacking a tryptophan residue against the substrate proline ring (Fülöp et al. 1998; Männistö et al. 2007). The β-propeller domain has been suggested to operate as a gating filter that controls the entrance of the substrates, a mechanism by which POP is proposed to distinguish between large proteins from unstructured peptides (Fülöp et al. 2000).

Fig. 1 POP is a cylinder shaped 80 kDa globular soluble protein constituted by peptidase and β-propeller domains. In a picture Z-Pro-Prolinal, a specific POP inhibitor, is bound at the active site of the enzyme (Protein Data Bank code 1qfs).

POP cleaves mainly peptides shorter than 30-mer at the carboxyl group of an internal proline residues (Fülöp et al. 1998, Polgar 2002). It is also able to hydrolyze after a residue of alanine, although at much slower catalytic rate. In addition, cleavages of Ala-Thr and Val-Gly bonds in octadecaneuropeptide and Cys-X bond in humanin have also been reported (Myöhänén et al. 2009). POP is the only proline-specific endopeptidase currently known in mammals (Polgár 2002). Proline is an imino rather than an amino acid, and thus most peptidases are unable to hydrolyze the peptide bond at that position (Fig.2) (Cunninham and O’Connor 1997; Polgár 2002).
The POP gene, *Prep*, has been cloned from several sources including human lymphocytes or a human T cell line MOLT4 (Kimura et al. 1999; Odaka et al. 2002). The structure and localization of the mouse POP gene has been accomplished. This gene contains 15 exons (Kimura et al. 1999; Garcia-Horsman et al. 2007; Männistö et al. 2007). The peptidase domain of the enzyme is coded by exons 1-3 and 10-15 and the propeller domain by exons 3-10. A national centre for biotechnology information (NCBI) gene data bank search reveals only one POP gene in human with similar structure of 15 exons. This gene is located at chromosome 6q22. Although POP appears to be a single copy of gene, different forms of POP, arising from post-translational modifications and/or alternative gene products, may account for the variety of functions and locations of the enzyme activity (Kimura et al. 1999; Garcia-Horsman et al. 2007).

2.2 Distribution

POP was first discovered 1971 in human uterus homogenates, where it cleaves the Pro-Leu bond of oxytocin (Walter et al. 1971). POP is mainly cytosolic, but lower activity/expression has been described e.g. in nuclei, mitochondria, microtubules, membranes and extracellular space (Irazusta et al. 2002; Schulz et al. 2005; Garcia-Horsman et al. 2007; Myöhänen et al. 2008; Myöhänen et al. 2009). Proliferating cells exhibit enhanced activity. Highest activities have been reported in cancerous tissues (Goossens et al. 1996). Presence of POP has been reported in immune cells, such as lymphocytes (T cells), macrophages and neutrophils (Gaggar et al. 2008; O’Reilly et al. 2009). Variable activity/expression, depending on the method used, have been measured.
from fibroblasts, epithelial cells, endothelial cells, thrombocytes, platelets, microglia, astrocytes, oligodendrocytes, heart, vasculature, kidneys, adipose tissue, testis, rectum, spleen, thymys, bone marrow and lung (Goossens et al. 1996; Odaka et al. 2002; Cavasin et al. 2004; Garcia-Horsman et al. 2007; Klegeris et al. 2008; Myöhänen et al. 2009). POP activities measured in blood, CSF, seminal fluid and prostate fluid are much lower than the activities measured in tissues.

The enzyme is found in all mature brain regions except for corpus callosum, and it has been detected in specific neuronal cells and absent in glial cells (Schulz et al. 2005; Männistö et al. 2007). The cerebral and cerebellar cortical neurons have been shown to be especially enriched of POP (Garcia-Horsman et al. 2007; Männistö et al. 2007; Myöhänen et al. 2009). Significant activity has been measured also in striatum, hypothalamus, hippocampus and amygdala. When POP distribution was investigated by high-throughput gene profiling, the POP mRNA levels were however similar in different brain areas (BioGPS).

Very often POP-like activity has not been fully identified (Garcia-Horsman et al. 2007). POP activity measured with specific substrates/inhibitors may not be exclusively associated to soluble POP, since possible posttranslational modifications and/or different/alternative gene products (membrane form, secreted form) may react similarly (Cunninham and O’Connor 1997; Garcia-Horsman et al. 2007).

2.3 Regulation

The mechanisms by which POP expression and its enzymatic activity are regulated are not currently known (Garcia-Horsman et al. 2007; Männistö et al. 2007). It is suggested that POP expression is developmentally regulated since mRNA/enzyme activity levels change with the animal age in tissue specific manner. Also environmental conditions seem to modify POP expression. POP regulation through a redox-sensitive modification has been suggested, since the enzyme is known to be inactivated (via formation of disulfide bonds) in oxidative conditions (thiol groups, oxidized gluthathione). Other
endogenous mechanisms that may modulate POP activity or expression are steroids, substrate phosphorylation and biological inhibitors in concert with polyamines. At the gene level there might exist multiple initiation sites, although there are no identified POP variants.

2.4 Physiological role of POP

Ubiquitous distribution of POP points to its importance in the general protein degradation process that focuses on peptide fragments containing pro-X bonds (Mantle et al. 1996). Extracellular POP is believed to inactivate peptide hormones and neuropeptides (Rößner et al. 2005; Schulz et al. 2005). Intracellular POP may be part of signaling pathways possible by regulating cellular phosphatases (Männistö et al. 2007; Klegeris et al. 2008). This peptide phosphorylation/dephosphorylation could alter peptide affinity to phosphatases, or even to POP, enabling activation or inactivation depending on the cellular conditions. Perinuclear POP associated with the tubulin cytoskeleton may have importance for physiological functions, such as axonal transport and protein secretion (Rößner et al. 2005; Schulz et al. 2005). Changes in POP mRNA levels affect on cell regeneration and tissue differentiation (Männistö et al. 2007). Decreased or increased enzyme activity has been implicated in many patophysiological processes (Mantle et al. 1996). However, the real physiological role of POP is unknown (Odaka et al. 2002).

2.5 Substrates and inhibitors

*In vitro* all naturally occurring proline containing small peptides are potential POP substrates (Garcia-Horsman et al. 2007). *In vivo* the situation is more complicated, since cellular location or secondary modifications may restrict the peptide-peptidase interaction. Many in vitro targets have not been confirmed *in vivo*. POP has been suggested to participate in the degradation/maturation of oxytocin, vasopressin, substance P, angiotensins, bradykinin, neurotensin, luteinizing hormone-releasing
hormone, and thyrotropin-releasing hormone (Yoshimoto et al. 1983; Kalwant et al. 1991; Cunningham and O’Connor 1997). The strongest in vivo evidence is for substance P, tyrotrophin releasing hormone (TRH), gonadotrophin-releasing hormone (GnRH) and arginin vasopressin (AVP) (Garcia-Horsman et al. 2007; Männistö et al. 2007). Also fragments of structural proteins (e.g. actin, myelin, collagen) and proteins connected to inositol pathway have been reported as POP substrates (Brandt et al. 2005; Gaggar et al. 2006; Garcia-Horsman et al. 2007).

There are reports of the existence of an endogenous POP inhibitor, but until this date it has not been fully identified (Yoshimoto 1982; Salers 1994; Cunningham and O’Connor 1997). Yoshimoto et al. have reported a POP inhibitor (∼MW 6.5 kDa) in porcine pancreas. This inhibitor was very stable against temperature, pH and trichloroacetic acid treatment. Also an endogenous inhibitor (∼MW 6.5 kDa) in neonatal rat pancreatic β-cells has been partly characterized by Salers. This cytosolic inhibitor inhibited fluorogenic substrate degradation by partly purified POP in a competitive manner. The inhibitor was detectable only in neonatal rats. A similar inhibitor has been reported in the sperm of Halocynthia roretzi (Cunningham and O’Connor 1997). The compound was purified and reported to have a molecular weight of 6.5 kDa. An octadecapeptide was isolated from bovine brain homogenates that demonstrated inhibitory capacity against POP obtained from Flavobacterium meningosepticum. Gebhard et al. have reported two proteins, PSKP-1 and PSKP-2 (MW 6.7 kDa and 6.6 kDa) isolated from the skin of Phyllomedusa sauvagii, as good prolyl oligopeptidase inhibitors (Gebhard et al. 2004). These proteins were found to possess in vitro inhibitory activity towards a prolyl oligopeptidase from bovine serum. However, they also reported residual activity of 24-32 % after the inhibition, which they concluded to be caused by another type of prolyl oligopeptidase in serum. An endogenous inhibitor is suggested to control proteolysis by POP.

Synthetic POP inhibitors have been of pharmacological interest as memory enhancers (Männistö et al. 2007). Most of these POP inhibitors are substrate-like compounds that resemble three amino acid long peptides, interacting with three substrate binding sites (S1, S2 and S3) of the enzyme. The majority of them contain a proline or proline
analogue residues at their P1 and P2 sites. Some of these inhibitors may have actions beyond the enzyme inhibition. Also natural compounds of microbial or plant origin have been tested as POP inhibitors (Kim et al. 2001).

3  MULTIPLE SCLEROSIS

MS is an autoimmune disease of central nervous system (CNS) characterized by focal T cell, B cell and macrophage infiltrates, demyelination and axonal injury (Bar-Or et al. 1999; Steinman 2001; Friese and Fugger 2005). This inflammatory and degenerative disease is possible triggered by a complex interplay of infectious, genetic and environmental factors (Ascherio and Munger 2007a,b; Sawai 2010). The disease usually begins between the ages 20 – 40 and affects women twice as often as men. Symptoms of the disease, e.g. paralysis, sensory disturbances, lack of coordination and visual impairment, are caused by loss of neurological function.

3.1 The course of the disease

The clinical course of MS varies greatly among individuals likely due to the complexity of the triggering factors (Schmidt 1999; Ascherio and Munger 2007a,b; Sawai 2010). MS is generally categorized as being either relapsing-remitting or primary-progressive in onset (Bar-Or et al. 1999). The RR form is characterized by series of attacks and recoveries; the progressive form involves gradual clinical decline. The course of RR-MS may later change to a secondary progressive form. MS commonly begins with an autoimmune “attack” against components of the myelin sheath (Sadovnick et al. 1996; Steinman 2001; Friese and Fugger 2005; Sawai et al. 2010). This “attack” may last from a few days to weeks, and it is followed by remission that can last from months to years. The earlier phase of disease is mainly mediated by an autoimmune reaction (focal inflammation), whereas the subsequent chronic phase of disease develops due to degeneration of myelin sheath and underlying axon (Steinman 2001). RR-MS, the most
common form of the disease, seems to follow cycles of immunological activation as a result of T cell attack (exacerbation), which is then followed by a suppressor response that down-regulates inflammation (remission) (Ziçaber et al. 1998; Polmán et al. 2005).

The inflammatory autoimmune reaction in CNS is mainly mediated by auto-reactive T cells, but other immune cells are also involved (Mahad and Ransohoff 2003). T cells, B cells or macrophages may be activated by a foreign microbe, self-protein or microbial superantigen (Steinman 2001). Once activated lymphocytes extravasate, they must pass through a barrier of extracellular matrix, comprised of type IV collagen, and vessels of blood-brain barrier (BBB). T cells are capable to bind to vessels of the BBB only when inflammation activates the blood-vessel endothelium. Bleaching BBB allows inflammatory cells to attack into the white matter. Myelin damage and impaired electrical conduction along the axon are caused by combined effects of cytotoxic cells, complement activation, autoantibodies and cytokines (Schmidt 1999; Steinman 2001). Resulting lesions are found predominantly in the periventricular white matter followed by the optic nerve and chiasm, pons, the cerebellar peduncles, medulla oblongata and the spinal cord. The axon loss in the spinal cord and spinal cord atrophy correlate most strongly with neurological impairment.

Experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, is generated by subcutaneous injection of antigenic peptides from myelin proteins e.g. myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) or by transfer of CD4\(^+\) (CD8\(^+\)) T cells reactive with these peptides (Bar-Or et al. 1999; Ransohoff 2009). Symptoms of the disease (tissue inflammation, motor weakness) develop in two weeks after immunization when immune cells accumulate in the animal’s spinal cord. These T cells educated in the periphery by exposure to antigen identify similar or identical antigens in the brain. Thus, T cell attack that is usually protective is now destructive creating multifocal perivascular inflammatory infiltrates in CNS.
3.2 Components associated with pathophysiology of multiple sclerosis

3.2.1 Inflammatory cells

The role of autoreactive T cells for the pathophysiology of MS is well established (Ziçaber et al. 1998; Mossberg et al. 2009). However, a number of studies suggest that components of innate immune system, such as monocytes/macrophages and granulocytes, may also mediate MS pathology.

T cells (cellular immunity) can be divided into CD4⁺ and CD8⁺ expressing T cells (Friese and Fugger 2005). CD4⁺ T-helper cells (Th) recognize peptides that are presented by major histocompatibility complexes (MHC) class II molecules on antigen presenting cells (APC). Differentiated no longer naïve Th cells can be divided into the functional subsets Th1 (proinflammatory) and Th2 (e.g. antibody class-switching) based on the cytokines they produce (Bar-Or et al. 1999; Friese and Fugger 2005). Former category protects against intracellular and latter against extracellular pathogens. CD8⁺ cytotoxic T cells mainly attack peptides from endogenously synthesized antigens presented by MHC class I molecules and may persist as memory cells. The focus in MS research has been mainly on CD4⁺ T cells, but also CD8⁺ cells seem to have significant role in the disease.

Efficient activation of naïve T cells is dependent on an antigen specific signal delivered through T cell receptor (TCR) and co-stimulatory signal that induce T cell to secrete cytokines (Bar-Or et al. 1999). Autoreactive T cells can be found in the peripheral blood of normal individuals. In MS blood, these cells are however in an enhanced activation state expressing IL-2 receptor on their surface as a mark of activation (DeFreitas et al. 1986; Bar-Or et al. 1999). They also may be less dependent on coactivation. In addition, the levels of these T cells are found to be higher in MS blood and CSF (Navikas and Link 1996). In the course of MS, activated T cells attack to the components of the myelin sheet (Steinman 2001). These include not only myelin basic protein (MBP),
proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG), but also stress proteins such as αB crystalline.

Regulatory T cells (Treg) are CD4+ T cells that express CD25, the alpha chain of IL-2 receptor, and high levels of the transcription factor forkhead box P3 (FoxP3) (Royal III et al. 2009). Circulating Treg cells are produced either centrally in the thymus or in the peripheral circulation and may convert to memory T cells after stimulation. The effect of Treg cells on symptoms of MS disease is thought to be ameliorating due to suppression of immune system.

B cells, that recognize antigen in its native form using B cell receptor (BCR) or membrane bound immunoglobulin, have been associated with MS through their ability to produce antibodies or autoantibodies against for example to myelin proteins and lipids (Franciotta et al. 2008). This response damages tissue by recruiting immune cells and by activation of the complement pathway (Steinman 2001; Franciotta et al. 2008). In MS, B cells take part to antigen uptake, processing, and presentation to T cells (Klawiter and Cross 2007). They produce cytokines, stimulate T cells and recruit and target cells to inflammatory sites. B cells also break up oligodendroglial and other central nervous system cells via antibody ± complement. Demyelination may be a result of myelin opsonization by antibodies.

Clonal expansion of B cells and the production of oligoclonal IgG in the brain and CSF of patients with MS have been suggested to be an evidence of the immune-mediated pathogenesis of the disease and suggest a possible infectious cause (Franciotta et al. 2008). In MS B cells aggregate lymphoid-like structures in the target organ. Some studies have found that many B cells that are infected with Epstein-Barr virus (EBV) accumulate in intrameningeal follicles and in white matter lesions, and according to one hypothesis might be the target of a cytotoxic immune response.

Granulocytes (neutrophils, eosinophils, basophils) are endowed with an oxygen radical-forming enzyme, the NADPH-oxidase, which reduces molecular oxygen to form several
reactive oxygen species (respiratory burst) (Mossberg et al. 2009). Initial radical formed by NADPH is superoxide anion.

Peripheral mononuclear neutrophils (PMNs) are able to affect both immune response and inflammation (Ziçaber et al. 1998). Activated neutrophils can produce oxygen and nitrogen species and cytokines or react by releasing proteases (Semple et al. 2009). PMNs in the peripheral blood (PB) of MS patients can be activated mainly by inflammatory cytokines, which induce expression of receptors for chemokines and other chemotactic peptides on PMNs (Ziçaber et al. 1998). Also the expression of CD10, CD13 antigen, and CD11b/CD18 molecules on PMN cell surface increases which often is associated with the course of exacerbation.

Monocytes, which become macrophages when they infiltrate into tissues, are suggested to be final mediators in MS since in vivo depletion of monocytes have been shown to lead marked suppression of the disease (Bar-Or et al. 2003). As a consequence lymphocyes did not infiltrate into the CNS parenchyma.

Microglia are scavenger cells that resemble tissue macrophages and are able to remove debris resulting from injury, infection and disease (McGeer and McGeer 2003). Tissue-based phagocytes are originally developed from monocytes, and when they migrate into tissues they provide a first line of defense. Microglia comprise 10%–12% of the total cell number of the brain (Dobos et al. 2010). These cells strongly interact with astrocytes, neurons, and blood vessels. When activated, their morphology change, and they start to secrete proinflammatory cytokines. INF-γ, mainly produced by T cells, is the main cytokine that is account to the activation of microglia (McGeer and McGeer 2003). Respiratory burst system of activated microglia is the most abundant source of oxygen free radicals.

The presence of reactive microglial cells has been described in variety of neurodegenerative diseases, including MS (Klegeris et al. 2008). Microglial neurotoxicity is likely mediated by a combination of different toxins i.e. glutamate,
quinolinic acid, TNF-α, soluble FasL, and ROS. Also various proteolytic enzymes have been identified as candidate neurotoxins (McGeer and McGeer 2003).

3.2.2 Cytokines and chemokines

Cytokines are signaling molecules that regulate immune responses and inflammatory reactions (Navikas and Link 1996). The balance between proinflammatory cytokines (e.g. IFN-γ, TNF-α, LT-α and 1L-12) and immunosuppressive cytokines (TGF-P, IL-10) may determine the pathology in MS (Navikas and Link 1996; Steinman 2001). Some cytokines are produced by a variety of cells, whereas others are secreted only by specific cells. They may act alone or in synergy with other cytokines. Specific cytokines are capable to stimulate cell proliferation, cytokine production, the expression of adhesion or MHC class II molecules. They also have a role in T-cell homing and induction of cell death. The concerted effects of proinflammatory cytokines may contribute to demyelination in MS.

Chemokines, small chemotactic cytokines, play a critical role in leucocyte recruitment to the site of inflammation. (Sørensen and Sellebjerg 2001; Mahad and Ransohoff 2003). Chemokines also modulate immunity by regulating T cell polarization, induction of respiratory burst, apoptosis, angiogenesis, mitosis, tumor metastasis, wound healing and secretion of cytokines and extracellular matrix proteases. Certain chemokines and chemokine receptors have been linked with relapse periods of MS, such as ligand/receptor pairs CCL5/CCR5 and CXCL10/CXCR3 (Semple e al. 2009). CCL2 has also been of interest to research (Mahad and Ransohoff 2003; Semple e al. 2009). Animal models have demonstrated increased CCL2 mRNA during EAE relapses, and a study with CCL2-/- and CCR2-/- mice showed both impaired immune cell recruitment during the course of EAE and attenuated clinical symptoms compared with wild-type animals. CCL2 is present in both active and chronic active lesions in MS. Figure 3 illustrates the inflammatory reactions in brain triggered by CCL2 (Fig.3)
Reboldi and colleagues investigated first stage of immune-cell entry to CNS by focusing on interleukin 17–producing T helper cells (T_H17) (Reboldi et al. 2009). These cells express CCR6, a chemokine receptor for CCL20, on their surface. The authors found that CCR6-knockout mice were resistant to developing EAE even though they show normal T_H17-cell responses: Transferred CCR6-expressing T cells from wild-type mice were sufficient to initiate inflammation in the CNS of CCR6-knockout mice before disease onset/immunization triggering a massive CCR6-independent recruitment of effector T cells across activated parenchymal vessels. After T cell transfer antigen activated CCR6-deficient T cells were again able to invade the CNS; T cells bind only to inflamed vessels of the BBB. These T cells accumulated in CSF secreting choroids plexus, whose epithelial cells express CCL20 which in turn signals through CCR6 for T_H17 cells. Thus, the CCR6-CCL20 axis in the choroid plexus seems to control immune surveillance of the CNS.

Figure 3 The roles of CCL2/CCR2 in brain inflammation. CCL2 induces the recruitment of macrophages and neural precursor cells, production of cytokines, and direct alteration of the expression of endothelial cell tight-junction proteins to increase blood–brain barrier (BBB) permeability (Semple et al. 2009).

The most important chemoattractants for neutrophils are ELR\(^+\) CXC chemokines (glutamic acid-leucine-arginine positive) including IL-8 (CXCL8) and growth related oncogenes GRO-\(\alpha\), GRO-\(\beta\) and GRO-\(\gamma\) (CXCL1, CXCL2 and CXCL3). These chemokines act through CXC chemokine receptor 1 and 2 (CXCR1 and CXCR2) (Weathington et al. 2006; Gaggar et al. 2008). Figure 4 illustrates the multiple functions mediated by CXCR2 signaling in CNS. CXCL13 is not found in normal CNS (Klawiter
and Cross 2006), but it is present only on infiltrating cells in actively demyelinating MS lesions. CXCL13 has a major role in the formation and maintenance of B-cell follicles in secondary and tertiary lymphoid organs. CXCL13 acts via CXCR5, which is expressed on B cells and subsets of T cells.

Fig. 4 CXCR2 signaling in the CNS. CXCR2 is the main receptor involved in neutrophil chemotaxis (Semple et al. 2009).

3.2.3 Matrix metalloproteinases

The matrix metalloproteinases (MMPs) comprises a large subfamily of endopeptidases that share structural domains (Hartung and Kieseier 2000). The mature CNS normally contains only moderate levels of most MMPs, but some of them become upregulated in neurological diseases, including MS (Bar-Or 2003). MMPs can degrade all protein components of the extracellular matrix, such as collagen, elastin, fibrinonectin, and laminin (Hartung and Kieseier 2000). Any imbalance in favor of inhibitors may lead to fibrotic processes, whereas any increase in enzymatic activity will result in tissue destruction or cell invasion.
In the course of MS MMPs are involved in the degradation of the extracellular matrix facilitating immune cell transmigration into the CNS (Steinman et al. 2001; Correale and de los Milagros Bassani Molinas 2003; Manicone and McGuire 2008). In addition, MMPs have been reported to degrade proteins of myelin sheath. The enzymes may also release of proinflammatory cytokines and participate in regulating the expression of the cell death signaling molecule FasL (Hartung and Kieseier 2000). Several MMPs including MMP-2, 3 and 9 have been shown to contribute to microglial toxicity (Klegeris et al. 2008).

In MS brain tissues MMP-2, MMP-7, MMP-9 and MMP-12 have been reported to be elevated (Hartung and Kieseier 2000; Bar-Or et al. 2003; Correale and de los Milagros Bassani Molinas 2003). MMP upregulation correlates with the disease course. MMP-2 and MMP-9 (Gelatinase A and B), detectable also in the CSF of MS patients, play a key role in penetration of the extracellular matrix (Steinman et al. 2001). The presence of MMP-9 in the perivascular infiltrate is associated with disruption of the type IV collagen-positive basement membrane, which is critical in the opening of the BBB.

There is growing body of evidence that MMPs are key to the pathogenesis of inflammatory demyelination (Hartung and Kieseier 2000; Bar-Or et al. 2003). In the CNS, the intracerebral injections or induction of MMP-2, MMP-7, MMP-8 and MMP-9 results in breakdown of the ECM, leucocyte recruitment, and opening of BBB in a rat model. In EAE study, increased levels of MMP-9 are detectable in the CSF of diseased animals. Also increased mRNA expression patterns of MMP-7 and MMP-9 have been found in the inflamed CNS. MMP inhibition by tissue inhibitors of matrix metalloproteases (TIMPs) can block TNF-α and thereby downregulate the induction of adhesion molecules (Steinman et al. 2001).

Bar-Or et al. found also a pattern of MMP expression in different cellular populations (Bar-Or et al. 2003). Certain MMP members were enriched in B cells, whereas others were prominent in T cells. However, the majority of MMPs were enriched in monocytes, which also demonstrated the most rapid BBB transmigration in a BBB model compared to T and B cells.
The regulation of MMP activity is controlled at three different levels: gene transcription, pro-enzyme activation and activity of TIMPs (Hartung and Kieseier 2000). The activation of zymogens involves multiple cleavage steps after which the activated forms are subject to inhibition by TIMPs. Proteinase inhibitors, such as α2-macroglobulin, also play a regulatory role. Corticosteroids and progesterone are known to suppress transcription.

3.2.4 Oxidative stress

The imbalance between cellular production of reactive oxygen species (ROS) and the inability of cells to defend against them is called oxidative stress (Gilgyn-Sherki et al. 2004). ROS oxidize important cellular components, such as lipids, proteins, and DNA causing mitochondrial and ultimately cellular death (Gilgyn-Sherki et al. 2004; Rintoul et al. 2006). The production of the reactive oxygen, and nitrogen species, is an inherent character of activated inflammatory cells. The most common cellular free radicals are hydroxyl radical (OH\textsuperscript{−}), superoxide radical (O\textsubscript{2}−), and nitric monoxide (NO\textsuperscript{−}). Free radicals may also activate transcription factors, e.g. nuclear transcription factor-κB (NF-κB) that upregulates the expression of some MS related genes. Post-mitotic glial and neuronal cells are particularly sensitive to free radicals.

It is thought that the inflammatory environment in CNS of MS patients leads to the generation of oxygen and nitrogen free radicals (Ortiz et al. 2009). Inflammation can lead to oxidative stress and conversely, increase on ROS could produce inflammation. A number of studies of patients with MS have shown increased free radical activity and/or deficiencies in antioxidant enzymes (e.g. glutathione peroxidase and superoxide dismutase) compared with healthy people (Naidoo and Knapp 1992; Gilgyn-Sherki et al. 2004). Nitric oxide metabolites (nitrates/nitrites), lipid peroxidation products (e.g. malondialdehyde), and diene conjugates are found at higher levels in patients with MS.
Both *in vitro* and *in vivo* studies have shown evidence of the protective role of antioxidant therapy against ROS destruction (Marracci et al. 2002). For example Marracci and coworkers demonstrated that alpha lipoic acid (ALA), an antioxidant capable of crossing BBB, can suppress and treat EAE in mice. ALA was administered to SJL mice 7 days after immunization with PLP 139-151 peptide and complete Freund’s adjuvant (CFA). As a result, spinal cord of ALA-treated mice had reduced demyelination and axonal loss. Also a number of CD3⁺ T cells and CD11b⁺ monocyte/macrophage cells within the spinal cord diminished markedly. The mechanism by which ALA inhibits destructive T cell trafficking into the spinal cord was suggested to be the inhibition of MMP activity.

### 3.2.5 Pathogens and molecular mimicry

The lesions found in multiple sclerosis are inflamed, but do not contain an evident pathogen (Steinman 2001). Sequencing the human genome and the genomes of various microbes, has demonstrated that biological organisms share many genes. Certain pathogens are comprised of proteins containing peptide sequences that mimic autoantigen epitopes (Bar-Or et al. 1999; Steinman 2001). Thus, the immune system, in targeting a structure on foreign microbe, may mistakenly self-attack. Many microbial protein sequences share homologies with structural components of myelin sheet. Antigen presenting cells (APCs) present these conserved peptides in periphery activating autoreactive T cells (TCR cross reactivity), which in CNS recognize the autoantigens in myelin sheath structure (Bar-Or et al. 1999). Also antibodies produced by B cells can cross react with components of the myelin sheath and microbial sequences (Steinman 2001). Molecular mimicry has been suggested both initiate and potentiate MS (Bar-Or et al. 1999).

Viral infections may trigger relapses in MS (Steinman 2001). Viruses such as herpesvirus 6, influenza, measles, papilloma virus and EBV virus all contain genes encoding sequences that share homology with myelin sheet peptides.
3.2.6 Genes

Susceptibility to MS is linked to genes in the major histocompatibility complex (MHC) on chromosome 63–5 (Steinman 2001). Both HLA class I and II alleles contribute to disease (Friese and Fugger 2005). However, the alleles for class II genes, HLA-DR and HLA-DQ, are the strongest risk factor (Steinman 2001). Other HLA complex genes are genes for TNF, components of the complement cascade and myelin oligodendroglial glycoprotein. A number of other genes have been implicated in MS pathology or susceptibility, including immunoglobulin Fc receptors, interleukin 1 beta and 6, apolipoprotein E (APO-E) and osteopontin genes (Steinman 2001; Gilgun-Sherki et al. 2004). Also polymorphisms in various human MMP genes (MMP-1, MMP-3, MMP-9 and MMP12) have been investigated, and as a result MMP9 and MMP12 polymorphisms are found to modify the disease course (Fernandes et al. 2009; Mirowska-Guzel et al. 2009). Polymorphism in the ACE gene is associated with susceptibility to MS (Platten et al. 2009).

3.2.7 Other factors

Glutamate is an excitatory neurotransmitter (Gilgun-Sherki et al. 2004). During inflammation lymphocytes, brain microglia and macrophages release excessive amounts of glutamate (Steinman 2001). Glutamatergic activity in the extracellular space may also be increased since oxidative stress reduces the efficiency of glutamate transporter (Gilgun-Sherki et al. 2004). Glutamate can cause necrotic damage to the cells in CNS via increased fluxes of calcium (Steinman 2001; Gilgun-Sherki et al. 2004). Myelin producing oligodendrocytes are especially vulnerable to glutamate excitotoxicity. Blocking AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) or kainate receptors has ameliorated symptoms in EAE.

Epidemiological studies have demonstrated that individuals with low vitamin D levels have a higher risk of MS (Royal III et al. 2009). This factor has been associated with high prevalence of MS in northern hemisphere. In EAE studies, vitamin D has been
demonstrated to suppress proinflammatory cytokine production and to increase secretion of anti-inflammatory cytokines. These immune modulatory properties of vitamin D are partly mediated through effects on Treg cells. The study by Royal et al. found correlations between percentages of Treg cell subtypes and levels of vitamin D metabolites. The protective effect of vitamin D seems to be more efficient in women.

A proteomic study by Han et al. reported the changes in proteins in three histological types of MS lesions (acute plaque, chronic active plaque and chronic plaque) compared to control sample (Han et al. 2008). The same study identified proteins involved in blood coagulation (tissue factor, PCI, thrombospondin, fibronectin and vitronectin) to be present in MS lesions (Han et al. 2008). It was also demonstrated that inhibition of certain coagulation factors ameliorates EAE. It is to note that this study found altered POP levels in MS lesions compared to healthy tissues.

4 PROLYL OLIGOPEPTIDASE IN INFLAMMATION

The classical immune system consists of inflammatory components such as antigen presenting cells (APC), T cells, B cells (acquired immunity) and granulocytes and natural killer cells (innate immunity) (Lühder et al. 2009). POP is not yet considered to be part of this system but it has been speculated it to be involved in activation of cell-mediated immunity, autoimmune, and inflammatory responses at various levels (Klegeris et al. 2008).

4.1 Immunological role of peptide hormone substrates

In addition to classical inflammatory components, there are inflammatory mediators which were originally discovered as regulators in the nervous or cardiovascular systems (Lühder et al. 2009). Substance P (SP), vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), bradykinin and renin-angiotensin system (RAS) components all
play a role in the immune system by modulating T cell responses, APC migration and also BBB function. These small peptides, most of them alleged POP substrates, may play an important role in immunology. Previously, POP has been suggested to regulate blood pressure by participating in the RAS system through metabolism of bradykinin and angiotensins I and II (Welches et al. 1993; Fülop et al. 1998; Polgár 2002), although there have been some concerns (Garcia-Horsman et al. 2007). Interestingly, also both proteomic and transcriptional analyses of multiple sclerosis lesions have revealed modulation of the renin-angiotensin and the opposing kallikrein-kinin pathways (Schulze-Topphoff et al. 2009).

4.1.1 Angiotensins

POP might be involved in degradation of biologically active angiotensin II and subsequent generation of angiotensin_{1-7} (Ang_{1-7}) (Skidgel 1992; Garcia-Horsman et al. 2007). Thus, it is appealing that POP may have influence on renin-angiotensin-system (RAS).

Angiotensin II (Ang II) mediates its effects mainly through AT1R and AT2R (Lühder et al. 2009). AT1R may activate multiple hemodynamic-independent cellular pathways resulting in proliferation, apoptosis, inflammation, extra-cellular matrix (EMC) remodeling and increased ROS production. A majority of these processes are attributed to ROS production, that Ang II enhances by up-regulating and phosphorylating tissue specific NAD(P)H subunits. Redox-sensitive inflammatory responses include e.g. increased expression of CCL2 leading to phagocyte chemoattraction via CCR2. In vitro RAS blockade has been shown to reduce Ang II induced release of chemokines in monocytes. Also dendritic cell (DC) and APC functions may alter via AT1R signaling.

The levels of angiotensin converting enzyme (ACE) are elevated in multiple sclerosis (Constantinescu et al. 1995; Linz et al. 1999). In MS serum increased ACE activity correlates with plaque volume in MRI scans (Lühder et al. 2009). ACE, that generates Ang II, participates in T cell stimulation by certain peptides and influences the
permeability of BBB. ACE inhibitors (captopril, telmisartan) and renin inhibitor (aliskeran) have been shown to suppress certain immune functions in EAE models (Constantinescu et al. 1995; Lühder et al. 2009). In a proteomic study, in which up-regulation of RAS components in brain lesions of MS patients was observed, both preventive and therapeutic treatment with ACE inhibitors suppressed autoreactive Th1 and Th17 cells and promoted CD4 positive FoxP3 positive Treg in an antigen specific manner (Platten et al. 2009). In another EAE study lymphocytes from captopril treated Lewis rats at the peak of disease severity had attenuated responses to stimulants MBP and concanavalin A (ConA) (Constantinescu et al. 1995). Telmisartan has been shown to reveal modulation of T cell responses in experimental autoimmune uveitis (Lühder et al. 2009).

Hemorphins are bioactive peptides derived from hemoglobin hydrolysis (Brand et al. 2005). The members of hemorphin family can also interact with the renin-angiotensin system, e.g. VV-hemorphin-7, an in vitro –POP substrate, is known to inhibit ACE enzyme.

This data suggest a pivotal role of the RAS in autoimmune inflammation of the central nervous system.

4.1.2 Bradykinin

Kinins belong to a family of bioactive octa- to decapeptides generated from kininogens in a stepwise cleavage process (Schulze-Topphoff et al. 2009). Bradykinin is susceptible to degradation by variety of exo- and endopeptidases, and it is inactivated when any of its peptide bonds are hydrolyzed (Skidgel 1992). In vitro both POP and ACE cleave bradykinin at the carboxy-terminal after proline residue. There is evidence that POP is involved directly or indirectly in bradykinin cleavage in vivo (Garcia-Horsman et al. 2007).
Kallikreins cleave kininogens to bradykinin that acts on the endothelium and facilitates the migration of lymphocytes to the CNS (Schulze-Topphoff et al. 2009). The inflammatory responses are passed on via two G protein–coupled receptors kinin receptor B1 (Bdkrb1) and B2 (Bdkrb2). Bdkrb1 does not express in immune cells under physiological conditions, whereas Bdkrb2 is ubiquitously expressed. Bradykinin mediates its effect primarily through Bdkrb2, and subsequent proteases generate des-Arg9-bradykinin, acting via Bdkrb1. Bdkrb1 expression has been found on brain endothelial cells, T lymphocytes and parenchymal CD3⁺ T cells within perivascular lesions from individuals with MS.

The activation of the kallikrein-kinin system in EAE models has been reported (Lühder et al. 2009). In the mouse model it was found upregulation of Bdkrb1, bradykinin, des-Arg9-bradykinin, kallikrein-1 and kallikrein-6 and low-molecular-weight kininogens in CSF and CNS tissue (Schulze-Topphoff et al. 2009). A specific Bdkrb1 agonist markedly attenuated clinical symptoms in SJL mice, whereas a specific Bdkrb1 antagonist resulted in earlier onset and greater severity of the disease. Bdkrb1 was identified as a specific modulator of immune cell entry (Th17) into the CNS and its expression on mononuclear cells from peripheral blood positively correlated with the expanded disability status (EDSS) of MS patients.

4.1.3 Substance P

An excitatory neurotransmitter Substance P (SP), also a suggested POP substrate, has mainly proinflammatory effects on multiple levels of immune responses (Nessler et al. 2006). A study using adoptive transfer EAE model linked the function of SP and autoimmunity. It showed that mice treated with a neurokinin-1 receptor (SP receptor) antagonist before disease onset had reduced severity of the disease. The outcome was related to a decreased expression of the adhesion molecules (ICAM-1 and VCAM-1) on CNS endothelia and decreased secretion of proinflammatory Th1 cytokines.
4.2 POP and chronic pulmonary inflammatory diseases

Chronic obstructive pulmonary disease (COPD) is primarily a systemic inflammatory disease of the lung that is induced by noxious agents (e.g., cigarette smoke). It results in airflow limitation that is not fully reversible and is characterized by emphysema and chronic bronchitis (Shapiro et al. 2005; O’Reilly et al. 2009). On the other hand, cystic fibrosis (CF), an autosomal recessive disorder, is caused by a mutation in the gene encoding for a membrane protein, the cystic fibrosis transmembrane conductance regulator (CFTR), which functions as an ion channel (Sheppard and Nicholson 2002). CF affects multiple organs, including lungs.

Neutrophils seem to be important mediators in COPD and CF (Weathington et al. 2006; Gaggar et al. 2008; O’Reilly 2009a). Chronic neutrophilic inflammation in the airways propagates damage by oxidant injury and the release of proteolytic enzymes causing tissue injury and end-organ dysfunction. POP has been identified in lung parenchyma, broncoalveolar lavage (BAL) fluid, pulmonary macrophages, and it is also present in the cytoplasm of neutrophils, where it is concentrated in granule-like structures (Gaggar et al. 2008; O’Reilly et al. 2009b).

The fragments of extracellular matrix (ECM) play an important role in inflammatory cell recruitment to the lung (Gaggar et al. 2008). Intratracheally administered collagen fragments have been reported to cause accumulation of pulmonary neutrophils. Non-specific-derived fragments have also been connected to neutrophil chemotaxis in murine models. In addition, elastin fragments ending with Pro-Gly have been described to cause fibroplast, monocyte, and to some extent neutrophil chemotaxis.

Chemical or enzymatic breakdown of collagen releases a tripeptide, N-acetylated proline-glycine-proline (N-α-PGP) that has been shown to be chemotactic for neutrophils both in vitro and in vivo (Weathington et al. 2006; Gaggar et al. 2008). The effect of N-α-PGP was tested in a transwell chemotaxis system, which demonstrated that N-α-PGP was active on neutrophils, whereas PGG as a control was not. Also a specific neutrophil recruitment into the airways was seen when C57 BI/6J mice were
exposed to N-α-PGP. Only the number of neutrophils in lungs increased when other cell types were unaffected. The initial neutrophil influx did, however, depend on ELR\(^+\) chemokines, but was maintained by N-α-PGP until neutrophils were absent. Also nonacetylated PGP has been shown to be a neutrophil chemoattractant, although \textit{in vitro} four to seven times less potent than N-α-PGP. Chemoattractance was also demonstrated in a murine model of pneumonic tularemia (Gaggar et al. 2008).

Structural homology to CXC chemokines is suggested to be the mechanism behind chemoattractance of ECM derived peptides (Weathington et al. 2006). Several ELR\(^+\) CXC chemokines (CXCL1, CXCL2 and CXCL3) contain a conserved PPGPH sequence immediately N-terminal to the third structural cysteine. IL-8 possesses the sequence ESGPH in this position (Downs et al. 2001). This GP motif can be found in all neutrophil specific chemokines, and the PGP motif is characteristic for several EMC proteins, such as collagen, collagen-like domains of elastin, and surfactant proteins A and D (Weathington et al. 2006). It has been shown that neutrophil chemotaxis to N-α-PGP and PGP is dependent on the CXC chemokine receptors (Weathington et al. 2006; Gaggar et al. 2008). Both ECM fragments act on IL-8 receptors CXCR1 and CXCR2 and they may cause release of superoxide via CXCR1 binding (Fig. 5).

![Fig. 5 N-α-PGP, a POP product, acts on IL-8 receptors CXCR1 and CXCR2](Migration.wordpress.com)
PGP production has been shown to correlate with the POP activity (Gaggar et al. 2008). In a murine model of airway inflammation, following intratracheal protease delivery to lungs, any protease alone did not generate PGP, but when either MMP-8 or MMP-9 was combined with POP, PGP was generated. A study by Gaggar et al. (2008) demonstrated that N-α-PGP and PGP levels were increased in sputum samples of CF patients. They also found that POP activity was increased 5-fold in CF sputum compared with healthy controls. In addition, they observed increased levels of HNE (human neutrophil elastase), MMP-8, MMP-9, MMP-11 and MMP-12. When CF sputum was incubated with collagen, PGP was generated. It was concluded that CF sputum contains the proteolytic enzymes necessary for generation of PGP from intact collagen. PGP levels in clinical samples decreased during inpatient therapy.

O’Reilly et al. investigated COPD sputum ex vivo and found similar results: COPD sputum was capable to generate both N-α-PGP and PGP, and this was dependent on synergistic actions of MMPs and POP (Reilly et al. 2009a). Additionally, these peptides were detected mainly in diseased sputum samples compared with healthy samples. PGP levels were also found higher in serum of COPD patients compared with controls. O’Reilly et al. hypothesized that N-α-PGP and PGP levels may increase during disease exacerbation. As a conclusion, they suggested these peptides as biomarkers or therapeutic targets for COPD.

The tripeptides generated by concerted action of MMPs and POP during inflammation are subsequently capable to attract neutrophils, which may lead to self-perpetuating cycle of neutrophilic inflammation (Gaggar et al. 2008). The airway epithelium serves as a barrier that protects collagen from protease cleavage. However, during disease progression, the epithelium gets damaged, and thus allows proteases to access the underlying ECM. Stimulated human neutrophils have also been shown to generate PGP, which proves that neutrophils contain all the enzymes necessary for this process (O’Reilly et al. 2009b). These cells also appear to contain an enzymatic activity which acetylates PGP to N-α-PGP.
POP is the only enzyme that is directly capable of releasing PGP from the often repeated “PPGP” motif in collagen, but it cannot liberate it from intact collagen (Gaggar et al. 2008). Intact collagen forms a helical trimmer and cleavage by MMP-1 or MMP-9 exposes previously hidden PGP-containing strands. Thus, MMPs seem to act in concert for generating an optimal substrate for POP (Fig. 6) (Weathington et al. 2006; Gaggar et al. 2008).

PGP generation can be partly inhibited by MMP-8 and MMP-9 antagonists, but POP inhibition alone completely blocks the generation (Gaggar et al. 2008). Thus, POP has a significant role in the pathology of pulmonary neutrophilic inflammation. Neutrophilic inflammation and matrix destruction and remodeling can be seen in a variety of chronic inflammatory diseases (O’Reilly et al. 2009b).

![Fig. 6](image)

Fig. 6 The generation of PGP, a POP (PE) product. Activated neutrophils secrete metalloproteinases (MMP-8 and MMP-9), which denature and cleave collagen to suitable fragments for POP cleavage to generate PGP. The PGP generated is chemoattractant on neutrophils. The process can go on in a cycle (Gaggar et al. 2008).

4.3 POP and bronchiolitis obliterans syndrome (BOS) after lung transplant

POP activity has been shown to be elevated in lavage fluid from lung transplant patients with chronic allograft rejection (Hardison et al. 2009). Hardison and coworkers reported increased levels of both IL-8 and PGP, the neutrophil chemoattractants, and MMP-8
and MMP-9 and POP, the enzymes responsible for generating PGP, in bronchiolitis obliterans syndrome (BOS) patient bronchoalveolar lavage (BAL) fluid.

Chronic allograft rejection accounts for poor rates of lung transplant patient survival (Hardison et al. 2009). The clinical correlate of this condition is known as bronchiolitis obliterans syndrome (BOS). Histologically it manifests as obliterative bronchiolitis (OB) damaging epithelial cells and sub-epithelial structures of airways. Neutrophils and chemokines, specifically ELR+ CXC chemokines, have notable roles in the development of this chronic pathology. Proteases were also suspected to be involved since there is a high degree of matrix remodeling during disease progression.

Hardison and coworkers demonstrated that the enzymes required for PGP generation are present at both increased levels and activity in BOS samples compared with other transplant populations (Hardison et al. 2009). Their data also confirmed the concerted activity of MMP-9 and POP. POP activity in the samples also correlated with PGP levels, and the change in PGP level correlated with the change in lung function. BAL samples demonstrated increased levels of IL-8 and PGP (only the nonacetylated form). By using specific antibodies against PGP and IL-8 they demonstrated that these chemokines are major chemoattractants in BOS BAL fluid. Their data also demonstrated a possible change in chemokine predominance seen during the development of BOS. PGP may thus be the main chemoattractant at the time of diagnosis. The study emphasized the influence of a matrix-derived neutrophil chemoattractant in posttransplantation BOS.

4.4 POP and rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease characterized by destruction of articular cartilage and subchondral bone (Hashimoto et al. 2001). Increased POP levels in synovial membrane preparations from patients suffering rheumatoid arthritis has been reported in earlier studies (Cunninham and O’Connor 1997). Hashimoto and coworkers investigated the activities of various proteinases (cysteine, serine, aspartic, and MMPs)
in cell-free knee synovial fluids of patients with RA and patients with osteoarthritis (OA; non-allergic inflammatory disease) and found more than 19-fold higher activity of cathepsin B and about 6-fold higher activity of POP compared to those found in synovial fluid of OA.

Previous documents have demonstrated that joint destruction occurs due to elevated levels of active forms of the proteolytic enzymes that degrade cartilage aggrecan proteoglycan and bone collagens (Hashimoto et al. 2001). Cathepsin B (cysteine protease) in RA fluid is able to degrade collagen, and this degradation was shown to be suppressed by the addition of a specific inhibitor. This indicated that cathepsin B may participate in joint destruction of RA. MMP levels detected in the RA synovial fluid as well as in the rheumatoid synovial tissues were low, possible due to an acidic micro environment. The characteristic differences in the synovial fluids between RA and OA remained unclear. The cells that secreted these enzymes were not identified. Authors concluded that the assays of cathepsin B and POP might be useful for diagnosis and prognosis of RA.

4.5 POP and delayed type of allergic inflammation induced by *Mycobacterium tuberculosis*

Kakewaga and colleges studied the proteinase release into the foci of *mucobacterium tuberculosis* (*M.tuber*) –induced delayed-type allergic inflammation in mice in order to determine what kinds of proteinases are secreted into the foci of allergic-inflammation involving delayed-type hypersensitivity reaction (Kagewaga et al. 2004). They observed significant activities of cathepsin B and POP in the washing-fluids of subcutaneous inflammatory foci of *M.tuber* –induced delayed-type allergic-inflammation, but not *M.tuber* –induced acute-inflammation. Thus, this secretion of cathepsin B and POP was likely due to the immune response involving delayed-type hypersensitivity, but not acute-inflammation. The activities of these enzymes in the washing fluids of inflammatory foci in the *M.tuber* –induced delayed-type allergic-inflammation increased time-dependently.
Delayed-type hypersensitivity, mediated by antigen stimulated antigen-specific T-lymphocytes, participates in various allergic-inflammatory diseases (Kagewaga et al. 2004). By secreting cytokines, these activated antigen specific T-lymphocytes activate macrophages, granulocytic cells, and lymphocytes. Th1-lymphocytes support the activation of macrophages, and the activated macrophages enhance the inflammatory response in delayed-type hypersensitivity e.g. by releasing Cathepsin B and POP. *M.tuber* was used in this study as a specific antigen, since it mediates Th1-lymphocyte-macrophage cooperation system via cytokine secretion to induce typical delayed-type hypersensitivity. Mice were injected subcutaneously with emulsion consisting of *M. tuber* and Freund’s incomplete adjuvant and after 14 days, the same emulsion was re-injected subcutaneously into the focus of inflammation. A specific cathepsin B inhibitor was demonstrated to suppress both swelling and cathepsin B activity in the footpad having *M. tuber*.-induced delayed-type allergic-inflammation.

4.6 POP and systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a chronic autoimmune connective tissue disease (Rahman and Isenberg 2008). Since some earlier studies have indicated that hydrolytic enzymes participate in the pathogenesis of immunoallergic disturbances, Hagihara and coworkers investigated the peptidase activities in plasma and tissues of the New Zealand Black (NZB) mouse as an animal model of human systemic lupus erythematosus (Hagihara et al. 1987).

In this study total POP activity was found to be lower in plasma of NZB mice than in the control mice (BALB/c mice) (Hagihara et al. 1987). On the other hand during maturational development POP activity in spleen increased gradually with age. The increase in POP activity in the spleen supported the previous report by Aoyagi et al, who also had noted changes in the POP activities in the spleen of an animal model of lupus erythematosus (hybrids of NZB and New Zealand White mice) (Hagihara et al. 1987; Cunninham and O’Connor 1997). The activity of POP had progressively increased with age in the hybrid mice with lupus, in contrast to decreased activities in
control mice. In mouse kidney tissue homogenates Hagihara and colleges found no difference in POP activity between two animal groups. The results indicate biochemically measurable relation between POP and the developmental immunological abnormalities.

4.7 POP and antifibrotic peptide Ac-SDKP

POP is considered to be the main enzyme involved in the generation of N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) most probably from thymosin-β₄ (Cavasin et al. 2004). Ac-SDKP is an antifibrotic tetrapeptide, which is further hydrolyzed almost exclusively by ACE. Treatment with ACE inhibitors can increase its plasma concentration 5-fold. Hence, many effects of ACE inhibitors seem to be similar to that of a Ac-SDKP surplus (Rasoul et al. 2004). Rasoul and coworkers demonstrated, using male Sprague-Dawley rats, that Ac-SDKP inhibits cell proliferation, left ventricle (LV) inflammatory cell infiltration (macrophages/monocytes and mast cells) and expression of TGF-β (transforming growth factor-β₁), CTGF (connective tissue growth factor) and collagen deposition in the LV. Ac-SDKP was also able to prevent the effects of Ang II infusion, but it did not alter blood pressure (Rasoul et al. 2004; Cavasin et al. 2004). Other studies have demonstrated that Ac-SDKP possesses angiogenic activity in vitro and in vivo. In animal models Ac-SDKP is able to enhance revascularization after myocardial infarction, or hind limb ischemia (Rossdeutsch et al. 2008). This hind limb revascularization was shown to be dependent on the induction of monocyte chemoattractant protein-I (MCP-1).

It is presumed that ubiquitously distributed thymosin-β₄ is the precursor of Ac-SDKP, since it possesses the sequence Ac-SDKP in its N-terminus (Cavasin et al. 2004). POP has been demonstrated to be the enzyme responsible for production of Ac-SDKP in vitro and in vivo. Since thymosin-β₄ has 43 amino-acids, it is likely that initial hydrolysis to smaller peptides (under 30 amino acids) is necessary for POP to produce the tetrapeptide in a second cleavage reaction. Ac-SDKP levels have been shown to be
significantly lower in rats treated with POP inhibitors, whereas ACE inhibitors significantly increase endogenous levels of Ac-SDKP.

Thymosin-β₄ cleavage by POP and formation of Ac-SDKP can happen locally in any organ (Cavasin et al. 2004). Ac-SDKP may prove to be an important mediator in inflammation acting as an anti-inflammatory cytokine.

5 PROLYL OLIGOPEPTIDASE IN NEURODEGENERATION

Neuroinflammatory hypothesis of neurodegenerative diseases proposes that inflammation contributes to the pathogenesis in a number of neurological disorders (Klegeris et al. 2008). However, it is not known whether neurodegeneration is a result of inflammation or from a more direct destructive function. Other factors, such as oxidative stress have been implicated in neuropathology (Schliebs 2004). Disturbances in mitochondrial function, cell signaling, neurotrophic support or protein folding ultimately lead to initiation of neuronal cell signaling cascades. The degenerative events are usually accompanied by counteracting regenerative mechanisms (Hashimoto 2003; Schliebs 2004).

Numerous studies have demonstrated that altered levels of POP activity are associated with neurodegenerative disorders (Cunningham and O’Connor 1997). Mantle and coworkers reported decreased POP activity levels in Alzheimer’s disease (AD), Lewy body dementia, Parkinson’s disease and Huntington’s disease in human cortical brain tissue samples (grey/white matter) (Mantle et al. 1996). They concluded that the reduction in POP activity in all these diseases is a mark of generalized process of neurodegeneration. Loss of enzyme activity may thus be an early response to cellular stress, e.g. resulting from oxidative conditions.
5.1 POP substrates and neurodegeneration

From a MSMS peptide analysis, Brand and colleagues studied the changes in peptide masses upon in vitro POP inhibition of brain homogenates and found fragments of myelin basic protein to be substrates of POP (Brand et al. 2005). This finding indicates that POP might be a part of the demyelination process.

Some authors have suggested that neuropeptide substrates (AVP, TRH, SP, α-MSH and neurtensin) are indeed implicated in neurodegenerative diseases, although mainly in memory function. However, some peptides like AVP, have been reported to protect from neuronal death (Tolde et al. 1997; Brand et al. 2007; Garcia-Horsman 2007). Consequently, the degradation of these peptides may accelerate the aging process.

POP has been suggested to participate in processing of the amyloid precursor protein (APP) (Roßner et al. 2005). Roßner and coworkers studied the effects of APP overexpression, increased β-amyloid concentrations and β-amyloid plaque formation on the expression or enzymatic activity of POP, in the brains of APP transgenic Tg2576 mice (Roßner et al. 2005). These mice are characterized by 5 to 7-fold overexpression of human APP695. They were able to observe increased hippocampal protein levels and enzymatic activity of POP in adult Tg2576 mice, compared to wild type mice. This hippocampal POP level in adult transgenic mice was as high as in aged control mice (“accelerated aging”). This phenomenon was not present in cortex or cerebellum. Their results indicated that hippocampal POP activity is modulated by soluble/oligomeric or fibrillar β-amyloid peptides. The increased POP expression in hippocampus was parallel with the development of memory deficits and it predicted β-amyloid plaque formation. Neurons most actively expressing POP in AD brain showed morphological characteristics of neurodegeneration.
5.2 Microglial toxicity

Supernatants of stimulated THP-1 cells (transformed human mononuclear cells) and human microglial cells have been shown to be lethal to cultured human neuroblastoma SH-SY5Y cells (Klegeris et al. 2008). Based on this fact, Klegeris and coworkers investigated microglia and their surrogate, THP-1 cells, in order to identify the proteins with potential of microglial toxicity. In these studies POP mRNA was found also to be significantly increased in both cell types.

When proteins were identified by SILAC (stable isotope labeling by amino acids in cell culture) methodology, POP was one of the proteins significantly up-regulated in the culture media compared to control cell line (Klegeris et al. 2008). Microglial stimulators, like LPS, INF-γ, or α-synuclein alone, did not up-regulate extracellular POP activity, but combinations of them did. The same supernatants were also analyzed by MTT assay (method of transcriptional and translational assay), and a statistically significant correlation between supernatant toxicity towards SH-SY5Y cells and POP activity was found. In these results cell survival decreased in a POP concentration dependent manner. Specific POP inhibitors were found to be only partially protective, which lead to the conclusion that other toxic components were also involved in this process.

5.3 Neuronal apoptosis

Abnormal apoptosis has been associated with the course of number of human diseases, including autoimmune and neurodegenerative disorders (Katsube et al. 1999). POP has been suggested to be a regulator of neuronal apoptosis, and some POP inhibitors, e.g. ONO-1603, indeed demonstrate neuroprotective character in various studies.

Katsube et al. reported that ONO-1603 delays age-induced apoptosis of rat cerebral cortical cells and cerebellar granule cells (Katsube et al. 1999). Age-induced apoptosis is a process where neuronal cells are cultured in the absence of medium change and
glucose supplement causing them to die apoptotically. This process in cerebellar neurons is inhibited by antagonists of the N-methyl-d-aspartate (NMDA) receptors and protected by antioxidants, which points to the importance of glutamatergic activity. However, POP activity seems to be unaltered during age-induced apoptosis (Katsube et al. 1999). Previously Katsube and coworkers have shown that this POP inhibitor protects immature cerebellar granule cells also from potassium deprivation-induced cell death, which is supposed to be mediated by increased muscarinic activity. These processes are closely associated with overexpression of GAPDH (Glyceraldehyde-3-phosphate dehydrogenase).

GAPDH has multiple roles in cellular activities (Katsube et al. 1999; Puttonen et al. 2006). Translocation of overexpressed GAPDH to nucleus is linked to apoptosis of neurons. GAPDH has also been found to bind the proteins that are up-regulated in certain neurodegenerative diseases. This implies that it may mediate the translocation of these disease gene products to the nucleus (Katsube et al. 1999). Additionally it has been suggested that the apoptosis seen in neurodegenerative diseases may be mediated by GAPDH. Thus, GAPDH may act as an intracellular sensor of oxidative stress.

Puttonen and coworkers investigated two POP inhibitors, JTP-4819 and Z-Pro-Prolinal, and their ability to inhibit 6-OHDA- and Ara-C-induced GAPDH translocation into the particulate cell fraction containing nuclei and mitochondria (Puttonen et al. 2006). The production of reactive oxygen species (ROS) as well as cell viability were determined in the presence of these inhibitors, as well as the content of p53, an indicator of cell stress, and Bcl-2, an antiapoptotic protein, were measured. This study demonstrated that Z-Pro-Prolinal was able to retain GAPDH in the cytosol after treatment with 6-OHDA in CV1-P fibroblasts, when there was no notable difference in GAPDH location in control SH-SY5Y cells. However, neither of these POP inhibitors was able to inhibit Ara-C-induced GAPDH translocation. The antioxidant effect of POP inhibitors was observed in 6-OHDA-treated CV1-P cells but not in SH-SY5Y cells. Both Z-Pro-Prolinal and JTP-4819 prevented toxin-induced elevation of ROS in CV1-P cells but did not enhance the viability of the cells. The level of Bcl-2 was elevated by Z-Pro-Prolinal in the particulate fraction of both CV1-P cells and SH-SY5Y cells whereas JTP-4819
increased the levels only in CV1-P cells. 6-OHDA induced increase in the levels of p53 was not inhibited by the POP inhibitors. It was concluded that POP may be involved in translocation of GAPDH to nuclei in cells undergoing apoptosis, and POP inhibitors may counteract oxidative stress factors (ROS) in certain cell types (Puttonen et al. 2006; Männistö 2007).

The antiapoptotic effect of POP inhibitors may not be due to POP dependant peptide cleavage inactivation (Katsube et al. 1999). For example the neuroprotective of effect of ONO-1603 seem to be associated with inhibition of GAPDH mRNA and protein overexpression. There is however complex interaction between GAPDH, IP₃ and POP, which all are linked to inositol pathway (Puttonen et al. 2006), where no direct peptide processing seems to be involved. IP₃-receptor activation is the signal to cells to commit apoptosis; releasing Ca²⁺ amplifies the reaction. The suggested mechanism by which POP inhibitors may inhibit GAPDH mediated nuclear translocation are either by inhibiting the formation of GAPDH-Siah1 complex and/or by the activating PI-3-kinase that translocates GAPDH back to the cytosol.

5.4 Activation induced cell death in T cells

An activation-induced cell death (AICD) is triggered by T cell receptor (TCR) stimulation with antigen that activates resting T cells (Odaka et al. 2002). When activated T cells are re-stimulated with antigen through TCR, T cells undergo apoptosis. This process is believed to play an important role in controlling the autoreactive T cells in thymus or in periphery.

POP is expressed in thymus and spleen (Odaka et al. 2002). Odaka and coworkers examined POP activity in subpopulations of murine T cells in order to define the role of this enzyme in T cells. Thymocytes were prepared from thymus glands of BALB/c mice, and partly used as unfractionated thymocytes. The earliest thymocytes express neither CD4 nor CD8 at their surface, and are classified as double-negative (CD4⁻CD8⁻) cells. During their development they become double-positive thymocytes (CD4⁺CD8⁺).
Mature single-positive (CD4⁺CD8⁻ or CD4⁻CD8⁺) thymocytes are released from the thymus to peripheral tissues. It was found that POP activities in mature thymocytes or peripheral T cells to be lower compared with immature thymocytes (Fig. 7). POP activity in stimulated peripheral T cells increased in a time-dependent manner.

![Graph showing POP activity in subpopulations of T cells (BALC/c mice).](image)

Fig. 7 POP activity in subpopulations of T cells (BALC/c mice). POP activities in the mature thymocytes or peripheral T cells are lower compared with immature thymocytes. POP activity is totally inhibited by ZPP (Odaka et al. 2002) (abbreviations: DN - double negative, DP - double positive, SP - single positive; ZZP - Z-Pro-Prolinal).

Odaka and coworkers also tested the hypothesis that T cells with high POP activity are sensitive to AICD in murine T cell hybridomas, thymocytes, and activated peripheral T cells using specific POP inhibitors (Odaka et al. 2002).

Murine T cell hybridoma N3-6-71 was found to express high POP activity (Odaka et al. 2002). When cells were pretreated or untreated (control) with POP inhibitor before stimulation with anti-CD3 antibody, most of the control T cells died, whereas AICD was inhibited in cells pretreated with POP inhibitor. Thus, pretreatment of murine T cell hybridomas with POP inhibitor resulted in their resistance to AICD. During AICD, POP activity in cells was unchanged. POP inhibition also interfered with inducible activation of caspase-8 and caspase-3.
When BALB/c mice thymocytes pretreated or untreated with POP inhibitor were stimulated with anti-CD3 and anti-CD28 antibodies, more than one third of untreated thymocytes died, while thymocytes pretreated with POP inhibitor showed decreased cell death (Odaka et al. 2002). Similar results were obtained in peripheral T cells stimulated with Con A. Thus, pretreatment with POP inhibitor prevented TCR mediated apoptosis in thymocytes and in activated peripheral T cells.

The results of this study suggest that T cells expressing high POP activity are susceptible to ACID. The authors considered that POP might be responsible for the regulation of metabolism of substance P, which in turn contributes to T cell proliferation in an autocrine fashion, during AICD.

6 CONCLUSION

A preliminary study in a Spanish cohort found altered POP activity levels and the levels of its endogenous inhibitor in plasma samples of RR-MS patients (Tenorio-Laranga et al. 2010). Furthermore, a number of studies have plausibly connected POP function to inflammation and tissue/cell destruction. Since POP is highly expressed in brain, it is presumable that it also plays a role in the course of MS disease. The importance of this role is more difficult to evaluate due to multiple components involved in the pathogenesis of MS disease. In addition, the importance of particular enzyme in vivo depends on its localization, access to substrate and the presence of other substrates and peptidases (Skidgel 1992). The activity of enzyme may become apparent only when other enzymes hydrolyzing same substrate are inhibited. However, it seems probable that POP could be one of the components associated with pathogenesis of MS.
PART II: EXPERIMENTAL PART

PROLYL OLIGOPEPTIDASE ACTIVITY IN MULTIPLE SCLEROSIS AND CHARACTERIZATION OF ITS ENDOGENOUS INHIBITOR

1 INTRODUCTION

Prolyl oligopeptidase (POP; prolyl endopeptidase, EC 3.4.21.26) is a member of the prolyl oligopeptidase family of serine proteases (clan SC, family S9) (Rawlings et al. 1991; Venäläinen et al. 2004). The enzyme consists of a peptidase domain, which contains its catalytic triad (Ser 554, His 680, Asp 641) and a seven-bladed β-propeller domain (Fülöp et al. 1998). POP mainly cleaves peptides shorter than 30 a.a. at the carboxyl site of an internal proline. The enzyme is presumed to participate in the maturation and degradation of many peptide hormones and neuropeptides, such as angiotensins, kinins, vasopressin and substance P (Cunninham and O’Connor 1997; Garcia-Horsman et al. 2007; Männistö et al. 2007). POP has been reported also to degrade structural protein fragments (Brandt et al. 2005; Gaggar et al. 2008; Tenorio-Laranga et al. 2010).

MS is a chronic autoimmune disease characterized by inflammatory demyelination and axonal degeneration leading to a severe and progressive neurological impairment (Mahad and Ransohoff, 2003). Inflammation and degeneration seem to dominate at different phases of the disease (Steinman 2001; Rovaris et al. 2006). In most MS patients, this disorder is characterized by a relapsing–remitting course (RR-MS) (Sawai et al. 2010).

In earlier studies POP has been shown to have a role in inflammatory diseases, such as rheumatoid arthritis and chronic obstructive pulmonary disease (COPD) (Hashimoto et al. 2001; Weathington et al. 2006). Also POP function is associated with neurodegeneration based on its possible role in regulation of neuronal apoptosis and
secretion by activated microglial cells (Männistö et al. 2007; Klegeris et al. 2008). Since POP may exhibit highest concentration among brain peptidases, and it has been reported to degrade fragments of myelin basic protein, it seemed relevant to investigate the role of POP in MS (Polgár 2002; Brandt et al. 2005).

A preliminary study in a Spanish cohort indicated that the plasma levels of POP activity and its endogenous inhibitor are altered in patients with relapsing remitting multiple sclerosis (RR-MS) (Tenorio-Laranga et al. 2010). The POP activity levels in plasma from patients were significantly lower compared to healthy controls, and such decreased activity could be reverted by the addition of reductants. The study also showed that the levels of the endogenous POP inhibitor were significantly increased in plasmas of patients with RR-MS. This study also found a relationship between POP plasma activity and severity of the disease. However, this study was limited to a relatively small number of cases.

The first objective of this study was to evaluate the POP activity levels in serum and cerebrospinal fluid (CSF) from a much larger sample of RR-MS patients and healthy controls in a Finnish population. The levels of the endogenous POP inhibitor in serum were also considered to be evaluated. For these objectives, sample of MS patients and healthy controls were obtained from the laboratory of Doc. PhD. Laura Airas from the Department of Neurology, Turku University hospital.

A second objective of this study was to isolate, characterize and identify the putative circulating endogenous POP inhibitor. In order to accomplish these goals, different biochemical and –biophysical techniques were applied.
2 MATERIALS

2.1 Compounds and reagents

Acetic acid glacial 100 % (Merck, Darmstadt, Germany, Lot K2911103)

Acrylamide/bis-Acrylamide 40 % (w/v) (Sigma-Aldrich Inc., St. Louis, USA, Lot 038K6060/039K6024)

Albumin standard. Source: bovine serum albumin (BSA) 2 mg/ml (Thermo Scientific/Pierce, Rockford IL, USA, Lot K6134878)

Ammoniumpersulphate for electroforesis (Sigma-Aldrich Inc., St. Louis, USA, Lot 113K0656)

Anti-bovine serum albumin (BSA)-agarose (antibody developed in rabbit, IgG fraction of antiserum) (Sigma-Aldrich Inc., St. Louis, USA)

N-Benzoxycarbonyl-Glycyl-Prolyl-7-amino-4-methylcoumarin (Z-Gly-Pro-AMC) (Bachem AG, Bubendorf, Switzerland)

Bio-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories, Hercules, CA, USA)

Chicken antihuman POP IgY antibody (Department of Pharmacology and Toxicology, University of Helsinki, Helsinki, Finland)

Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, USA)

1.4-Dithio-DL-threitol (DTT) (Bachem AG, Bubendorf, Switzerland, Lot 9004958)
Ethanol 99 % (Etax Aa, Altia Oyj, Rajamäki, Finland)

Formaldehyde 37 % (Merck, Darmstadt, Germany, Lot K24900203/ Sigma Aldrich Inc., St. Louis, USA, Lot SZB93500)

Hydrochloric acid 37 % fuming (Merck KGaA, Darmstadt, Germany)

Immuno pure antibody, host: rabbit, anti-antigen: chicken IgY (H+L) (Pierce Biotechnology, Rockford, USA, Lot JG1153873)

JTP-4819. (–)-(2S)-1-benzylaminocarbonyl-[2(S)-2-2-glycoloyl-pyrrolidinyl]-2-pyrrolidine carboxamide (Department of Pharmaceutical Chemistry, University of Kuopio, Kuopio, Finland)

2-Mercaptoethanol (Sigma-Aldrich, France, Lot 083K0054)

Methanol (Mallinkrod + Baker R.V., Deventer, Netherland)

Potassium phosphate monobasic (Sigma-Aldrich, Steinheim, Germany, Lot 097K0067)

Recombinant porcine POP (rPOP) (Department of Pharmacology and Toxicology, University of Helsinki, Helsinki, Finland)

Sodium acetate anhydrous (Merck, Darmstadt, Germany Lot 410TA666068)

Sodium acetate trihydrate (Merck, Darmstadt, Germany, Lot 2323185)

Sodium carbonas decahydrate (Riedel-de-Haën Gmbh, Seelze, Germany, Lot 7107A)

Sodium chloride (Merck, Darmstadt, Germany, Lot K39486504849)
Sodium dihydrogen phosphate monohydrate (Merck KGaA, Darmstadt, Germany, Lot A916846743)

Sodium dodecyl sulphate 99 % (Sigma Aldrich Inc., St Louis, USA, Lot 014K0116)

Sodium phosphate dibasic dehydrate (Sigma-Aldrich, Steinheim, Germany, Lot 3320)

Sodium sulphate (Sigma Aldrich, St. Louis, USA, Lot 089K0005)

Sodium thiosulphate pentahydrate (Sigma Aldrich, St. Louis, USA, Lot 049K0310)

Tris(hydroxymethyl)aminomethane (Tris) (Fluka Chemie AG, Buchs, Switzerland, Lot 93350)

TEMED (N,N,N,N’-tetramethylenediamine) (Sigma Aldrich/YA-Kemia OY, Helsinki, Finland, Lot 87689)

Triton-X-100 (Merck KGaA, Darmstadt, Germany)

Tween®20 (Sigma Aldrich Inc, St Louis, USA, Lot 125K0102)

2.2 Solutions

2.2.1 Solutions for prolyl endopeptidase activity measurements

Incubation buffer (0.1 M Na-K-Phosphate buffer, pH 7): To 0.1 M Na₂HPO₄ was added 0.1 M KH₂PO₄ adjusting pH at 7

DTT stock 100 mM: DTT dissolved in MilliQ water
JTP-4819 stock 2 µM: JTP-4819 dissolved in MilliQ water

Purified porcine POP stock 0.1 mg/ml: rPOP dissolved in phosphate buffer

Purified porcine POP solution for experiments 5 µg/ml: rPOP stock solution diluted in incubation buffer

Substrate solution (10 mM Z-Gly-Pro-AMC): Z-Gly-Pro-AMC dissolved in DMSO

2.2.2 Solutions for affinity chromatography

Activation buffer (1mM hydrochloric acid): 37 % hydrochloric acid diluted in MilliQ water

Ligand solution (0.2 M sodium carbonate, 0.5 M sodium chloride, pH 8.3): salts dissolved in MilliQ water, pH adjusted

Stopping solution (1 M sodium-acetate buffer, pH 4.2): sodium-acetate dissolved in MilliQ water and pH adjusted with acetic acid

Washing buffer (0.5 M ethanolamine, 0.5 M sodium chloride, pH 8.3): substances dissolved in MilliQ water, pH adjusted with hydrochloric acid

Deactivation buffer (0.1 M acetate, 0.5 M sodium chloride, pH 4): substances dissolved in MilliQ water, pH adjusted with acetic acid

Equilibration buffer (50 mM sodium phosphate, 100 mM sodium chloride, pH 7): salts dissolved in MilliQ water, pH adjusted

Elution buffer (50 mM sodium dihydrogen phosphate, 1 M sodium chloride, pH 7): salts dissolved in MilliQ water, pH adjusted
2.2.3 Solutions for albumin removal

Acidic solution (50 mM sodium acetate, 2 M sodium chloride, pH 4): substances dissolved in MilliQ, pH adjusted

Alkaline solution (50 mM Tris, 2 M sodium chloride, pH 10): substances dissolved in MilliQ water, pH adjusted

Neutral solution (50 mM sodium phosphate, 100 mM sodium chloride, pH 7)

2.2.4 Solutions for anion exchange chromatography

Buffer A (50 mM sodium phosphate, pH 7): sodium phosphate dissolved in MilliQ water, pH adjusted

Buffer B (50 mM sodium phosphate NaH₂PO₄, 500mM sodium chloride, pH 7): salts dissolved in MilliQ water, pH adjusted

Washing buffer (50 mM sodium phosphate, 2 M sodium chloride, pH 7): salts dissolved in MilliQ water, pH adjusted

2.2.5 Solutions for hydrophobic interaction chromatography

Buffer A (20 mM sodium phosphate, 1.0 M sodium sulphate, pH 7.2): salts dissolved in MilliQ water, pH adjusted

Buffer B (0.25 mM sodium phosphate, pH 7.2): sodium phosphate dissolved in MilliQ water, pH adjusted
2.2.6 Solutions for electrophoresis

Ammoniumpersulfate (APS) 20 % stock: APS dissolved in MilliQ water

Loading buffer: 2% SDS, 70% glycerol, 10mM 2-mercaptoethanol, 0.005% bromophenol blue, 100 mM Tris · HCl, pH 6.8

Running buffer (10 x concentrate): 3 % Tris, 14.4 % glycine, 1 % SDS in MilliQ water (Institute of Biotechnology, University of Helsinki, Helsinki, Finland)

Running buffer: 10 % running buffer concentrate, 1 % SDS in MilliQ water

Sodium dodecyl sulphate 10 % (SDS, C12 grade): SDS dissolved in MilliQ water

2.2.7 Solutions for protein staining

Coomassie Staining:

Coomassie blue 2 %: acetic acid glacial/methanol/water (7:40:53), 2 % bromophenyl blue

Silver staining:

Developing solution: 2 % sodium carbonate, 0.04 % formaldehyde in MilliQ water, formaldehyde added just before use

Fixing solution: 99 % ethanol/ acetic acid glacial/MilliQ water (45:5:50)
Silver nitrate 0.1 %: silver nitrate dissolved in MilliQ water

Sodium thiosulfate 0.02 %: sodium thiosulphate dissolved in MilliQ water

Stop solution (1 % acetic acid): acetic acid glacial diluted in MilliQ water

Tris·HCl 1 M, pH 6.8: Tris dissolved in MilliQ water, pH adjusted

Tris·HCl 1.5 M, pH 8.8: Tris dissolved in MilliQ water, pH adjusted

2.2.8 Solutions for Western blotting

Milk solution 5 %: dry skimmed milk dissolved in TTBS 0.1 %

Tris-buffered saline (10 x TBS concentrate, pH 7.4): 3 % Tris, 8 % sodium chloride, 0.2 % potassium chloride in MilliQ water, pH adjusted with hydrochloric acid (Institute of Biotechnology, University of Helsinki, Helsinki, Finland)

TTBS 0.1%: 10 % 10 x TBS concentrate, 0.1% Tween®20 in MilliQ water

Western blotting buffer: 10 % 10 x running buffer concentrate, 20 % methanol in MilliQ water

2.2.9 Other solutions

Sodium hydroxide 1 M: 5 M sodium hydroxide diluted in MilliQ water

Sodium hydroxide 5 M: sodium hydroxide dissolved in MilliQ water (Institute of Biotechnology, University of Helsinki, Helsinki, Finland)
2.3 Gels for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Resolving gel 7%: 7% acrylamide, 375 mM Tris, 0.1% SDS, 0.1% APS, 0.0825% TEMED in MilliQ water

Resolving gel 12%: 12% acrylamide, 375 mM Tris, 0.1% SDS, 0.1% APS, 0.0413% TEMED in MilliQ water

Staking gel 5%: 5% acrylamide, 125 mM Tris, 0.1% SDS, 0.1% APS, 0.099% TEMED in MilliQ water

2.4 Patients and controls

Fourteen patients who had confirmed clinical diagnosis of MS according to modified McDonald criteria (Polman et al. 2005) were used in this study. Concretely all the subjects were clinically classified as having relapsing-remitting form of the disease according to the criteria described by Lublin (Lublin et al. 1996). Eleven of the patients were females, three were males. The age range was from 19 to 42 years with the mean age of 28.6 years (Table 1). All the patients were on interferon-β therapy.

Twenty eight healthy volunteers (19 females and 9 males) from the age of 19 to 80 years old (mean 47.3 years) were also recruited for this study as control (Table 1). None of them was diagnosed with any neurological illness or any chronic disease.

<table>
<thead>
<tr>
<th>Table 1 Age range of patients</th>
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<tr>
<td>Age (years)</td>
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<tr>
<td>MS patients</td>
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<tr>
<td>Healthy controls</td>
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2.5 Serum and CSF samples

Paired serum and CSF samples were collected during the years 2006-2008 and immediately stored at -80°C after acquisition. The samples were collected from the Hospital of Turku and provided for the present study in collaboration with the Department of Neurology from the University of Turku. The study was approved by the Ethical Committee of Southwest Finland and followed local and European regulations.

3 METHODS

3.1 Assays for prolyl endopeptidase (PE) activity

Serum samples (n=42) were analyzed after one freezing cycle. POP activity was assayed by measuring the fluorescence from the amino methyl coumarin (AMC) release due to the cleavage of the substrate Z-Gly-Pro-AMC. It had to be noted that there are two different peptidases in serum with prolyl endopeptidase (PE) activity: POP and a POP specific inhibitor insensitive endopeptidase or also called Z-Pro-Prolinal insensitive prolyl oligopeptidase (ZIP) (Breen et al. 2004). ZIP function has been identified as fibrinogen activated protein α (FAPα; also called seprase) (Birney and O’Connor 2001; Collins et al. 2004).

For PE activity measurements, serum aliquots (25 µl) were incubated in 0.1 M Na-K-Phosphate buffer (KH2PO4/Na2HPO4· 2H2O, pH 7.0) by triplicate in 96 well microtiter plate and Z-Gly-Pro-AMC was added after 20 minutes of pre-warming at 37°C to reach the final concentration of 200 µM. Free AMC fluorescence was determined by using Wallac 1420 Victor2TM multilabel counter (PerkinElmer Life Sciences) with excitation and emission filters of 355 and 460 nm, respectively. The kinetic was carried out during 90 min at 37°C after the substrate addition. ZIP activity was determined as the remaining activity after adding the POP specific inhibitor JTP-4819 (diluted to 50 nM).
POP activity was subsequently calculated by subtraction of ZIP activity from PE activity.

The effect of reductant agents on serum POP activity (n=44) was also investigated following the same procedure described above but with the addition of DTT to 5 mM.

The cerebrospinal fluid (CSF) samples (n=21) were analyzed in the presence of reductants (5 mM DTT) after one freezing cycle by duplicate following the same procedure described for serum samples, but the CSF volume was 100 µl per well. All the PE activity detected was due to POP, since the addition of inhibitor decrease the liberation of AMC to zero. Therefore additional assays using POP inhibitors were not necessary.

3.2 Measurement of the endogenous POP inhibitor in serum samples

Serum samples (n=40) were analyzed after second freezing cycle. The levels of the endogenous POP inhibitor were determined as the decrease of the recombinant POP (rPOP) activity upon addition of 25 µl of serum. The assays were done by triplicate using 75 ng of rPOP. The serum PE activity was less than 5 % in all the cases. Bovine serum albumin (BSA 0.1 mg/ml) was added to reaction mixture in order to reduce variability among the assays. The inhibitory capacity is expressed as % of decrease in POP activity compared to basal rPOP value.

3.3 Characterization of the endogenous POP inhibitor

3.3.1 Protein concentration determination

The protein concentration in the samples was determined by Bradford method using BSA as a standard.
3.3.2 Molecular size estimation by ultrafiltration

An aliquot of serum sample was loaded onto upper chamber of a filtration devise with PVC filter of 10 kDa cut off (Microcon Ultracel) and centrifuged at 10,000 g for 30 min at 4°C. The filtered fraction was kept on ice, and the retained fraction washed three times with 1-2 volumes of 100 mM Na-K-phosphate buffer, pH 7. After the process both fractions were assayed for POP inhibitory capacity.

3.3.3 Albumin removal

The albumin removal was carried out using anti serum albumin antibody coupled to agarose bead. An aliquot was placed in the antibody-agarose mixture diluted with neutral buffer (50 mM NaH$_2$PO$_4$, 100 mM NaCl, pH 7) and incubated during 15 min at 4°C. After that the mixture was centrifuged at 500 g during 2 min and the supernatant was pipetted out and applied to a fresh agarose bead batch one more time. To regenerate the antibody-agarose mixture, it was carefully washed at low pH (50 mM Sodium acetate, 2 M NaCl, pH 4), high pH (50 mM Tris, 2 M NaCl, pH 10) and neutralized with neutral buffer (50 mM NaH$_2$PO$_4$, 100 mM NaCl, pH 7).

3.3.4 The effect of temperature on inhibitory capacity of serum

The proteins in serum were inactivated by heat in a water bath. Serum samples were kept in a bath for 15 min at 45°C, 60°C or 5 min 100°C, after that the pre-heated fractions were assayed for the endogenous inhibitor level as described before.
3.3.5 Generation of POP-sepharose coupled chromatographic column

Purified in E.coli expression system, recombinant porcine POP in solution (0.1 mg/ml) was buffer exchanged to ligand buffer (0.2 M NaCO₃, 0.5 M NaCl, pH 8.3) using Millipore falcon filters (MWCO 30,000 kDa). Hi Trap NHS-activated HP column (0.7 × 2.5 cm) was activated with ice cold HCl and 1 ml of POP in ligand buffer solution was loaded onto the column. The coupling reaction was carried out for 20 min at room temperature (RT).

The coupling reaction was stopped by washing and incubating the column with 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3 during 20 min at RT. The ethanolamine buffer was washed with 0.1 M Acetate, 0.5 M NaCl, pH 4 and equilibrated with 50 mM NaH₂PO₄, 100 mM NaCl, pH 7.

3.3.6 Purification of the endogenous POP inhibitor by affinity column

An aliquot (200 µl) of the albumin free sample was loaded in the POP-sepharose coupled column. The column was sealed and incubated on ice during 10 min. After the incubation the column was washed with neutral buffer (50 mM NaH₂PO₄, 100 mM NaCl, pH 7) and eluted in batch with 50 mM NaH₂PO₄, 1 M NaCl, pH 7. The whole process was repeated with 2 more aliquots. All the resulting washed fractions or eluted fractions were pooled and concentrated. The resulting fractions (wash and elution fractions) were analyzed by SDS-polyacrylamide gels and for endogenous POP inhibitor level.

3.3.7 Anion exchange chromatography

Albumin free serum samples, containing endogenous POP inhibitor, were subjected to anion exchange chromatography using HiTrap DEAE FF (weak anion exchange, 0.7 x 2.5 cm, Amersham bioscience AB, Uppsala, Sweden) or alternatively HiTrap QFF
column (strong anion exchange, 5 x 10 mm, Amersham Biosciences Inc., Piscataway, NJ, USA) that were equilibrated with buffer A (50 mM NaH₂PO₄, pH 7). The purification process was done in ÄKTA prime protein purification system (Amersham bioscience, Uppsala, Sweden). The sample (1 ml) was loaded onto the column and the elution was performed at a flow rate of 1 ml/min using buffer A for 10 min, linear gradient from 0 mM to 500 mM NaCl in 50 mM phosphate buffer, pH 7 for next 10 min followed by buffer B (50 mM NaH₂PO₄, 500 mM NaCl, pH 7) for 10 (-20) min. The protocol was carried out at 4°C. The elution was monitored by UV absorbance at 280 nm using the Prime view software (Amersham biosciences, Uppsala, Sweden). Based on the UV absorbance profile (protein amount) fractions were chosen for POP activity and inhibitory capacity assays.

### 3.3.8 Hydrophobic interaction chromatography

Albumin free serum samples were subjected to hydrophobic interaction chromatography (HIC) using HiTrap Phenyl-sepharose™ High Performance column (0.7 x 2.5 cm, Amersham Biosciences AB, Uppsala, Sweden). The procedure was carried out similarly to anion exchange chromatography with following changes. The column was equilibrated with buffer A (20 mM NaH₂PO₄, pH 7.2, 1 M Na₂SO₄) and the elution was performed using buffer A, linear gradient of 1 to 0 M Na₂SO₄ in 20mM – 0,25 M phosphate buffer, pH 7.2 followed by buffer B (0,25 mM NaH₂PO₄, pH 7.2). The protocol was carried out in RT.

### 3.3.9 SDS-PAGE and western plotting

Samples were diluted with loading buffer (100 mM Tris · HCl, pH 6.8, 70 % glycerol, 2 % SDS, 0,005 % bromophenol blue, 10 mM β-mercaptoethanol) and loaded into 7 or 12 % polyacrylamide/bisacrylamide Tris·HCl discontinuous gels. Gels were stained for protein or transferred electrophoretically to nitrocellulose membrane for plotting.
For Coomassie protein staining, gels were fixed and stained with 2 % Coomassie blue in acetic acid : methanol : water (7:40:53) overnight. Background was destained with several changes of ethanol : acetic acid : water (40:5:45), until clear background. Silver staining was done by incubating the gel during 30 min ethanol : acetic acid : water (40:5:45), washed with water for 30 min (two changes), sensitized with 0.02 % sodiumthiosulphate for 1-2 min, and incubated with 0.1 % silver nitrate for 20 min at RT. Gels were then rinsed twice with distilled water, and bands visualized by incubating for 1-2 min with 2 % sodium carbonate, 0.04 % formaldehyde.

Western blotting was performed under standard conditions using primary antibody chicken anti-POP IgY diluted to 1:5000 (with 0.1 % TTBS), and the anti-chicken-horseradish-peroxidase complex diluted to 1:50000 (horseradish peroxidase conjugated rabbit anti-chicken secondary antibody solution) (Pierce Biotechnology, Rockford, USA). Protein visualization was performed using Super signal West Pico -kit (Thermo scientific, Rockford, USA) following manufacturer’s instructions. The immunoreactivity was detected by Gene gnome (Vacutec Life Sciences, JHB, South Africa) and GeneSnap Imaging (Syngene, Cambridge, UK).

3.4 Data analysis

The kinetic data was processed by Microsoft Office Excel (Microsoft Corporation). Statistical analyses were performed using GraphPad Prism version 5.02 for Windows, GraphPad Software, San Diego California USA. A two-tailed t-test was used for assessing the significance of the difference between healthy and diseased sample groups. A linear regression analysis was used for evaluating a correlation between different factors. Results are expressed as means ± S.E.M. Statistical significance was defined as $p < 0.05$. 
4 RESULTS

4.1 POP activity levels

POP activity levels in the serum samples were found to be lower to those observed in plasma samples on the previous study for both healthy controls and patients with RR-MS (Fig. 1a). Nevertheless, the levels of POP detected in MS serum were consistently lower when compared to controls. The statistical significance of this difference was however low due mostly to the low levels of absolute activity in both groups (p=0.4249).

Serum POP activity levels measured in the presence of the reductant agent DTT were notably higher in both groups (Fig. 1b). The POP activity in MS serum seemed to be slightly higher compared with the healthy controls. However, the statistical significance of the difference was still low (p=0.2588).

By the assay procedure used it was not possible to measure the POP activity levels in CSF without DTT because of the very low amounts of the active enzyme. With DTT the activity was detectable, but still very low. However, the POP activity levels in CSF seemed to be decreased in CSF from MS patients compared to that of control patients. Again, the difference between the groups had a statistical low significance (p=0.3795) (Fig. 1c).

A correlation between POP activity levels and the age and sex of a patient could not be found in this study neither in serum nor in CSF (data not shown).
Fig. 1 POP activity levels in serum (a,b) and cerebrospinal fluid (CSF) samples (c) from MS patients and healthy controls. a, basal POP activity. b and c, POP activity in the presence of DTT (5 mM). Data represents the mean ± SEM of kinetic fluorescence assay measurements on 25 µl of serum or 100 µl of CSF.

4.2 Endogenous POP inhibitor

Serum inhibitory capacity over recombinant porcine POP (rPOP), which indicates the presence of an endogenous POP inhibitor, was one of the interests of this research. Figure 2 shows the difference in the inhibitory capacity of serum between MS patients and healthy controls. The data suggested that MS serum might have stronger inhibitory
capacity over rPOP compared with the effect of control serum, but this difference had
low statistical significance (p=0.4878). There was no correlation between the age or sex
of a patient and the inhibitory capacity of serum (data not shown).

![Graph showing inhibitory capacity of serum over recombinant porcine POP (rPOP).](image)

Fig. 2 Inhibitory capacity of serum over recombinant porcine POP (rPOP). Inhibitory
capacity (mean ± SEM) was measured after preincubating rPOP (75 ng) with 25 µl of
serum, and it is expressed as % of decrease in POP activity compared to basal POP
activity value.

4.3 Characterization of the endogenous POP inhibitor

The inhibitory capacity of different volumes of serum over recombinant porcine POP
was measured after pre-incubating recombinant POP (75 ng) with 0, 12.5 µl, 25 µl, 50
µl and 75 µl of serum. A clear positive correlation was found between the volume of
serum used and the inhibitory capacity in serum (Fig. 3). Bigger volumes of serum
possessed bigger inhibitory capacity over rPOP.
Fig. 3 Dose response curve of serum POP inhibitor over recombinant porcine POP (rPOP). Inhibitory capacity (mean ± SEM) was measured after preincubating rPOP with different amounts of serum as indicated.

To try to establish the nature of this POP inhibitor, the effect of DTT (5 mM) and Triton-X-100 (1 %) on the inhibitory capacity was measured (Fig. 4). The inhibitory capacity in serum did not decrease with DTT demonstrating that the inhibition was not a redox process. On the other hand the addition of Triton X-100 potentiated the inhibitory effect of serum, suggesting that the inhibition might be dependent on ionic interactions between inhibitor and POP.

Fig. 4 Effect of DTT and Triton X-100 on the serum inhibitory capacity over recombinant porcine POP (rPOP). Inhibitory capacity was measured after preincubating rPOP (75 ng) with 50 µl of serum, in the presence of DTT (5 mM) and Triton-X-100 (1 %). The mean of inhibitory capacity is shown ± SEM, and it is expressed as % of decrease in POP activity compared to basal POP value.
The serum was subject of different treatments in order to learn about the biochemical features of the endogenous POP inhibitor. Albumin, the most abundant protein in serum, was removed from serum samples by anti bovine serum albumin antibodies. This albumin free serum was compared with original serum in terms of its inhibitory capacity over recombinant porcine POP. The loss of albumin did not affect to serum’s capacity to inhibit POP (Fig.5). The serum was fractionated by ultrafiltration with a cut-off filter of 10 kDa. The inhibitory capacity did not decreased in the >10 kDa fraction. On the other hand, the <10 kDa fraction showed no effect on POP activity. These assay results indicated that the endogenous POP inhibitor is bigger than 10 kDa in size and probably a protein rather than a short peptide.

Fig. 5 The inhibitory capacity of serum, albumin free serum and molecular filtered fractionations of serum over recombinant porcine POP (rPOP). Albumin was removed by anti bovine serum antibodies and fractionated by ultrafiltration with molecular weight cut off 10 kDa filter. Inhibitory capacity (mean ± SEM) was measured after preincubating POP (75 ng) with 50 µl of intact, albumin free, and >10 kDa and <10 kDa fractions. Values are expressed as % of decrease in POP activity compared to basal POP activity.

The endogenous POP inhibitor, which was now expected to be a protein according to its molecular weight, was further investigated by affinity chromatography. An albumin free serum sample was loaded onto the POP-Sepharose coupled column (recombinant POP coupled via primary amines) which allowed the inhibitory factor (probably a protein or large molecule) in serum to interact with linked POP and be retained in the column. The retained fraction was eluted in batch, and the resulting wash and elution fractions were further investigated. The inhibitory factor was expected to be present in the eluted
fraction (the interacted proteins). The fraction obtained from the wash was loaded again to the column in order to maximize the yield. Still, the efficiency of the column was low, since only a small fraction of total amount of inhibitor was bound to the column as demonstrated by inhibitory capacity assay. The bound fraction showed only slight inhibitory capacity whereas it was moderate in the wash fraction. The elution fraction (bound proteins), as a pure sample, were subsequently subjected to SDS-PAGE and silver staining.

From this fraction the bands that appeared after staining were very weak. There was a band around 80 kDa and also a weak band at higher mol.wt, which leaded to think of the possibility of protein complex of some sort. The sample was concentrated by acetone precipitation and re-analyzed by SDS-page and silver staining. However, the protein content was still too low.

The hypothesis of the protein character of the endogenous POP inhibitor was tested by preheating the serum samples before determining the inhibitory capacity in serum. From the results (Fig. 5) it can be seen that the inhibitory capacity remained at the original levels at 45ºC and 60ºC, but at 100ºC the capacity was decreased to one third of the original value. Since generally proteins start to denature above 40ºC and lose their biological activity, the presumed inhibitory protein was expected to inactivate due to heat in this experiment. However, these results suggested that if the inhibitor is a protein, it has a considerable resistance to heat. Heat resistant proteins indeed have been described.
Fig. 5 The effect of temperature on the inhibitory capacity of serum on recombinant porcine POP (rPOP). Inhibitory capacity (mean ± SEM) was determined after preincubating POP (75 ng) with 50 µl of serum/preheated serum and it is expressed as % of decrease in POP activity compared to basal POP value.

The proteins of albumin free serum were subsequently fractionated by chromatography using an anion exchange column (HiTrap DEAE FF). The resulting fractions were investigated for inhibitory capacity (and for POP activity/protein) in order to find the fraction(s) that possess the endogenous POP inhibitor. Fractions of interest were used as a sample for the next chromatographic step. The following section presents the results of the most relevant purification steps.

Figure 6 shows the DEAE chromatogram of an albumin free serum sample. Both POP activity and inhibitory capacity were assayed from several fractions. POP activity (triangles) was found in two peaks, which essentially coincided with the inhibitor capacity profile (squares). There was however a slight shift in the peak position for POP activity and inhibitory capacity. This was considered as a possible mark of the existence of protein complex POP-inhibitor. High inhibitory capacity fractions 16 and 17 were chosen for further investigation.
Fig. 6 DEAE chromatogram of an albumin-free serum sample and subsequent measurements of POP activity and inhibitory capacity from fractions 1-4 and 14-29. POP activity and inhibitory capacity were measured as described in methods in 50 µl of sample.

The complexity of the sample fractions (16 and 17) was evaluated by silver staining of SDS-PAGE, which revealed that the fractions were too complex for protein identification by mass spectrometry (data not shown). Western plot was used to determine the presence of POP protein in the fractions. Figure 7 demonstrates that the peak did not contained POP at measuring levels. Accordingly, the POP activity measured in these samples was likely attributed to ZIP. The western result strongly argues against the presence of a POP inhibitor complex.

Fig. 7 Western blotting of the fractions 16 and 17 from DEAE chromatography. Recombinant porcine POP (rPOP, 0.02 µg) was used as a positive control. Sample proteins were probed with specific chicken anti-POP antibody linked to horseradish-peroxidase conjugated rabbit anti-chicken and detected with chemiluminescent substrate. (MWm – molecular weight marker)
However, in order to confirm a lack of POP and inhibitor complex in this conditions, POP inhibitor peak fractions from DEAE were mixed with a sample of pure recombinant POP and re-subjected to DEAE to look for co-purification (Fig. 8). POP activity was only eluted with the inhibitor fractions. However, western plots did not revealed POP presence in these fractions (Fig. 9).

Fig. 8 DEAE chromatogram of an albumin free serum sample mixed with recombinant porcine POP (rPOP, 1 µg) and subsequent measurement for POP activity from all the fractions. POP activity was measured as described above using sample volume of 50 µl.

Fig. 9 Western blotting of the fractions 15-19 from DEAE chromatography. Recombinant porcine POP (rPOP, 0.02 µg) was used as a positive control. Original FPLC sample was mixed with extra rPOP (1 µg). Sample proteins were probed with chicken anti-POP antibody linked to horseradish-peroxidase conjugated rabbit anti-chicken and detected with chemiluminescent substrate. (MWm – molecular weight marker)
The results from silver staining of these fractions (15 – 17) showed no band at 80 kDa corresponding to POP (Fig. 10). However, the fractions contained multiple other proteins.

Fig. 10 Silver staining of fractions 15–17 from DEAE chromatography. Recombinant porcine POP (rPOP, 0.02 µl) was used as a positive control. Original FPLC sample was mixed with extra rPOP (1 µg). (MWm – molecular weight marker)

Since the results obtained so far were not conclusive to isolate the serum inhibitor hydrophobic interaction chromatography (HIC) was subsequently tested. HIC is a typical high selectivity technique for purifying biological macromolecules. Chromatography of albumin free serum was performed using a Phenyl Sepharose column as shown in Fig.11. After chromatography, the inhibitory capacity of fraction concentrates 20-23, 25-27 and 30-33 was assayed finding a peak of inhibitory capacity on fraction 22, which coincides with a protein peak (Fig. 11). Fractions 20, 21, 23 and 25 (25 as a negative control) were subjected to SDS-PAGE (7 % resolving gel) and silver staining. The polyacrylamide gels revealed that the fractions were still very complex with too many protein bands, making difficult to identify the protein corresponding to the inhibitor. The fractions 21 and 22 were pooled and further processed.
Fig. 11 Phenyl Sepharose chromatogram of an albumin free serum sample and subsequent measurement for inhibitory capacity from concentrated fractions 20-23, 25-27 and 30-33. Inhibitory capacity was measured as described above using sample volume of 50 µl.

Chromatography upon a Q Sepharose column was then performed. The pooled sample from previous HIC run (21 and 22) was buffer exchanged and applied to the column (Fig. 12). Inhibitory capacity was tested in concentrated pools of fractions 1-2 (1), 9-10 (2), 15-19 (3), 20 (4) and 21-23 (5) according to the protein peaks eluted (Fig 13). The pooled fraction number 3 demonstrated the highest inhibitory capacity (10 %) and was subjected to SDS-PAGE (12 % resolving gel) and protein staining together along with the original sample. Figure 14 presents electrophoresis gel after staining. The original sample from phenyl sepharose run showed still be complex. However, analysis of the Q sepharose fraction revealed a single band after coomassie-blue staining and expressed few minor additional bands after silver staining. This fraction was thus considered as a highly purified fraction. The two major bands from this fraction (1. and 2. in Fig. 14) were excised and analyzed by mass spectrometry for identification.
Fig. 12 Q sepharose chromatogram of an albumin free serum sample (pooled fractions 21 and 22).

Fig. 13 Inhibitory capacity of 5 pooled fractions of Q sepharose run. Inhibitory capacity was measured as described above using sample volume of 50 µl. [fractions: 1-2 (1), 9-10 (2), 15-19 (3), 20 (4) and 21-23 (5)]
Fig. 14 Coomassie blue-silver staining of fractions demonstrating inhibitory capacity as a mark of the endogenous POP inhibitor. The fraction samples were from Phenyl sepharose chromatography and from subsequent Q sepharose chromatography.

The mass spectrometry analysis identified the band number 1 as $\alpha_2$-macroglobulin, a known panprotease inhibitor in serum. The second band was identified as human albumin. This indicates that the removal of albumin was not quantitative, as it has been observed before. The presence of albumin this far on the purification however, can not be explained.

5 DISCUSSION

The POP activity levels in physiological fluids have been shown to be significantly lower than in tissues (Garcia-Horsman et al. 2007). When measuring POP activity in blood, the activity levels (PE/POP) in serum samples are lower compared to plasma samples, although the additives used to prevent coagulation process in plasma are shown to decrease POP activity (Breen et al. 2004; Momeni et al. 2003; Tenorio-Laranga et al. 2010). Citrate inhibition of POP can be almost 50 %, whereas effect of
EDTA is moderate (Tenorio-Laranga et al. 2010). It was considered these problems could have been circumvented by using serum instead plasma as a sample. However, in turn the observations here indicated that POP activity in serum is very low, making more difficult to reach statistical significance when comparing diseased and healthy samples. Furthermore, POP activity levels in CSF were found particularly low.

Proline-containing oligopeptide substrates were previously thought to be specific for POP, until in 1997 it was shown a second enzyme (ZIP) in bovine plasma that cleaves POP substrates in vitro (Cunningham and O’Connor; Birney and O’Connor 2001). However, the actual substrates of ZIP in vivo have not been described. This enzyme has later been identified as a fibroblast activated protein (FAP, seprase), although whether the serum ZIP really is a product of the FAP gene has not been confirmed (Collins et al. 2004, Garcia-Horsman et al. 2007). The POP activity has been shown to be 40 % of the PE activity in plasma (Breen et al. 2004, Tenorio-Laranga et al. 2010). In the present study the proportion of POP activity in serum accounted just 2 % of the PE total activity. This is not in agreement with previous information. However, when POP activity was measured in the presence of a reductant as DTT, the activity rose to be over 50 % of the total activity. Oxidative deactivation of POP is well documented and it is thought to be due to oxidation of certain cysteine residue, or residues, involved in substrate binding or product exit from active site (Garcia Horsman et al. 2007). From the data in this study it can be concluded that in serum POP is substantially deactivated most probably dependent on cysteine oxidation. The results also indicate that this process is related to coagulation, which is the main difference between serum and plasma preparation. It is concluded that in order to measure POP levels in serum is recommended to perform assay in the presence of a reductant agent.

Many factors can alter POP activity and thus assay results and the conclusions of the results. Sample collection, preparation and assay conditions have been shown to affect POP activity and stability (Momeni et al. 2003). Storage at room temperature (20°C) causes rapid decline in enzyme activity. Storage at – 80°C has not been shown to decrease POP activity, but freezing and thawing cycles of samples does. The first freeze-thawing cycle produces the biggest reduction in enzyme activity being less
notable in subsequent cycles. Furthermore, the activity is sensitive to organic solvents, usually used to dissolve synthetic substrates and inhibitors, and they have been shown to influence the PE assay sensitivity. In this study DMSO was used, since it shows no significant effect on activity values.

All the differences observed in POP activities (serum, serum with DTT, CSF) between healthy controls and patients with RR-MS in this study were of very low statistical significance. However, the differences in blood POP activities between study groups were similar to preliminary study in the Spanish cohort (Tenorio-Laranga et al. 2010). When serum POP activity measurements were performed with DTT, it showed clearly that the POP enzyme was present in serum, but that it had lost its enzymatic activity most probably due to harsh oxidative conditions possible as a result of clothing process. This is substantiated by the fact that the effect of DTT on POP activity in serum was notable both in MS and in control samples, whereas in the preliminary study, in plasma, this DTT effect was more clearly observed in MS compared to control samples, although the trend was similar. Furthermore, the POP activities in serum with DTT seem to be similar to the POP activities in plasma with DTT. It might as well be that the additives in plasma may (indirectly) protect the enzyme from oxidation after sample collection or they may even restore some activity.

The POP activities in CSF samples were very low, which was in line with the previous studies measuring such activities in various diseases (Hagihara and Nagatsu 1987; Momeni et al. 2003). It would have been interesting to be able to measure the enzymatic activity without the reductant in order to evaluate the oxidative state in CNS. The results showed that there was a POP enzyme present in CSF, but that it has oxidized (inactivated) in both study groups to the level under the detection limit of the procedure since DTT activated to measuring values. It is worth noting that the variability of the activity numbers measured in CSF samples was relatively wide, while this was not the case for serum samples when DTT was present.

Even though the oxidative stress is linked to MS, the notable difference in serum POP activities with and without DTT in both study groups suggests that serum samples in
some point may have been exposed to oxidative/inactivating conditions before the activity measurement. POP activity has been shown to be sensitive to thiol reagents and oxygen oxidation; the effects that are mediated by the oxidation of the Cys255 residue of the catalytic triad (Polgár 2002). The activity measurements were done in batches, and there was no observable difference in day-to-day POP activity values. The serum used in the assays was not collected in the purposes of this study, instead it was material saved after clinical laboratory testing in the years of 2006 to 2008.

Another factor that can decrease POP activity in the patient samples is the level of the endogenous POP inhibitor. In the preliminary study there was a significant difference between healthy controls and MS patients in the inhibitory capacity of plasmas (Tenorio-Laranga et al. 2010). In this study the difference was similar; MS serum seemed to possess the bigger inhibitory capacity over purified recombinant POP than control serum. However, the difference was too small to be statistically significant. Nevertheless, this inhibitory effect could not be reversed by DTT, thus the presence of this inhibitory factor itself, is not sufficient to explain the POP inactivation seen in the results. The inhibitory factor inactivates POP enzyme in a reversible manner since POP can be still activated with higher affinity substrates.

In addition almost all the patients were on interferon-β (INF-β) therapy. Currently it is not known if this cytokine medication has an effect on POP activity levels. A study by Bayer et al. that investigated INF-β regulated proteins (by 2-DE-DIGE and MS) in CNS, did not report altered POP expression (Beyer et al. 2009). The changes detected were mainly cytoskeletal and transport proteins. However, it is known that POP interacts with cytoskeletal proteins, and probably also with transport factors (Schulz et al. 2005).

The preliminary study found significant decrease in POP activity according to age in healthy subjects, but this was not true in the case of MS patients, where the activity was already low in young patients with no further change at older age (Tenorio-Laranga et al. 2010). In this study such a difference could not be observed, most due to the fact of the low activities in all the samples. Also the mean age was higher among the control
patients. It is worth of noting that activity measurements were done in reaction mixtures, so the POP concentration (activity) \textit{in vivo} should be bigger.

This part of the study was performed initially measuring the levels of POP activity and the levels of its endogenous inhibitor in all samples blind, that is, the origin of every sample was not known until all samples were assayed. The different phases in MS disease might cause fluctuation to the measured parameters, when in control samples these levels are expected to be more stable. A longitudinal study or a defined sample collection period would sharpen the results. In this study there was no correlation between the age of a patient and measured parameters, but the preliminary study reported decreasing POP activity levels along aging.

The strong point of this study was the biochemical characterization of the endogenous POP inhibitor in serum. There was no previous information on the nature this factor. It was suspected however, that inhibitor would be a peptide or a polyamine based on previous suggestions (see Garcia-Horsman et al. 2007). The studies that have investigated an endogenous inhibitor in animal tissue samples have isolated a small peptide (Salers 1994; Yoshimoto 1982). However, one of the first experiments in this study characterized the inhibitor to a molecular weight of above 10 kDa. This was the first difference from previous studies. However, the results here found that the factor is temperature resistant, similarly to previous studies, supporting the possible peptidic nature. These two findings seem to be contradictory.

In trying to isolate the inhibitor, there were applied different chromatographic methods. Affinity chromatography showed to be effective in binding the inhibitor, but it was found to have of extremely low yield which precluded a confident identification and the addition of further purification step.

The chromatographic sequence, DEAE, Q-Sepharose and phenyl-Sepharose was found to be adequate for a good purification with enough yield for identification. These steps finally leaded to the identification of the serum POP inhibitor as human albumin or $\alpha_2$-macroglobulin. The activity assay of pure recombinant POP in the presence of purified
albumin (BSA) shows no difference with the control ruling out this protein as POP inhibitor. Thus, it was concluded that the inhibitory factor in serum was indeed \( \alpha_2 \)-macroglobulin. Despite that this has to be verified by direct assay it is very likely that this protein is indeed actual inhibitor.

\( \alpha_2 \)-Macroglobulin has been described as inhibitory protein, that denaturates at high temperature, 66°C (Kalwant and Porter 1991; Borth 1992; Yamamoto et al. 1996). Furthermore it is known that substrate binding can increase temperature resistance. The relative resistance to 100°C might have been due to partial renaturation at the temperature of inhibition assay (37°C). Alternatively, the remaining inhibitory capacity after high temperature incubation may also lead to think on the possibility of other molecule in serum inhibiting POP, which may not been revealed after purification process, and remains to be discovered. It can be speculated that this molecule, if small, might be bound to a bigger protein precluding its filtering through a 10 kDa filter.

On the other hand \( \alpha_2 \)-macroglobulin belongs to a family of \( \alpha \)-macroglobulins that comprise 8-10 % of total serum protein (Borth 1992). It can mediate reversible or irreversible inhibition of proteins of diverse biological functions. \( \alpha_2 \)-Macroglobulin binds virtually any proteinase, whether self or foreign, primarily through \( \varepsilon(\gamma\text{-glutamyl}) \) lysine bonds. In blood it functions as an inhibitor of coagulation by inhibiting thrombin and inhibits fibrinolysis by inhibiting plasmin and kallikrein (DeBoer et al. 1993). It serves as a humoral defence barrier against pathogens in the blood and tissues of vertebrates (Borth 1992). Human \( \alpha_2 \)-macroglobulin, coded by gene A2M, is implicated e.g. in pathogenesis of Alzheimer’s disease based on its ability to mediate the clearance and degradation \( \beta \)-amyloid (Blacker et al. 1998). It is also expressed in brains and upregulated with its receptor LRP, during acute phase brain injury.

It is also important to mention that \( \alpha_2 \)-macroglobulin has been found altered in MS (Jensen et al. 2004). Significantly lower concentrations of native \( \alpha_2 \)-macroglobulin and significantly higher concentrations of transformed \( \alpha_2 \)-macroglobulin were found in plasmas of MS patients. Also immunochemical alteration due to impaired \( \alpha_2 \)-
macroglobulin stability has been reported from patients with MS (Gunnarsson et al. 2000).

Kalwant and Porter have previously reported that $\alpha_2$–macroglobulin inhibits POP moderately at 10 mM concentration (in the presence of DTT) (Kalwant and Porter 1991). If the inhibitory capacity seen in this study was all caused by $\alpha_2$–macroglobulin, $\alpha_2$–macroglobulin inhibits POP notably. But this needs to be proven by additional tests.

The therapeutic aspect of this POP inhibition is complicated, since by affecting $\alpha_2$–macroglobulin levels multiple other factors will also be affected, but it opens a new line of the relationship between POP function and MS.

6  CONCLUSION

The first part of this study investigated POP activity levels and the levels of its inhibitor in patient samples from healthy controls and patients with RR-MS. Although, low statistical significance was found, the data are somehow in concordance with the previously reported results in a Spanish cohort. By themselves, this result justify a more comprehensive study of plasma POP activities in a Finnish larger sample to confirm the hypothesis of link between the level of POP activity and the cause or effect of the disease.

The second part of the study was a step by step characterization of the endogenous POP inhibitor in serum samples. After biochemical characterization and series of chromatographic runs, a mass spectrometry analysis revealed the inhibitor to be $\alpha_2$ – macroglobulin, a panprotease inhibitor in serum. This is of relevance not just because confirm the observed effect of $\alpha_2$ – macroglobulin on POP, but because it substantiate a relationship between POP and MS. The results found justify further studies on the role of POP in complex mechanisms of homeostasis and how this is broken in disease.
REFERENCES


Borth W: α2-Macroglobulin, a multifunctional binding protein with targeting characteristics. FASEB J 6: 3345–3353, 1992


Cavasin MA, Rhaleb NE, Yang XP, Carretero OA: Prolyl oligopeptidase is involved in release of the antifibrotic peptide Ac-SDKP. Hypertension 43: 1140–1145, 2004


DeFreitas EC, Sandberg-Wollheim M, Schonely K, Boufal M, Kaprowski H: Regulation of interleukin receptors on T cells from multiple sclerosis patients. Immunology 83: 2637–2641, 1986


Fülöp V, Zoltán S, Polgár L: Catalysis of serine oligopeptidases is controlled by a gating filter mechanism. EMBO Rep 1: 227–281, 2000


Hartung HP, Kieseier BC: The role of matrix metalloproteinases in autoimmune damage to the central and peripheral nervous system. J Neuroimm 107: 140–147, 2000


Mahad DJ, Ransohoff RM: The role of MCP-1 (CCL2) and CCR2 in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE). Semin Immunol 15: 23–32, 2003


