Investigating novel approaches to linear epitope mapping; biophysical characterization of tau protein and its interactions with anti-tau antibodies

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Abstract

Intraneuronal filamentous lesions which are characteristic for Alzheimer´s disease, the so called neurofibrillary tangles (NFT´s), are misfolded β-sheet like aggregates of tau protein. Their presence is closely linked to neurodegeneration and progression of the disease.

Tau protein has been investigated as a promising target for therapeutic intervention of AD. Several monoclonal antibodies specific to tau protein were previously identified and recovered from human blood serum. As potential candidates for development of biopharmaceuticals, the anti-tau antibodies and their interactions with tau epitopes are to be investigated in detail.

This work aims to develop novel methods for linear epitope mapping and to characterize binding sites of identified anti-tau antibodies on tau protein.

Contents

List of Abbreviations

AA	Amino acid
Ab	Antibody
ACN	Acetonitrile
AD	Alzheimer´s disease
BPI	Base peak intensity chromatogram
C_H	Constant domain of Ab heavy chain
C_L	Constant domain of Ab light chain
CSF	Cerebrospinal fluid
dRI	Refractive index increment
ESI	Electrospray ionization
Fab	Fragment antigen-binding
Fc	Fragment crystallizable
GC	Gas chromatography
GFC	Gel filtration chromatography
GFS	Gel Filtration Standard
GPC	Gel permeation chromatography
HDX	Hydrogen-deuterium exchange
HPLC	High-performance liquid chromatography
IDP	Intrinsically disordered protein
Ig	Immunoglobulin
LC	Liquid chromatography
LS	Light scattering
mAb	Monoclonal antibody
MALDI	Matrix-assisted laser desorption/ionization
MALS	Multi-angle light scattering

MAP	Microtubule associated protein
MQ	MilliQ ultrapure water
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MT	Microtubule
MTB	Microtubule binding
MTBR	Microtubule binding region
Mw	Molecular weight
NC	Negative control
NFT	Neurofibrillary tangles
NR	Non-reducing
PHF	Paired helical filaments
PLGS	ProteinLynx Global Server
PTM	Post translational modification
RI	Refractive index
rms	Root mean square
RP	Reverse phase
SDS PAGE	Sodium dodecyl sulfate polyacrylamide electrophoresis
SEC	Size exclusion chromatography
SPA	Staphylococcal protein A
SPG	Streptococcal protein G
UPLC	Ultra performance liquid chromatography
UV-vis	Ultra violet-visible
V_H	Variable domain of Ab heavy chain
V_L	Variable domain of Ab light chain

1. Introduction

1.1 Alzheimer's disease

1.1.1 AD

Alzheimer's disease is a progressive, irreversible, neurodegenerative disease characterized by gradual loss of cognitive functions, memory impairment and neuropsychiatric changes of affected individuals, inevitably leading to death. It is the most common form of dementia affecting more than 25 million individuals worldwide, ranking among the top 10 causes of death. With no known cure for the disease, AD represents the largest unmet medical need in the area of neurology and a great burden on patients, caregivers and society. Annual costs for the AD treatment exceed hundreds of billions of dollars. AD is becoming an important health and socioeconomic problem which calls for a solution.

The causes of AD are not fully understood. Complex changes in brain are involved in both the onset and the progression of the disease. It is probable that processes leading to the brain damage start years before any of the symptoms appear. Even though the age is the greatest risk factor (AD mostly affects people above 60 years old), AD is not a part of normal aging. It has been proven that genetics plays an important role in development of AD, especially in cases of early on-set AD (< 60 years of age). Many other factors, such as mental stimulation, physical activity and condition, nutrition etc., seem to be involved in development and course of the disease. (Citron 2010)

1.1.2 Pathological hallmarks of AD

Two abnormal structures present in the brain are primary suspects when it comes to the cause and progression of AD; neurofibrillary tangles (NFT´s), formed by accumulation of abnormal filaments of tau protein in neurons, and amyloid plaques which are extracellular deposits of β-amyloid protein. These neurotoxic structures are responsible

for impairing the normal function of the brain cells and their death. The progression of the disease and its symptoms seem to be linked to the gradual spreading of the protein deposits throughout the brain. Even though plaques and tangles are hallmarks of the disease and play an indispensable role in AD, it is impossible to declare whether these structures drive the disease, are only bystanders or a result of unsuccessful repair attempts of the body. There are multiple alternations in biochemical pathways and processes besides the formation of the pathological structures. It is very challenging to precisely identify and describe the causes and impacts of individual changes. Nonetheless an extensive research has been conducted during the past decades, mainly focusing on elucidating the mysteries behind the abnormal protein aggregation. Understanding the mechanisms behind the formation of tangles and plaques and their role in AD brings high hopes for finding means of pharmacological intervention and developing effective treatments for the disease. (Kolarova et al. 2012)

1.2   Tau protein

1.2.1   Tau protein

Tau proteins belong to a microtubule-associated protein (MAP) family. This intrinsically disordered, soluble protein is mainly expressed in brain cells where it plays an important role in microtubule stabilization. Tau proteins are known to promote the assembly of tubulin monomers into microtubules which are essential for structural support of cells and serve as tracks for axonal transport. Tau also seems to assist in establishing links between the microtubules and other cytoskeletal components (e.g. actin). Misfolding and aggregation of tau protein have been proven to significantly contribute to neurodegenerative processes. (Buée et al. 2000; Elie et al. 2015)

1.2.2 Structure of tau protein

Tau is defined as an IDP. It contains a high amount of polar and charged amino acid residues which in turn results in lack of well defined tertiary structure of free protein. The structural flexibility of tau is required for the physiological function of the protein.

Tau protein is mainly expressed in neurons, however, it can also be found in peripheral tissues in trace amounts. Tau is translated from a single gene, which stretches over 100 kb on the long arm of chromosome 17. The gene contains 16 exons, from which the exons 2, 3 and 10 are alternatively spliced, ultimately giving a rise to six different isoforms of tau protein which are specific to adult human brain. The sequence length of tau protein ranges from 352-441 amino acids and varies based on the presence/absence of one or two N-terminal inserts and combination of either three or four repeats in the C-terminal part of the molecule. There is only one tau isoform present in the fetal brain, corresponding to the shortest, 352 AA's long version of the protein. This isoform is not expressed in adult brains. The expression of tau isoforms differs during the human brain development, suggesting that specific physiological functions linked to each of the isoforms.

The N-terminal part of tau is referred to as a projection domain. This region of the molecule is quite acidic and is followed by a proline-rich sequence. The two 29-amino acid long inserts (E2, E3) are responsible for different length of the N-terminal part of tau. The N-terminal domain projects from the surface of the microtubule and can interact with other cytoskeletal elements and plasma membrane. The projection domain plays a crucial role in stabilization and organization of axons and is involved in various signal transduction pathways. The C-terminal part of tau is rather basic and contains so called microtubule binding domain. This part of molecule is responsible for binding to the microtubules and promoting their polymerization. The MTB domain consists of repetitive regions (R1-R4) that are made of highly conserved sequence of 18 amino acids. The repeats are separated by less conserved 13- or 14-amino acid long spacer regions. Adult tau isoform contains four repeats and is much more efficient at promoting microtubule assembly in comparison to the fetal tau which only contains three repeats (lacking R2). The interregion between R1 and R2, which is unique to adult

brains, is the most potent sequence when it comes to inducing microtubule polymerization and is therefore responsible for the big difference in the binding affinities between the 3R and 4R forms of tau. (Buée et al. 2000)
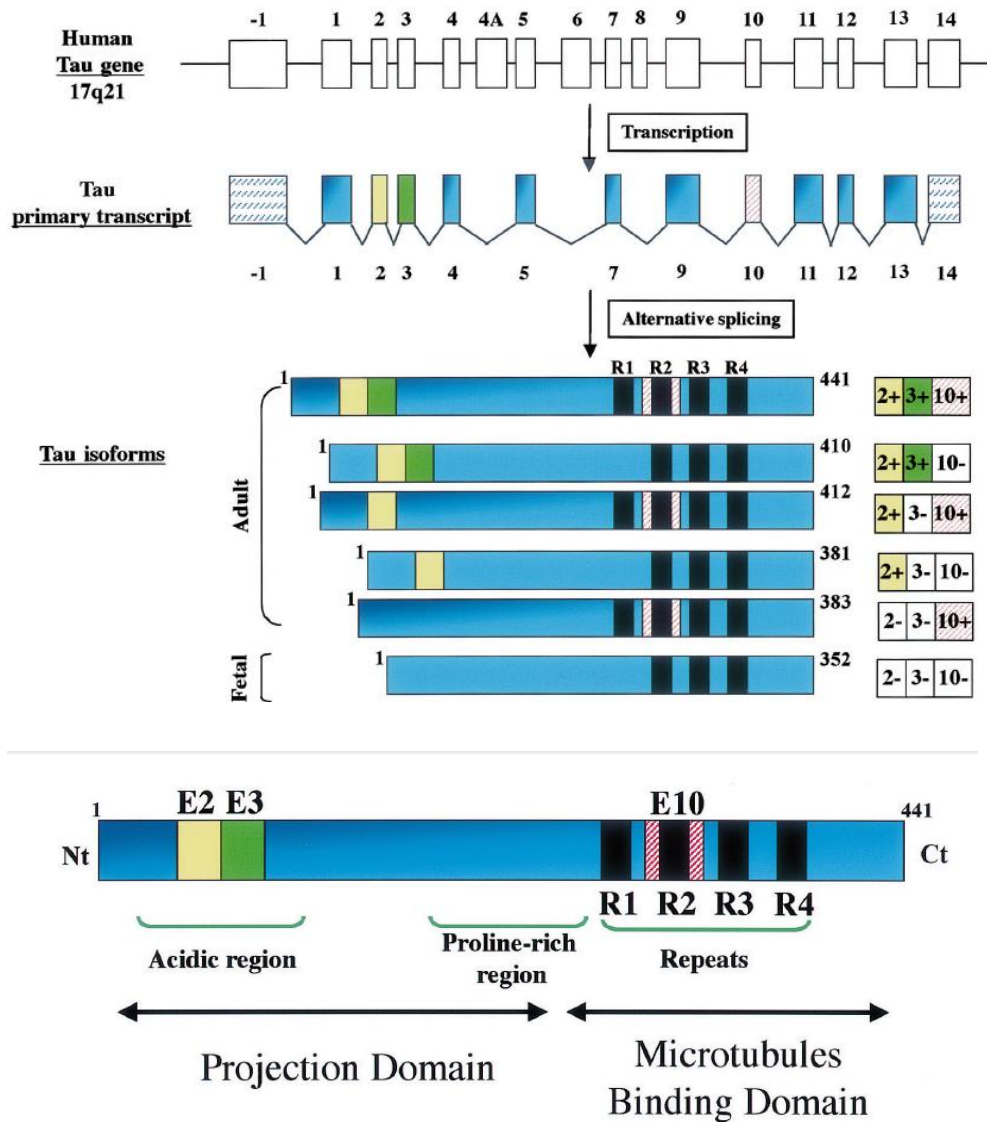
Figure 1: Schematic representation of the human tau gene, tau primary transcript and the six human tau isoforms (top). The longest isoform of tau protein and its functional domains (bottom) (Buée et al. 2000).

1.2.3 Tau phosphorylation

Posttranslational modifications (PTM´s), including phosphorylation, can alter the structure and hence the biological activity and fate of protein molecules. In vivo,

phosphorylation has many different effects on the proteins. It can be a prerequisite for proper folding, modify their enzymatic activity via induced conformational changes, function as a recognition signal for further modifications and protein localization, and influence its interactions with other molecules (Salazar, Höfer 2009). Tau phosphorylation has been proven to directly affect the affinity of the protein for microtubules and is believed to be a regulatory mechanism through which the cells control the tubulin assembly and levels of tau in its unbound and bound state. Research has revealed that tau is more efficient at promoting microtubule binding when less phosphorylation is present.

The sequence of the longest isoform of tau contains 45 serine, 35 threonine and 5 tyrosine residues which means almost 20% of the protein can be phosphorylated (Tenreiro et al. 2014). Tau requires certain levels of phosphorylation to maintain its function and approximately thirty phosphorylation sites have been identified in 'normal' tau. With very few exceptions, all of the phosphorylated residues are localized outside the microtubule binding domain, mainly clustered within the proline-rich domain and the MTBR flanking regions. The different states of tau phosphorylation and the equilibrium of tau binding to the microtubules are regulated by coordinated actions of specific kinases and phosphatases.

Tau is found in all cell compartments, however, in different phosphorylation states. The variability in the degree of tau phosphorylation is altered during the brain development via changes in expression of different isoforms of tau and the ratio between the phosphatases and kinases, which are responsible for the dephosphorylation and phosphorylation respectively. High levels of phosphorylation are observed in fetal brains and gradually decrease with aging. Abnormally high levels of phosphorylation of tau in adult brains are associated with protein misfolding and neurodegenerative diseases (Kolarova et al. 2012).

1.2.4 Dysregulation of tau phosphorylation

Abnormally high levels of phosphorylation can cause conformational changes and misfolding of tau, leading to loss of its capacity to bind tubulin and hence the inability

of the protein to fulfill its physiological role. Tau is involved in providing microtubules their character such as length, stability and interactive capacity. Dysregulation of tau phosphorylation leads to detachment of tau proteins from microtubules and increase in the concentration of the free cytosolic tau. The decrease in levels of the normally functioning, MT bound tau leads to break down of microtubule networks which has a direct impact on the cytoskeletal architecture and the axonal transport in neuronal cells. The set of events triggered by tau dissociation from tubulin is linked to protein aggregation and neurodegenerative processes. Hyperphosphorylated tau is associated with formation of intracellular proteinous inclusions which are pathological hallmarks of AD and various related neurodegenerative disorders, collectively called tautopathies (Tenreiro et al. 2014).

1.2.5 Tau pathology

It has been demonstrated that tau proteins polymerize into intraneuronal filamentous inclusions called paired helical filaments (PHF´s). PHF are insoluble aggregates of multiple isoforms of hyperphosphorylated tau which accumulate in certain brain cell populations of diseased individuals. PHF´s constitute intraneuronal lesions that are characteristic for Alzheimer´s disease, the so called neurofibrillary tangles (NFT´s). These misfolded, β-sheet like protein aggregates are believed to greatly contribute to the degradation of neuronal cells and hence the manifestation of the clinical symptoms of AD.

Aggregated tau proteins have been also found in neurons of aged non-demented individuals. However, the overall levels of the tau phosphoprotein and its aggregates are several times higher in case of diseased brains than in the seemingly healthy ones. The detection of the inclusion bodies in different parts the central nervous system of AD patients well correlates with the severity of dementia and the progression of the disease.
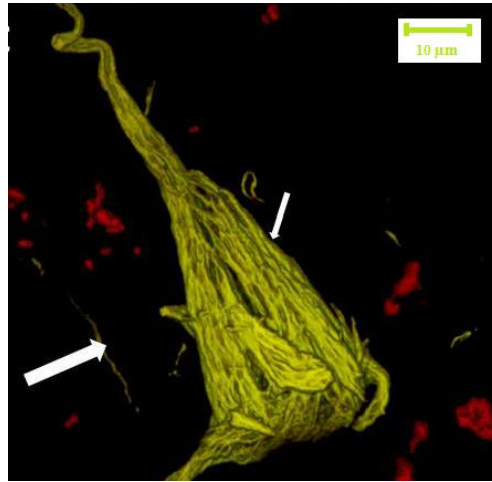
Figure 2: An image of neurofibrillary tangle, strongly labeled with anti-tau antibody (green) and counterstained with thiazine red (Luna-Munoz et al. 2013).

1.2.6 Self-assembly of tau

The self-assembly of tau proteins seems to occur via interaction between the "sticky" MTBR domains. The β-structure in monomeric tau is mainly concentrated in regions of R2 and R3 which have been shown to form filaments on their own in vitro. The repeats lying within the MTBR region quite probably play an important role in the tau self assembly. The exposure of these domains due to conformational changes of the tau protein (mainly the flanking C-terminus and N-terminus regions) caused by alternation of phosphorylation and other PTM´s (e.g. glycosylation, ubiquitination) is possibly involved in the formation of filaments. Many other factors such as ratio between the free cytosolic tau and the MTB bound form (which is closely related to the activity of specific de/phosphorylation enzymes) as well as the presence of various cofactors and ions, might play a role in the formation of the inclusions. Proteolytic cleavage of tau protein has also been proposed as possible promoter of tau self-assembly into aggregates (Bergen et al. 2000; Kolarova et al. 2012)
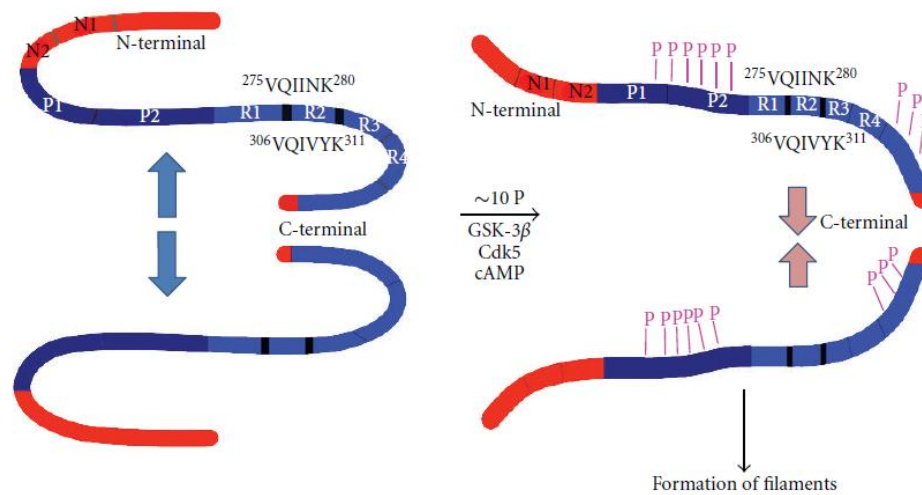
Figure 3: Schematic representation of possible mechanism of tau self-assembly through MTBR aided by conformational changes of the protein due to hyperphosphorylation.

1.2.7 Controversy behind tau hyperphosphorylation

Phosphorylation has been reported as the main PTM present on aggregated tau proteins in NFT´s. It was long believed that abnormal phosphorylation of tau is responsible for transition of normal proteins into the neurotoxic species. Recent evidence, however, brings some controversy to the matter of tau hyperphosphorylation and its role in the induction of the aggregation. Heavily phosphorylated normal tau proteins which do not aggregate into PHF´s exist in fetal and adult brains. It has also been shown that recombinant non-phosphorylated tau can form filamentous inclusions in vitro in presence of specific polyions. These facts suggest that other factors are possibly involved in the aggregation process.

The reasons behind high levels of phosphorylation of tau constituting PHF´s are not clear. Neither is even the fact whether the tau phosphorylation level increases prior to the formation of PHF´s and is therefore a reason for the formation of aggregates of whether the hyperphosphorylation is a consequence and occurs after the polymerization of tau. One of the possible explanations for the hyperphosphorylation of tau is that the body unsuccessfully attempts to induce the dissociation of tau aggregates by additional phosphorylation. Negatively charged phosphate groups repulse each other and should be

helpful in dissociation of aggregates. It is possible that hyperphosphorylation is just a byproduct of a failed protection mechanism and not a cause of the aggregation itself. (Buée et al. 2000)

The biochemical processes and reasons behind the changes of tau proteins into their neurotoxic form are without a doubt very complex. In order to be successful in the search for the treatment for AD and other neurodegenerative diseases it is necessary to unravel the mechanisms responsible for protein aggregation and to connect the individual pieces of information to obtain a bigger picture that would help us to understand the complex set of events that lead to the development of a disease.

1.3 Antibodies

1.3.1 What are antibodies?

Antibodies are globular proteins which are produced by the immune system of vertebrates in response to antigens that might be harmful to the body. These molecules are exclusively secreted by B-cells, have an ability to specifically bind to an antigen and to trigger an immune response against the non-self invaders. The human antibody repertoire is potentially as high as 10^{11} unique molecules. Each B-cell clone secretes antibody with affinity for a unique epitope.

Antibodies, collectively called immunoglobulin, account for about 20% of all plasma proteins and represent one of the most abundant protein groups in blood. There are different classes of Ig´s, however, in general these molecules have similar structural features and properties. Antibodies are large, roughly Y shaped proteins with molecular weight of approximately 150 kDa. They are composed of two types of polypeptide chains, contain two identical sites for antigen binding and one site for the effector binding (e.g. receptor on lymphocytes). Antibody molecules share the same features, however, are still unique. The binding versatility, specificity and thus biological activity of the Ig´s are given by their structure and chemical composition (Janeway 2001).

1.3.2 Structure of an antibody

A typical Ig molecule is composed of two identical heavy chains (H, Mw ~ 50kDa) and two identical light chains (L, Mw ~ 25 kDa). Each of the chains contains separate Ig domains, each consisting of approximately 110 amino acid long sequence folded into β sheet structure. Each light chain has one variable (V_L) and one constant (C_L) domain. The heavy chains each consist of either three or four constant domains (C_H) and one variable domain (V_H) (Wang et al. 2007).

The variable regions which are located at the N-termini of the light and heavy chains combine to form two identical antigen-binding sites (Fab´s) on each of the tips of the 'arms' of the Y shaped immunoglobulin. The constant regions on the C-terminal site of the heavy chains form the so called fragment crystallizable (Fc). This region accounts for binding to the effector molecule or cell. The Fc stem part of the Ig contains conserved glycosilated sites. The levels of glycosilation are isotype and species dependant and play an important role in the immunogenic activity of antibodies.
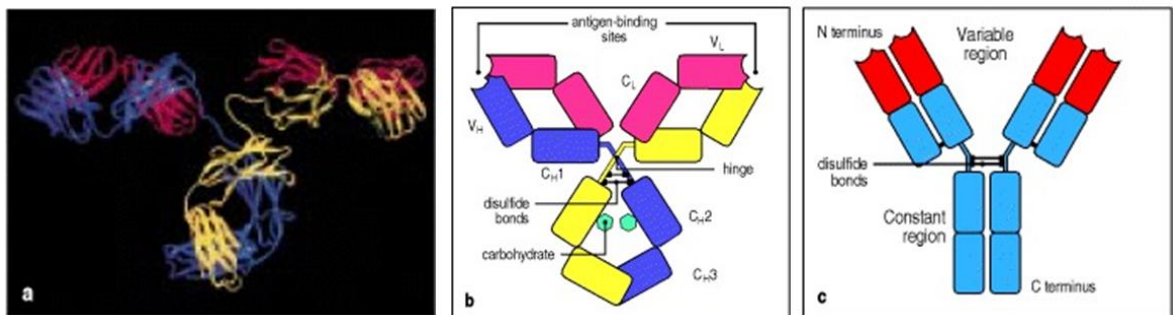
Figure 4: a) Structure of IgG antibody based on X-ray crystallography showing the backbones of the polypeptide chains. b) Schematic representation of the four-chain composition and the separate domains comprising each chain. c) Simplified schematic representation of an antibody molecule (Janeway 2001).

The two antigen-binding Fab´s are linked to the constant Fc portion of the antibody via a flexible stretch of polypeptide chain known as a hinge region (the hinge region in IgM´s and IgE´s classes -see Figure 4 above- is replaced by an extra C_H domain). The flexibility of the connecting portion of Ig´s allows the arms of the molecule to acquire a range of different angles and positions upon binding to an antigen.

Typically, an Ig molecule has four interchain disulfide bonds. Two of them link the heavy chains together at the hinge region and one disulfide bond connects the light and heavy chain of each of the two antibody arms. Non-covalent interactions also contribute to the link between the chains and the overall structure of the molecule (Alberts 2002).

1.3.3 Antibody classes

Immunoglobulins are divided into five major classes according to their C region. The five so called isotypes are the IgG, IgE, IgD, IgM and IgA, each having a distinctive heavy chain (γ, ε, δ, μ and α) and a different biological activity. All Ig´s except IgM (pentamer) and IgA (dimer) are monomeric. Among the five classes is by far the most abundant one the IgG class.

There are two types of light chains found in immunoglobulins, the kappa (κ) and lambda chains (λ). Either of the chain type can be a part of an Ig molecule, however, both light chains are always of the same type, never exist as a combination of two. The light chain distribution chain varies among different species. (Wang et al. 2007)

1.3.4 Specificity and variability of antibodies

The variability in the AA sequences of the Fab´s at the N-terminal regions provide structural bases for the incredible diversity when it comes to binding to epitopes on different antigen molecules. Each variable domain contains a set of three hypervariable regions which form three hypervariable loops at the far end of the domain. The loops of the light and heavy chains cluster together to form antigen-binding sites. Even small changes in the amino acid sequence result in changes in specificity and/or binding affinity of antibodies. Rearrangements of distinct gene segments responsible for expression of both the V and C regions are the reasons behind the binding flexibility of these molecules. As in the case of majority of biologically active proteins, the 3D structure of Ig´s is crucial for proper function of the molecule.
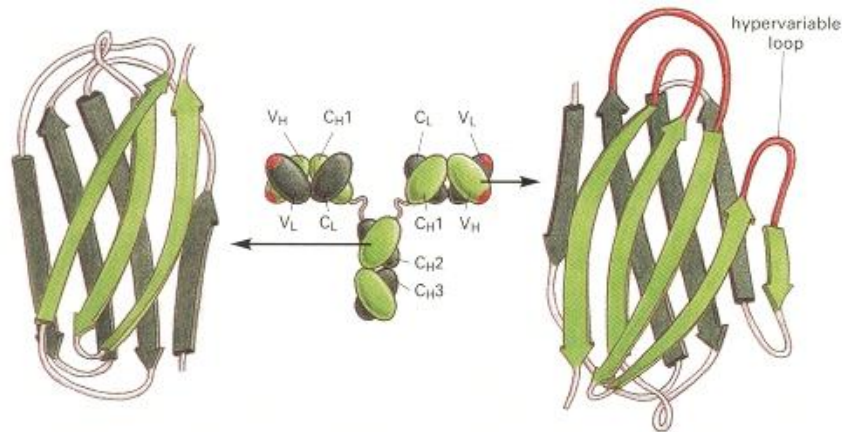
Figure 5: Schematic representation of a folded IgG antibody molecule is shown in the middle, structure of a constant domain is shown on the left and of a variable domain on the right. Hypervariable loops (in red) at the far end of the variable domain form a part

1.3.5 Antibody-antigen interactions

The antigen-binding site of the antibody, so called paratope, binds to the complementary regions of the antigen molecule, so called antigenic determinant or epitope. Antigens are in general molecules, very often protein or polysaccharide moieties, containing one or more (in most cases) antigenic determinants that can be recognized by specific antibody populations. Similarly to binding of the enzyme to a substrate, antibody-antigen complex formation is a reversible process that occurs via relatively weak non-covalent interactions such are for example hydrogen bonding, electrostatic interactions or van der Waals forces. The affinity of the Ig´s for the antigen depends on the fit between the two interacting partners and can be, in a simplified way, described by the key and lock model which requires chemical and structural compatibility of epitope and paratope (Janeway 2001; Alberts 2002).

In case of polypeptide chains, the epitopes are defined by a sequence of amino acid residues which are involved in binding with the antibody. This sequence might either be continuous (we are then talking about a linear epitope) or discontinuous (so called nonlinear or conformational epitope) (Lu et al. 2009).

1.4 Immunotherapy

1.4.1 Antibodies as therapeutic agents

Ig´s secreted by clones which originate from one unique parent B-cell and are directed against a specific epitope on antigen molecules are called monoclonal antibodies (vs. polyclonal antibodies which target the same antigen but do not originate from the same B-cell line). The use of monoclonal antibodies, namely the IgG class mAb´s, as therapeutic agents targeted against various diseases has been gaining considerable interest in last couple of decades. Nowadays IgG´s and their derivatives represent the fastest growing class of biotherapeutic agents (Beck et al. 2010).

In general, protein based drugs require lower effective doses than traditionally used small molecule pharmaceuticals. Further, IgG products are highly specific which provides benefits in form of fewer side effects and a possibility of targeted delivery of therapeutic and diagnostic agents. They can be used not only for the delivery of active agents but also to trigger or stimulate immune response of the organism against the harmful entities.

Nowadays, deeper knowledge and technology allow scientists to engineer antibodies and modify their properties such as affinity for epitopes, cytotoxicity or immunogenicity etc. (Wang et al. 2007).

1.4.2 Tau as a target for immunotherapy

It was long believed that intracellular proteins are not accessible to antibodies and therefore are not suitable candidates for immunotherapy. Since this hypothesis has been proven to be wrong, an ongoing research has been focusing on development of therapeutic agents which target pathological aggregates of hyperphosphorylated tau. The mechanisms behind clearance of intracellular proteins by antibodies are still a matter of discussions.
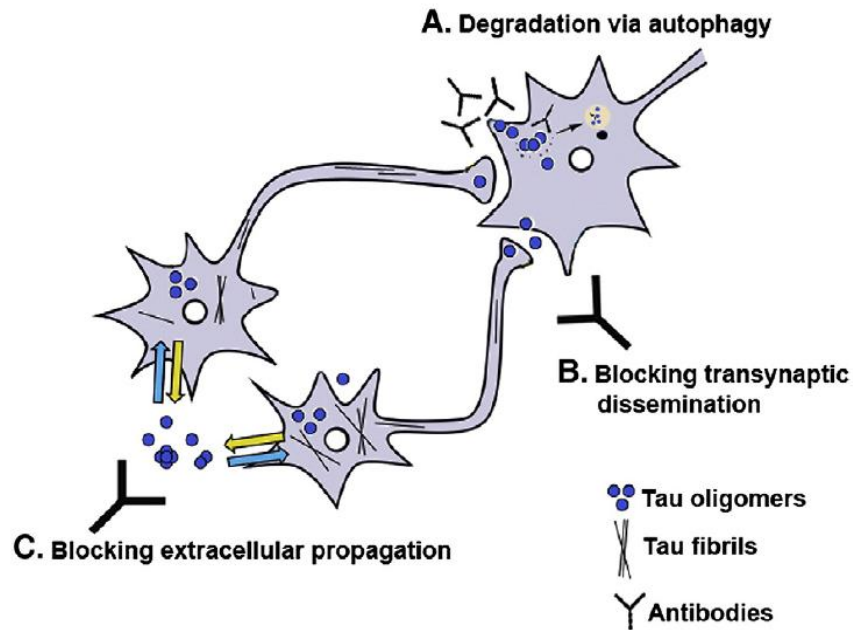
Figure 6: Possible mechanisms of clearance of intracellular aggregates by antibodies (Ubhi, Masliah 2011).

It has been proven that the human immune system is able to generate antibodies targeted against both the phosphorylated and non-phosphorylated tau protein. They are found mainly in serum and in lower amount also in the CSF of AD patients. The low amounts of Ab´s in CSF in respect to serum suggests that they do not originate in brain but are likely to be produced in blood in response to a leakage of tau into the circulatory system. The role of anti-tau antibodies is still unknown. Their natural occurrence itself, however, indicates that tau has an immunogenic potential and might be a suitable target for development of immunotherapy for AD. Number of potential active and passive immunization approaches is being investigated (Rosenmann et al. 2006).
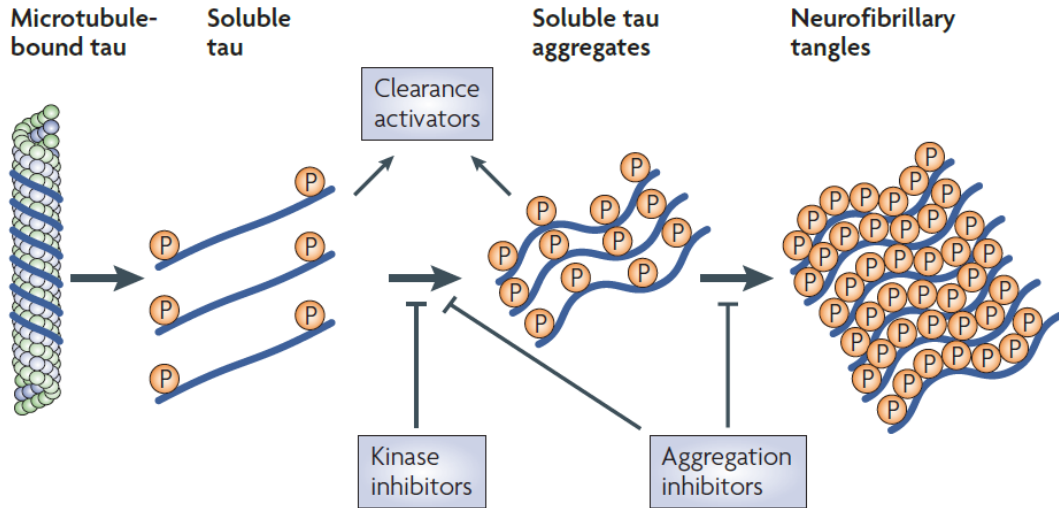
Figure 7: Different strategies that aim to interfere with neurodegenerative processes caused by formation of NFT's are investigated. These include inhibition of hyperphosphorylation (kinase inhibitors), inhibition of tau aggregation, enhancement of clearance of the tau protein and degradation of aggregates (Citron 2010).

Since the abnormal phosphorylation of tau protein has been associated with the formation of NFT´s, extensive studies have been aiming to identify and describe specific phospho-tau epitopes which are linked to the aggregation of the protein. The research has been, to a certain extent, successful and currently investigated approaches are based on targeting of individual phospho-epitopes that are believed to play a role in tau pathology. There are, however, many question marks surrounding the pathology of tau and mechanisms underlying the formation of the intracellular protein inclusions.

Selection of a therapeutic agent that is effective and safe is a great challenge. The potential agent must only target the pathological form of tau and allow the "normal" tau protein to fulfill its physiological function. Considering the number of potential phosphorylation sites and different tau isoforms, identifying the relevant antigens is most definitely not an easy task. Another issue appears when it comes to the delivery of the drugs. The active agents must be able to cross the brain-blood barrier. In summary, development of effective and safe immunotherapy remains a great challenge, however, also a great hope for the AD patients (Ubhi, Masliah 2011).

1.5 Epitope mapping

Understanding the interactions between the antibody and the specific epitope on an antigen molecule is crucial for development of safe and effective pharmaceuticals. So called epitope mapping aims to identify and describe the binding sites of antibodies on the antigenic molecule and therefore represents an indispensable part of research. There are several techniques which are employed for epitope mapping. Among the most commonly used and precise ones are nuclear magnetic resonance, X-ray crystallography and mass spectrometry.

Due to high sensitivity and rapid analysis, MS is emerging as a powerful tool for epitope mapping experiments. Even though different methods have been established to identify the epitope sites on atomic level, there is always a demand for development of novel approaches (Lu et al. 2009).

2. Scope and Goals of the thesis

2.1 The aim of the project

Several monoclonal antibodies specific to tau protein were identified and recovered from human blood serum (Janssen, La Jolla, CA). As potential candidates for development of biopharmaceuticals, the anti-tau mAb and their interactions with tau epitopes are to be investigated and described in detail.

The aim of this work is to develop novel methods for linear epitope mapping and to elucidate the areas of interaction on tau on the primary structure level.

2.2 General workflow

The basic idea behind the new epitope mapping approaches is to digest the tau protein with a specific enzyme thus generating a unique pool of peptides. Peptides that are a part of the tau binding site for the investigated antibody form a complex with the molecule and therefore 'select' themselves from the pool. Interacting peptide species are then identified with ESI-MS, aiding the identification of linear epitopes on the tau protein.

| rTau protein | Digestion with endoproteinase | Binding with mAb | Separation of bound species/fraction collection | Detection and identification with ESI-MS |
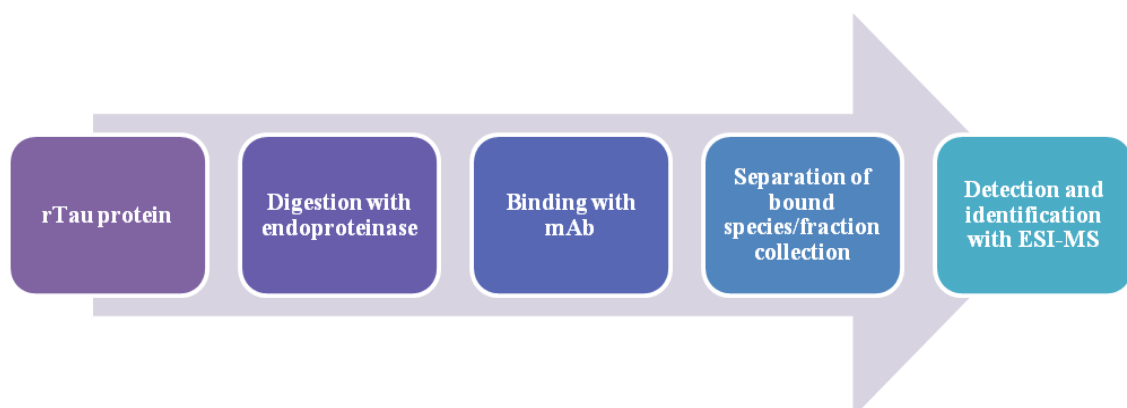
Figure 8: Scheme describing the general workflow of the epitope mapping experiments

2.3 Epitope mapping experiments

Two approaches (A and B) based on similar principles, however, employing different techniques are described in the thesis:

Approach A   **Approach B**

Tau protein
- recombinant tau protein

Enzymatic Digestion
- in-solution digestion

Binding with mAb
- binding partners free in solution

Separation and Fractionation
- SEC-MALS

Detection and Identification
- ESI-MS

Tau protein
- recombinant tau protein

Enzymatic Digestion
- in-solution digestion

Binding with mAb
- mAb immobilized on resin

Separation and Fractionation
- elution of mAb-antigen complex at low pH

Detection and Identification
- ESI-MS

Figure 9: Schematical representation of the workflow of the epitope mapping approaches A and B.

Additionally, a 'proof of concept' experiment was performed in case of the approach B. Detailed design and results of all the experiments are described in sections 5 and 6.

3. Methods

3.1 Enzymatic digestions of proteins

3.1.1 In-solution digestions

In order to more precisely characterize/map proteins with mass spectrometry, an analysis of peptides created via enzymatic digestion of desired molecule is performed. These peptides can be generated by using endopeptidases, proteolytic enzymes that can selectively cleave peptide bonds within amino acid sequence of proteins.

Different endoproteinases possess different specificities. This fact provides us with a tool to generate unique peptide pools containing limited population of protein fragments which can be theoretically predicted and identified by comparison to existing databases.

The in-solution digestion provides a simpler; more straight-forward alternative to another traditional approach called the in-gel, electrophoresis based enzymatic digestion. This method combines both, fragmentation of the protein of interest and the separation of the created peptides in one step. The digest products generated via the in-solution, gel free method are separated in another step, using various separation techniques such as LC (Rawlings, Barrett 2009; Turk et al. 2012).

Experimental conditions for successful digestion of proteins with endoproteinases are highly dependent on the enzyme. The essential parameters to be optimized are the temperature, the pH value and the composition of the solvent, and the means of inhibiting/deactivating the enzyme after the digestion itself (mainly to avoid autodigestion of the enzyme). The availability of the cleavage sites as well as the primary structure of the protein at the cleavage site (e.g. the amino acids in its proximity causing possible steric or electrostatic hindrances) are important factors to consider.

In most cases, proteins can only be successfully digested under denaturing and reducing conditions (followed by alkylation of cystein residues to prevent renaturation), upon which the 3D structure of the molecule disassembles and the AA sequence becomes

exposed and available to the activated enzyme. In general, considering the robustness and the reliability of experiments, the sequence coverage and the reproducibility of the digestions are the two most important factors (Kolsrud et al. 2012).

3.1.2 Peptidases

Peptidases (by systematic name peptide hydrolases) play a crucial role in various intra and extracellular processes involved in cell cycle, cell signaling, inflammatory response etc. Enzymes which are able to hydrolyze peptide bonds are separated into two general groups based on where within the amino acid chain they cleave. Exopeptidases are enzymes that break bonds at the terminal sites of polypeptide chains. Endopeptidases (also called proteinases) cleave the internal peptide bonds. The latter group is used in protein digestion for MS analysis (Garcia-Carreon 1997).

There is a great variety of proteinases present in living organisms. Based on different mechanisms of catalytical action, four major mammalian protease groups are classified: serine, cysteine, metallo- and aspartic proteinases (Rawlings, Barrett 2009).)

3.1.2.1 Serine protease family

Serine protease family represents the most abundant and diverse proteinase group (comprising almost one third of all known proteases). These enzymes contain the Ser/His/Asp catalytic triad (with alternative variations of the two latter amino acids), where serine acts as a nucleophile. The well known and most commonly used enzymes belonging to this group are trypsin, chymotrypsin, Lys-C and elastase (Ekici et al. 2008).

Lys-C proteinase specifically hydrolyzes peptide bonds at carboxyl side of lysine residues. Optimal activity of enzyme is reported at 37°C and pH 7.0-9.0. The cleavage by Lys-C can be partially inhibited if lysine is followed by proline residue.

3.1.2.2 Metalloproteinases

Metalloproteinases are another widespread group of enzymes. These enzymes require an assistance of a divalent cation which is in the majority of cases zinc (almost all wild

type metalloproteinases are monozinc enzymes). Catalytic centers vary with the type of metalloproteinase. The -His-Glu-Xaa-Xaa-His- motif, which is responsible for zinc binding is, however, very well conserved in most monozinc proteinases. The origins of the group, the degree of conservation of the sequence and the separation of metallo-proteinases into distinct families are still a matter of further research. Examples of enzymes belonging to this group are thermolysin, astacin, Asp-N etc. (Fukasawa et al. 2011; Rawlings, Barrett 2009).

Asp-N endopeptidase specifically hydrolyzes peptide bonds at the amino teminus of aspartic and cysteic acid (oxidized cystein) residues. Optimal activity of enzyme is reported at 37°C and pH 6.0-8.5.

3.2 SDS PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis is a common method used for separation of proteins based on their size. As in other electrophoretic techniques, charged molecules migrate through medium under the influence of electric field. Their mobility depends on both, the type of the separation system and their own properties.

In case of SDS-PAGE, polyacrylamide gel is used as matrix. This polymerized matrix creates a network of pores through which the molecules travel. The size of pore hence the resistance of the gel can be controlled by adjustment of the concentration of the gel monomer. Other important factors influencing mobility of molecules are the strength of the electric field, the buffer system used, the shape of the molecules, their charge and size.

Proteins are a very diverse group of molecules with different properties and geometries (amino acid content and distribution playing a crucial role). In order to separate the proteins based on their size only, other factors affecting the electrophoretic mobility, such as the higher order structures and surface charges, must be taken out of the equation. Sodium dodecyl sulfate (SDS) is used for that purpose. This anionic detergent, in combination with heat, breaks noncovalent bonds, linearizes protein chains and imparts a uniform mass to charge ratio to the molecules. SDS is an amphipathic, negatively charged molecule that consists of hydrophobic carbon chain and a

hydrophilic sulfate group. The hydrocarbon chains of SDS permeate the interior of proteins and bind to the hydrophobic amino acid residues. Polypeptide chains therefore become covered with negatively charged molecules of the detergent which imparts an even charge and shape to proteins. The negatively charged molecules then travel through the polyacrylamide gel towards the anode and become separated based on their size.
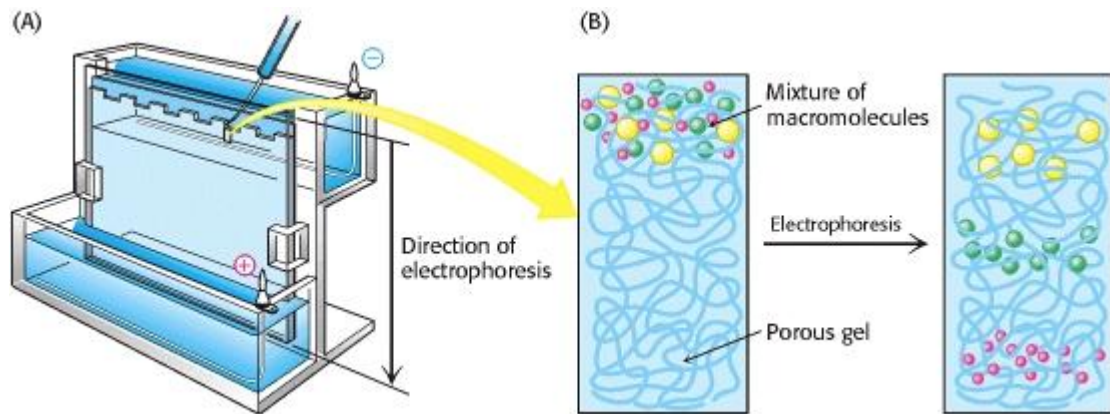
Figure 10: The apparatus used for SDS PAGE (shown on the left). Simplified representation of the mechanism of the protein separation during the electrophoresis (shown on the right) (Berg et al. 2001).

SDS is responsible for breaking the noncovalent bonds. To disrupt covalent bonding (disulfide bridges between cysteins) reducing agents such as 2-mercaptoethanol are used in addition to the detergent. The two 'versions' of SDS PAGE are called non-reducing and reducing SDS PAGE.

Vast majority of proteins are not visible on the gel after the separation. To visualize the position of protein bands, different staining methods are employed (most commonly the Comassie Brilliant Blue stain). This anionic dye binds to proteins in a noncovalent and nonspecific manner. This type of staining is also considered as quantitative procedure since the intensity of the stained band is directly proportional to the amount of protein.

In general the smaller molecules migrate faster through the pores of the gel and therefore travel a greater distance than the bigger ones. To determine the molecular weight of analytes, molecular weight size markers are used. By comparing the migration pathways of molecules with known molecular weights, the mass of the analytes can be estimated (Berg et al. 2001).

3.3 UV-vis measurements

As other spectroscopic methods, UV-vis measurements are based on observing the interaction of molecules with electromagnetic radiation. Measurement of absorption of light at specific wavelengths can be used to acquire important information about samples; for example the concentration of the solute or its conformational transitions. Small amounts of material are required for the analysis and can be easily recovered after the measurements.

The absorbance (A) is related to the intensity of the light absorbed before (I_0) and after (I) passing through the sample solution (eqn.1). The absorbance of the solute is linearly dependent on its concentration according to the Lambert-Beer law (eqn. 2).

$$\text{eqn. 1:} \quad A = - \log_{10}(I/I_0)$$

$$\text{eqn. 2:} \quad A = \varepsilon cl$$

where:

c…………molar concentration [$mol.L^{-1}$]

l…………pathlength [cm]

ε………..molar absorption coefficient e [$L.mol^{-1}.cm^{-1}$]

The concentration of the solute can be simply determined directly from the measured sample absorbance, applying the eqn. 2.

The absorption of wavelength highly depends on the chemical and structural nature of molecules. Macromolecules, such proteins, absorb light in the near ultra violet and visible range of electromagnetic spectrum. Proteins typically show absorption maxima at around 280 nm due to absorption of light by aromatic acid residues. These are namely tyrosine, tryptophan and phenylalanine.

Useful information can be obtained by measuring the absorbance at 'far-UV' range (180-230 nm). Peptide bonds typically absorb light at around 214 nm. The concentration can be determined based on this absorbance value in cases where the

peptide sequence contains little or no aromatic acids. Additionally, the disulfide bonds absorb at 260 nm (Schmid 2005).

3.4 Antibody immobilization

Capturing antibodies on solid matrix is a common procedure used mainly for protein purification and antigen screening purposes. Most commonly, resin coated with protein A or protein G is used as affinity medium to which antibodies bind in a nonspecific manner. The solid support must be chemically and physically inert. Cross-linked agarose based supports are very often used.

Protein A (SPA-staphylococcal protein A) and G (SPG-streptococcal protein G), which are used as affinity reagents for antibody immobilization, originate from bacterial cell walls and have high affinity for the Fc portion of various antibodies. The affinities depend on the subclass, source and type of antibodies.
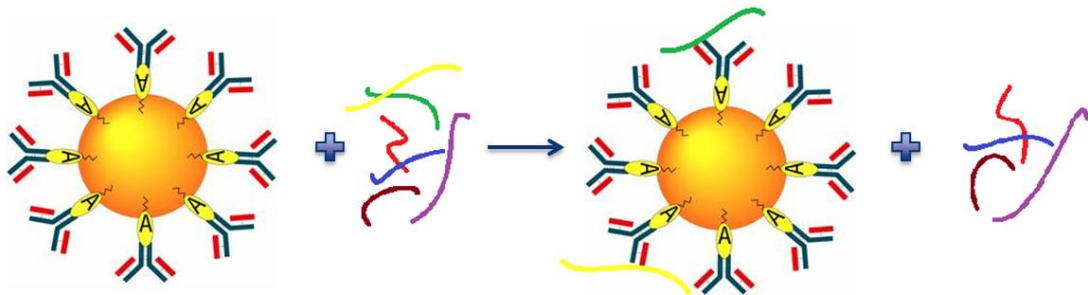
Figure 11: Antibodies immobilized on solid support via an A protein linker can be used for binding specific antigen molecules (http://www.the-scientist.com/?articles.view/articleNo/12801/title/Ultimate-Abs/)

Binding between proteins and antibodies is pH sensitive. Formation of the complex is favored mainly in neutral or slightly basic pH. During the procedure, antibodies are first loaded on the resin at slightly basic environment, followed by various washing steps and binding of antigen of interest. The antibody-antigen complex can be eluted from the resin by addition of acidic buffers (Aybay 2003).

3.5 Reverse-Phase High Performance Liquid Chromatography

Reverse-phase high performance liquid chromatography is a high resolution separation technique widely used for separation of peptides, proteins and other biomolecules. The analyte in a liquid solvent (the mobile phase) is passed through a column containing a solid adsorbent matrix (the stationary phase) under high pressure.

Based on the properties of individual molecules and their interactions with the adsorbent material, the sample mixture can be separated into its components as it flows through the column. Different interactions/degree of interactions of analyte components with the stationary phase represent the basic principles of separation of molecules in HPLC.

In case of the reverse-phase HPLC, the molecules are separated based on their hydrophobicity. The stationary phase is made of non-polar, hydrophobic material (the base material are very often silica particles modified with straight chain alkyl groups such as C8, C4, C18) and the starting mobile phase is aqueous with polar character. In general, the affinity of more hydrophobic molecules to the column is stronger than the ones with less hydrophobic character.

To elute the molecules from the column a gradient is applied. The polarity of the mobile phase is gradually decreased by addition of an organic solvent (e.g. acetonitrile) which in turn reduces the retention of the more hydrophobic species on the column. The polar molecules will therefore elute at the beginning of the gradient and the hydrophobic ones later on along with the increasing percentage of the organic solvent.

Essential parameters influencing the separation of the analyte mixture are the choice of the stationary and mobile phases, the gradient and the temperature. HPLC is very often used in combination with other techniques for direct detection and detailed analysis of separated samples (e.g. MS).

An abbreviation UPLC stands for ultra performance liquid chromatography. It is an advanced version of HPLC instrumentation that operates under much higher pressures which in turn results in more rapid flow rates. UPLC systems improve the analysis with

respect to resolution, sensitivity and shorter time frames (Aguilar 2000; Boysen, Hearn 2001).

3.6   Size Exclusion Chromatography

Size exclusion chromatography is considered as one of the HPLC modes. The separation of molecules takes place within a column through which the dissolved analyte passes under high pressure. The packing material of the column and the principle of the separation of molecules is however different from the 'classical' HPLC where molecules are separated based on their hydrophobic character.

The stationary phase in SEC is an inert porous material through which molecules travel in different speeds depending on their size. Smaller molecules are able to penetrate the porous material to a greater extent and therefore travel slower and elute later. The larger molecules cannot penetrate small pores and therefore will travel through the column within a shorter time frame. The molecules do not bind directly to the stationary phase which means that the buffer does not affect the resolution. This is a significant advantage when it comes to variation of experimental conditions.

In general two types of SEC exist. So called gel filtration chromatography (GFC), which is used in case of measuring the molecular weight distribution of molecules that are soluble in aqueous solvents (e.g. proteins, polysaccharides). The second type is called gel permeation chromatography (GPC). It is used for analysis of molecules which are soluble in organic solvents (e.g. of synthetic polymers).

SEC is often used for fractionation of multiple components of a sample based on their size. It is used for purification, isolation of individual components and for determining the molecular weight distribution within the samples (Price, Nairn 2009).

3.7   Multi-angle Light Scattering (MALS)

Multi-angle light scattering is a technique used for determination of absolute mass and measurement of rms radius of molecules based on their property to scatter light. MALS belongs to very few so called absolute methods in the sense that it does not rely on

relative Mw standards for determination of molecular masses. The masses are directly determined from the angular dependence of scattered light intensity as a function of concentration. Out of all the absolute methods (ultracentrifugation, membrane osmometry and mass spectrometry), MALS is applicable over the broadest range of Mw´s.

This non-destructive and quite fast technique can be use in both, a batch and/or chromatography mode. More commonly used chromatographic mode includes a separation step prior to the LS analysis and allows investigation of individual species within the sample (LS provides weight-averaged molar mass for all molecules present within an analyzed fraction). Size-exclusion chromatography with on-line light-scattering, refractive index and UV absorbance detectors connected in series, is a very useful instrumental setup used for determination of sizes and absolute masses of molecules (Oliva et al. 2004).

3.8   Mass Spectrometry

3.8.1   Basic principles of MS

Mass spectrometry is a powerful analytical technique with wide range of applications. Being able to determine an intrinsic property of the molecule, its mass, with high sensitivity, this method holds an important place among other analytical techniques (Mann et al. 2001).

MS instrument is able to separate and detect gas phase ions based on their mass to charge ratio. Mass spectrometer consists of an ionization source, a mass analyzer and a detector. There are many different types of instrumentation suited for analysis of various samples, they all, however, have the same general purpose.

The ionization source is used to produce gas phase ions from the sample molecules. Ions can be both, negatively or positively charged (most MS experiments are performed on positive ions) (Baldwin et al). Mass analyzer then uses electric and magnetic fields to separate the charged particles based on their mass to charge ratio and to guide them to

the detector, where the incoming ions are detected. The signals are then transferred into a computer and read out.

To introduce the sample into the system, different tools are used. Most often, the MS instrument is connected to LC or GC line, combining a powerful separation techniques with direct analysis of the sample (Finehout, Lee 2004).

3.8.2 MS of biomolecules

MS was for a long time constrained mainly to the analysis of volatile molecules which were easily transferable into the gas phase and ionized by hard ionization techniques such as electron impact. To investigate large polar molecules such proteins in a gas phase has been a great challenge for mass spectrometry (Maleknia, Johnson 2009-).

It wasn´t until the late 1980´s along with the discovery of novel ionization methods, that MS became a powerful and indispensable analytical tool in life sciences. The so called soft ionization methods allow successful ionization and transfer of large polar biomolecules into the gas phase which is an essential part of successful MS analysis. The two mentioned methods are MALDI (matrix-assisted laser desorption/ionization) and ESI (electrospray ionization) (Mann et al. 2001).

In MALDI, the analyte is co-crystallized with matrix (usually small organic molecule) and applied on a metal plate. By irradiating the solid phase sample with short laser pulses, predominantly singly charged gas phase ions are produced. The charging mainly occurs via proton transfers. The results highly depend on the choice of matrix and successful incorporation of the analyte. Since intact proteins are prone to fragmentation this technique is used mainly for analysis of peptides.

In electro spray ionization (ESI), multiply charged ions are produced. Charged droplets are generated under atmospheric pressure by passing a solubilized sample through a narrow needle to which a high voltage is applied. ESI commonly generates a range of charged states via different extent of protonation.

Four types of mass analyzers are commonly combined with ESI and/or MALDI. Quadrupole mass analyzer, Time-of-flight (ToF) analyzer, Quadrupole ion traps and Fourier transform ion cyclotron resonance (FTICR) analyzer. Different mass analyzers are often combined within the same MS instrument (Wysocki et al. 2005).

3.8.3 MS and proteins

Nowadays, MS has a broad range of applications. It is routinely employed in simple identification and quality control of purified proteins, identification of proteins in complex mixtures, determination of post-translational modifications, elucidation of higher order structures and even observation of molecular interactions (HDX-MS). Peptides are generally best analyzed as positive ions. Tandem MS (MS/MS) experiments have become indispensible in the field of protein sciences. Tandem MS has proven to be a powerful tool for peptide sequencing. In this technique the ionized peptide species are further fragmented to produce daughter ions. The charged fragments are subsequently sorted in a mass analyzer and can be then assigned to the parent molecules. Knowledge of the gas phase peptide chemistry and the choice of instrumentation are essential for the protein identification.

Special algorithms are used to identify proteins based on the obtained mass spectral data of peptides. Recorded MS/MS spectra are compared against theoretical spectra of candidate peptides within a protein database. Different methods are used to assign a score and determine the probability of a potential match.

A very popular approach to protein identification is so called bottom-up sequencing. Proteins are proteolytically digested prior to their analysis with MS. The protein identification is based on sequence analysis of the MS/MS spectra of the fragments generated via a digestion (Sobott, Robinson 2004).

4. Materials and Instrumentation

4.1 List of chemicals

Compound	Chemical Formula	Mw [g.mol^{-1}]	Supplier	Purity [%]
Acetic Acid	CH_3COOH	60.05	Merck	100.0
Acetonitrile	CH_3CN	41.05	Sigma-Aldrich	≥ 99.9
Asp-N	-	24 500	Sigma-Aldrich	-
di-Sodium hydrogen phosphate dihydrate	$Na_2HPO_4.2H_2O$	177.99	Merck	99.5
Formic Acid	$HCOOH$	46.03	Sigma-Aldrich	98.0
Gel Filtration Standard (GFS)*	-	670 000, 158 000, 44 000, 17 000, 1 350	Bio-rad	-
Hydrochloric Acid	HCl	36.46	Sigma-Aldrich	≥ 25.0
InstantBlue staining	-	-	Expedeon	-
Lys-C	-	30 000	WAKO	-
MES Running buffer	-	-	Invitrogen	-
NuPAGE LDS sample buffer	-	-	Invitrogen	-
Sodium Azide	N_3Na	65.01	Merck	≥ 99.0
Sodium Chloride	$NaCl$	58.44	Merck	99.5-100.5
Sodium dihydrogen phosphate monohydrate	$NaH_2PO_4.H_2O$	137.99	Merck	99.0-102.0
SeeBlue Plus2 Prestained Standard	-	-	Invitrogen	-

* GFS standard: thyroglobulin, γ-globulin, ovalbumin, myoglobin, and vitamin B12

4.2 List of instruments and materials

Instruments/Materials	Model/Type	Manufacturer
Mass Spectrometer	Synapt G2 Si	Waters
	Xevo G2-S QToF	Waters
HPLC (on line with Xevo MS)	Waters 2695	Waters
HPLC/Analytical Column	218TP_RP-C18	Vydac
UV-Vis spectrophotometer	Shimadzu UV-2450	Shimadzu
	SoloVPE CARY60 UV Vis	CTechnologies
Multi-angle static light scattering detector (MALS)	DAWN 8+	Wyatt
Differential refractive index detector (dRI)	Optilab T-Rex	Wyatt
HPLC (on-line with SEC-MALS)	Agilent Series 1200/Agilent Series 1260	Agilent
SEC/Analytical Column	TSKgel G3000SWxl	Tosoh
UPLC (on-line with Synapt MS)	Aquity UPLC	Waters
UPLC/Analytical Column	Aquity UPLC - BEH300/RP-C4	Waters
Centrifuge	5415D	Eppendorf
Power Supply	Power pack HC	Bio-rad
pH meter	Consort C3010	Consort
Gel Imager	Gel Doc Imager	Bio-rad
Heating Block	VWR - digital heat block	VWR
Resin	MabSelect SuRe Resin	GE lifesciences
Filters	Illustra MicroSpin Columns	GE lifesciences
	Vacuum Filters 0.22 um	Corning
	10k Amicon filters	Millipore
	30k Amicon filters	Millipore
SDS-PAGE Precasted Gels	NuPage 4-12 % BIS-TRIS	Invitrogen

4.3 List of solutions

4.3.1 In-solution Enzymatic Digestions

CH$_3$COOH stock solution; 20 mM:

3.4 uL of concentrated stock solution of CH$_3$COOH in 3 ml of MQ

Lys-C stock solution A; 0.70 mg/ml:

20 ug of the Lys-C enzyme in 30 uL of 20 mM of CH$_3$COOH

Lys-C stock solution B; 0.25 mg/ml:

20 ug of the Lys-C enzyme in 80 uL of 20 mM of CH$_3$COOH

Asp-N stock solution B; 0.20 mg/ml:

2 ug of the Asp-N enzyme in 10 uL of 20 mM of CH$_3$COOH

4.3.2 SDS PAGE

Running buffer for SDS PAGE; 20 x:

50 mL of MES running buffer solution in 950 mL of MQ

4.3.3 SEC-MALS

Mobile phase; 150 mM NaPi, 50 mM NaCl, pH 7.0

14.6 grams of sodium dihydrogen phosphate monohydrate, 34.6 grams of di-Sodium hydrogen phosphate dihydrate and 5.8 grams of sodium chloride filled up to 2000 mL with MQ. pH adjusted with concentrated HCl/NaOH. Solution filtered over a 0.22 um filter.

Sodium azide stock solution; 2.5%

5 g of sodium azide were dissolved in 200 ml MQ

Column storage solution; 100mM NaPi, 0.05 % NaN$_3$, pH 7.0

2.2 grams of sodium dihydrogen phosphate monohydrate, 6.1 grams of di-sodium hydrogen phosphate dihydrate and 10 mL of 2.5% sodium azide stock solution filled up to 500 mL with MQ. pH adjusted with concentrated HCl/NaOH. Solution filtered over a 0.22 um filter.

4.3.4 HPLC/UPLC

Mobile phase A; 0.1% Formic acid in H$_2$O

1 mL of concentrated stock solution of formic acid in 999 mL of ultrapure H$_2$O

Mobile phase B; 0.1% Formic acid in ACN

1 mL of concentrated stock solution of formic acid in 999 mL of acetonitrile

Mobile phase C; 70% ACN in H$_2$O

700 mL of acetonitrile and 300 mL of ultrapure H$_2$O

Mobile phase D; 10% MeOH in H$_2$O

100 mL of methanol in 900 mL ultrapure H$_2$O

4.4 List of Samples

4.4.1 Approach A

Table 1: : Overview of the samples used in epitope mapping experiment A

#	Sample	Concentration [mg/mL]	Mw$_{theor.}$ [kDa]	Buffer
1	**Histag-rTau441-ctag F8W (transformation, E.coli)**	0.9	47.46	20 mM NaPi/150 mM NaCl (pH=6)
2	**HumanIgG1 CB tau 28.1 (Expi293F transient)**	0.5	~ 152	PBS (pH=7.4)
3	**IgG CR8020/SEC-MALS control**	2.0	~ 150	20mM Acetate buffer with 75mM NaCl and 5% Sucrose, pH 5.5

4.4.2 Approach B

Table 2: Overview of the samples used in epitope mapping experiment B

#	Sample	Concentration [mg/mL]	Mw$_{theor.}$ [kDa]	Buffer
1	**Mouse CB tau 28.1**	8.2	~ 150	PBS (pH=7.4)
2	**Mouse CB tau 27.1**	8.3	~ 150	PBS (pH=7.4)
3	**Mouse CB tau 24.1**	9.4	~ 150	PBS (pH=7.4)
4	**CR8043**	25.1	~ 150	20mM Acetate buffer with 75mM NaCl and 5% Sucrose, pH 5.5
5	**Histag-rTau441 (transformation, E.coli)**	5.8	47.46	PBS (pH=7.4)

Table 3: Overview of the synthetic peptide samples used in the 'proof of concept' experiment B

#	Peptide	Concentration [mg/mL]	Antibody Epitope	Mw$_{theor.}$ [Da]	AA Region	Buffer
1	A6984	1.0	None – NC	6553	003-054	H$_2$O
2	A6907	1.0	CB tau 24.1	7341	186-253	H$_2$O
3	V1089-23	0.5	CB tau 24.1	2355	228-245	H$_2$O
4	B1002	1.0	CB tau 27.1	3380	299-328	H$_2$O
5	A8119	1.0	CB tau 27.1	2419	299-318	H$_2$O
6	B1014	1.0	CB tau 28.1	3391	048-077	H$_2$O
7	A7731	1.0	CB tau 28.1	2364	052-071	H$_2$O

Synthetic peptides were supplied by Janssen La Jolla, CA and Pepscan.

Peptide Sequences:

1) A6984: GSGMAEPRQEFEVMEDHAGTYGLGDRKDQGGYTMHQDQEGDT

   DAGLKESPLQTPTED (GSG - not a part of tau sequence)

2) A6907: GEPPKSGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKKVAVVRTP

   PKSPSSAKSRLQTAPVPMPDL

3) V1089-023: VVRTPPKSPSSAKSRLQT (phosphoserine)

4) B1002: HVPGGGSVQIVYKPVDLSKVTSKCGSLGNI

5) A8119: HVPGGGSVQIVYKPVDLSKV

6) B1014: LQTPTEDGSEEPGSETSDAKSTPTAEDVTA

7) A7731: TEDGSEEPGSETSDAKSTPT

5. Experimental setup

5.1 Approach A

5.1.1 Buffer exchange/Sample concentration:

hCB tau 28.1 and rTau samples were concentrated to 5.2 mg/mL and 2.2 mg/mL respectively, using the amicon ultra centrifugal filters (30K and 10K respectively). Additionally, rTau sample was buffer exchanged from the 20 mM NaPi/150 mM NaCl (pH = 6) into PBS (pH = 7.4) to achieve optimal pH for Lys-C enzymatic activity (pH = 7-9) and therefore to ensure a complete digestion.

To obtain enough material for a control sample of hCB tau 28.1 for SEC-MALS run, another aliquot of the antibody was concentrated resulting in CB Tau 28.1 solution with c = 3.3 mg/mL.

Slope spectroscopy (SoloVPE instrument) was used to determine the final concentrations of all the solutions after the filtration/concentration procedure. Extinction coefficients used for the calculation of the concentrations were 0.305 mL/(mg·cm) in case of the recombinant tau and 1.634 mL/(mg·cm) for the hCB tau 28.1 antibody.

5.1.2 In-solution Enzymatic Digestions

The digestion reaction was carried out at the temperature of 37°C on a heating block.

Reaction mixture of molar ratio 1Lys-C:50rTau was prepared by addition of 4 uL of Lys-C solution A (c = 0.7 mg/mL) to 95 uL of rTau (c = 2.2 mg/mL).*

The reaction proceeded overnight (~15 hrs). To inhibit the enzyme and terminate the digestion, the sample mixture was incubated at 60°C for 30 minutes which should lead to denaturation and deactivation of Lys-C.

*In case of all the digestions of rTau, no denaturation step is required. Tau is and IDP. The structure of the   free protein is opened and therefore all the cleavage sites are available for digestion.

5.1.3   SDS PAGE

Precasted gels NuPage 4-12% BIS-TRIS and MES running buffer were used for the separation of fragments generated via the digestion of rTau. Gel was run under non-reducing conditions.

Sample solutions and controls were first mixed with appropriate amount of LDS Sample buffer and incubated at 98˚C for 10 min. Both non-incubated and rTau incubated at 37 ˚C overnight were used as controls (for detailed information about exact amounts, see the Table 4 below).

20 uL of each sample and 10 uL of the SeeBlue Plus2 Prestained Standard (See Blue Plus2 Prestained Standard ladder is shown in Appendix, chapter 9.1) were loaded on the 4-12 % BIS-TRIS gel. The gel was ran at 200 V for 40 minutes.

To visualize the bands, gel was stained with Comassie based staining solution, the InstantBlue, for 1.5 hours. Gel was destained in MQ for 4 hours to achieve optimal contrast of the background and sample bands.

Table 4: Overview of the sample preparation for SDS PAGE

#	Sample name	[Sample] (ug/uL)	Load (ug/lane)	Incubation (10 min)	Sample load (uL)	H2O (uL)	LDS buffer 4x (uL)
1	Sample Buffer	NA	NA	NA	0	0	10
2	Sample Buffer	NA	NA	NA	0	0	10
3	Sample Buffer	NA	NA	NA	0	0	10
4	Marker Standard	NA	NA	NA	10	0	0
5	Tau fresh - Control	0.50	5	98 °C	10.0	5.0	5
6	Lys-C Digest_inc.	2.00	10	98 °C	5.0	10.0	5
7	Tau Inc. at 37 °C	0.50	5	98 °C	10.0	5.0	5
8	Marker Standard	NA	NA	NA	10	0	0
9	Sample Buffer	NA	NA	NA	0	0	10
10	Sample Buffer	NA	NA	NA	0	0	10

5.1.4 SEC-MALS

The sample solution (sample A) was prepared by mixing 36.3 uL of rTau/Lys-C Digest with 53.7 uL of hCB tau 28.1(c = 5.2 mg/ml). Bearing in mind that antibody must be in excess in order to bind all the peptides, the amounts of the samples used for binding experiment were calculated according to molar ratio 1 rTau:1.2 hCB tau 28.1. The mixture was incubated at room temperature for 30 minutes prior to the SEC-MALS analysis.

Control sample of the rTau/Lys-C Digest (sample B) was prepared by mixing 36.3 uL of rTau/Lys-C Digest with 53.7 uL of PBS buffer. Control sample of the hCB tau 28.1 (sample C) was prepared by mixing 45 uL of CB 28.1 (c = 3.3 mg/ml), 25 uL of CB 28.1 (c = 5.2 mg/ml) and 20 uL of PBS. 60 uL of each of the samples A, B and C were injected on SEC-MALS. Additionally, GFS, BSA and IgG CR8020 were analyzed for purposes of the system and column quality control.

Table 5: Overview of the samples injected on SEC-MALS

#	Sample	Conc.$_{Initial}$ [g/L]	Required Amount [ug]	Extinction coefficient [mL /(mg.cm)]	Volume$_{total}$ [uL]	Volume$_{injection}$ [uL]
1	MQ1	NA	NA	NA	500	20
2	GFS1	NA	NA	NA	250	20
3	CR8020	2.0	40	1.44	250	20
4	rTau/Lys-C Digest + hCB tau 28.1	NA	48.4 + 186	~1.634	130	60
5	rTau/Lys-C Digest	2.0	48.4	0.137	130	60
6	hCB tau 28.1	3.1	186	1.634	90	60
7	MQ2	NA	NA	NA	500	20
9	BSA	2.0	40	0.66	200	20
10	GFS2	NA	NA	NA	250	20
11	MQ	NA	NA	NA	500	20

G3000 SEC column was used for the separation. Time of each run was 20 minutes with detector flow of 1 ml/min. UV 280 nm, MALS and dRI detectors were used.

ASTRA software was used for the analysis and processing of the results

5.1.5 Fraction collection/Concentration measurements

Samples A and B were manually collected as eluting from the SEC-MALS. 1 ml of the mobile phase was collected each minute starting at time 0 min and ending at time 17 minutes. Fractions were collected as 1A-17A and 1B-17B for samples A and B respectively.

To identify fractions containing the absorbing species, UV-vis instrument (Shimadzu) was used (quartz cuvettes with pathlength of 10 mm). UV absorbance between 190 nm and 350 nm was measured. Absorbance at 280 nm and 214 nm were the values relevant for the fraction identification. Absorbance at 350 nm was subtracted from the measured values and used to correct for the background noise.

5.1.6 LC-MS

Control samples of rTau digested with Lys-C and the fractions which showed UV absorbance were to be analyzed. All the samples were stored at -20 ˚C for two days prior to the MS measurements.

Xevo G2-S QToF MS instrument connected to Waters 2695 HPLC was used for the analysis. Reverse phase C18 analytical column was used for the separation of peptides.

900 uL ml of the fractions 9A, 10A, 11A, 13A, 14A, 15 A and 800 uL of 12B, 13B, 14B, 15B were then injected on HPLC column and analyzed with ESI MS. Lys-C/rTau digests, both the non-incubated and the sample incubated at 60 ˚C prior to the binding experiment, were also analyzed with ESI-MS for control purposes. 200 uL of each of the control 0samples (c = 0.05 mg/mL) corresponding to ~ 10 ug of the material were

injected. In between each of the sample run a blank injection was performed. 200 uL of the 0.1 % FA in H_2O (corresponding to mobile phase A) were used for that purpose.

Flow was set to 0.2 ml/min. Run time for the sample injections was 100 minutes and for the blank injections 20 minutes. Column temperature was set to 40 °C.

5.1.6.1 Technical parameters:

Table 6: HPLC Separation/Gradient information

Time [min]	Flow [ml/min]	% A	% B	Curve
Sample Injections				
initial	0.2	99.0	1.0	Initial
5.0	0.2	99.0	1.0	/ 6
60.0	0.2	65.0	35.0	/ 6
70.0	0.2	50.0	50.0	/ 6
80.0	0.2	1.0	99.0	/ 6
82.0	0.2	1.0	99.0	/ 6
83.0	0.2	99.0	1.0	/ 6
84.0	0.2	99.0	1.0	/ 6
85.0	0.2	1.0	99.0	/ 6
86.0	0.2	1.0	99.0	/ 6
87.0	0.2	99.0	1.0	/ 6
100.0	0.2	99.0	1.0	/ 6
Blank Injections				
initial	0.2	99.0	1.0	Initial
5.0	0.2	99.0	1.0	/ 6
6.0	0.2	1.0	99.0	/ 6
11.0	0.2	1.0	99.0	/ 6
12.0	0.2	99.0	1.0	/ 6

13.0	0.2	99.0	1.0	/ 6
14.0	0.2	1.0	99.0	/ 6
15.0	0.2	1.0	99.0	/ 6
16.0	0.2	99.0	1.0	/ 6
20.0	0.2	99.0	1.0	/ 6

Table 7: MS instrument/Operation mode information

MS Analysis – Basic Operation Parameters	
ESI mode	Positive
Scan range	50 - 2000 m/z
Capillary Voltage	1.5 kV
Sampling Cone Voltage	30.0 V
Collision Energy (MS/MS):	6.0 V
Desolvation temperature	250 °C
Source temperature	100 °C
Desolvation gas flow	1000 L/hr

After the measurement, PLGS software was used to identify the peptides. Processing parameters for the experiment are shown in the Table 8 below.

Table 8: PLGS data processing parameters

Data Preparation	
Lock Mass	556.2771 Da/e
Lock Mass Window	0.25 Da
Low Energy Threshold	250 counts
Elevated Energy Threshold	100 counts
Intensity Threshold	750 counts

5.2 Approach B/Proof of concept experiment

To proof the reliability of the epitope mapping experiment B, the peptide pool created via the digestion of rTau was substituted by synthetic peptides which were known to bind to the anti-tau antibodies. By comparing the experimental results with theoretical hypothesis, the approach can be validated.

5.2.1 Synthetic peptide pool preparation

Seven different synthetic peptides of known sequence were selected to be bound with anti-tau antibodies. Based on the knowledge of the tau eptiopes, following peptides were chosen: A6907 and V1089-023 which both theoretically bind to mCB tau 24.1, B1002 and A8119 both theoretically binding to mCB tau 27.1, B1014 and A7731 both theoretically binding to mCB tau 28.1 and A6984 which should not bind to any of the antibodies and was selected as a negative control.

Each peptide was to be present in the amount of 120 pmoles in the final MS injection (injection volume = 10 uL). The binding ratio of the antibody:peptide pool was to be 0.8mAb:1Peptide pool (calculated based on average molecular mass of the peptides), to make sure that the antibody is in excess and that the potential competition among peptide species wouldn´t be an influencing factor.

Five separate peptide pools were required for the experiment (4 x binding with the immobilized antibody, 1 x MS control peptide pool). The peptide pool was prepared as one solution. After taking all of the mentioned factors into consideration, following volumes of peptides were added to 478.5 uL of 1PBS:1MQ buffer:

Table 9: Overview of volumes of individual synthetic peptides used for preparation of the peptide pool

#	Sample	Volume [uL]
1	A6984	59.0
2	A6907	66.0
3	V1089-23	42.5
4	B1002	30.5
5	A8119	22.0
6	B1014	30.5
7	A7731	21.5

5.2.2 Immobilization of antibodies

Four different antibodies were to be immobilized on the resin; CB tau 28.1 (Sample 1), CB tau 27.1 (Sample 2), CB tau 24.1 (Sample 3) and CR8043 (Sample 4).

Antibody CR8043 is a flu antibody which theoretically shouldn´t bind to the tau protein, hence to any of the synthetic peptides. CR8043 was used as another negative control in the experiment along with the peptide A6984.

100 uL of the MabSelect SuRe resin was to be used for the antibody immobilization. The amount was calculated based on the resin capacity (20 mg/mL), the percentage of the resin in the slurry (78%) and the amount of the antibody to be immobilized (~ 1512 ug).

Immobilization of antibodies was performed in three basic steps:

Washing the resin:

The PBS buffer used during the antibody immobilization was 1x diluted with H_2O to achieve lower ionic strength (prepared by mixing 10 mL of 1PBS and 10 mL of MQ).

Resin slurry was calculated to be ~ 78% (in 20% EtOH). 100 uL of the resin were pipetted into 1.5 mL Eppendorf test tube, 3x as much of 1PBS:1MQ was added and the mixture was mixed and consequently centrifuged for 5 minutes at 1500 g. The supernatant was carefully discarded and the resin was washed twice more using the same procedure. Finally, twice as much volume of 1PBS:1MQ (in respect to the resin slurry = 200 uL) was added to the resin.

Four test tubes each with 100 uL of resin were prepared in order to perform four separate binding experiments

Note: In all the experiments using the antibody immobilization method, 1PBS:1MQ buffer and low incubation temperatures were used. Previous experiments showed favorable binding of CB tau 28.1 in lower ionic strength and temperature.

Antibody immobilization:

184.4 uL of CB tau 28.1, 182.2 uL of CB tau 27.1, 160.9 uL of CB tau 24.1 and 60.3 uL of CR8043 - each corresponding to ~ 1512 ug of the material - were added to the individual test tubes containing washed resin. The solutions were incubated overnight at 4 ˚C, constantly shaken on a roller shaker.

Removal of unbound material:

After the incubation, the mixtures were centrifuged at 1500 g for 5 minutes, the supernatant was removed and the resin in each of the test tubes was resuspended with 500 uL of 1PBS:1MQ (5 x the resin volume). This procedure was repeated once more. In the final step, as much of supernatant as possible was removed, yielding the washed resin with immobilized antibody.

5.2.3   Binding of antibodies with peptides

To each individual test tube containing the antibodies immobilized on resin, 150 uL of the synthetic peptide pool of was added. The solutions were incubated for 4 hrs, constantly shaken at 4 ˚C.

5.2.4   Fraction collection

Illustra microspin columns were used for the separation purposes. Prior to the filtration, the filters were twice washed/equilibrated with 400uL of 1PBS:1MQ.

Various fractions were collected at different stages of the experiment:

Fractions A - collecting the supernatant:

After the incubation, the mixtures were centrifuged for 5 minutes at 1500 g and the supernatants were collected as fractions 1A, 2A, 3A and 4A. Fractions 1A-4A were then filtrated on the illustra microspin columns prior to the MS experiments to separate the liquid and the resin which potentially remained in the solution. Fractions A should contain unbound peptide species.

Fractions B and C - washing the resin:

To wash the resin, 400 uL of 1PBS:1H2O (4 x the resin volume) were added to each test tube, gently mixed and centrifuged at 1500 g for 5 minutes. The supernatants were collected (1B, 2B, 3B, 4B). The resin was washed once more using the same procedure. Fractions were collected (1C, 2C, 3C, 4C). Fractions B and C should contain low amounts of unbound or loosely bound peptides.

(the final concentrations of peptides in fractions B and C are up to three times lower than in the other fractions due to the higher volume of buffer used during washing)

Fractions D and E - Eluting the antibody/peptide complex from resin:

To elute the antibody/peptide complex from the resin beads, 0.1% formic acid in H_2O was used (pH = 2.6). To each of the microtubes, 150 uL of 0.1% FA were added and the

mixtures were incubated for 30 minutes at room temperature. After the incubation, the solutions were centrifuged at 1500 g for 5 minutes and the supernatants were collected as 1D, 2D, 3D, 4D. The last step was repeated once more with shorter incubation time (15 minutes) and the supernatants were collected as 1E, 2E, 3E, 4E.

5.2.5 LC-MS

Synapt G2 Si MS instrument connected to Aquity UPLC was used for the analysis. Reverse phase C4 analytical column was used for the separation of peptides.

All the collected fractions were injected and analyzed with ESI-MS. All together 18 samples were to be analyzed each with total volume of 140 uL. 10 uL of each sample were to be injected. As a control, the synthetic peptide pool was used. 10 uL of the control sample corresponding to ~ 120 pmoles of each peptide in the mixture was injected.

In between each of the sample run a blank injection was performed. 10 uL of the 0.1 % FA in H_2O (mobile phase A) was used for that purpose.

Flow was set to 0.3 ml/min. Run time for the sample injections was 30 minutes and for the blank injections 18 minutes. Column temperature was set to 40 °C.

5.2.5.1 Technical parameters

Table 10: UPLC Separation/Gradient information

Time [min]	Flow [ml/min]	% A	% B	Curve
Sample Injections				
initial	0.3	99.0	1.0	Initial
5.0	0.3	99.0	1.0	/ 6
15.0	0.3	65.0	35.0	/ 6
18.0	0.3	45.0	55.0	/ 6
19.0	0.3	1.0	99.0	/ 6

21.0	0.3	1.0	99.0	/ 6
22.0	0.3	99.0	1.0	/ 6
23.0	0.3	99.0	1.0	/ 6
24.0	0.3	1.0	99.0	/ 6
25.0	0.3	1.0	99.0	/ 6
26.0	0.3	99.0	1.0	/ 6
30.0	0.3	99.0	1.0	/ 6
Blank Injections				
initial	0.3	99.0	1.0	Initial
3.0	0.3	99.0	1.0	/ 6
4.0	0.3	1.0	99.0	/ 6
5.0	0.3	1.0	99.0	/ 6
6.0	0.3	99.0	1.0	/ 6
7.0	0.3	99.0	1.0	/ 6
8.0	0.3	1.0	99.0	/ 6
9.0	0.3	1.0	99.0	/ 6
10.0	0.3	99.0	1.0	/ 6
11.0	0.3	99.0	1.0	/ 6
12.0	0.3	1.0	99.0	/ 6
13.0	0.3	1.0	99.0	/ 6
14.0	0.3	99.0	1.0	/ 6
18.0	0.3	99.0	1.0	/ 6

Table 11: MS instrument/Operation Mode

MS Analysis – Basic Operation Parameters	
ESI mode	Positive
Scan range	50 - 2000 m/z
Capillary Voltage	1.5 kV
Sampling Cone Voltage	30.0 V
Extraction Cone Voltage	3.0 V
Collision Energy (MS/MS):	4.0 V
Desolvation temperature	400°C
Source temperature	120 °C
Desolvation gas flow	1000 L/hr

Each of the spectra was analyzed using MassLynx software to check on the presence of peptides in individual fractions.

5.3 Approach B/Epitope mapping experiment

Peptide pools created via enzymatic digestions of recombinant tau protein were to be bound with several anti-tau antibodies which were immobilized on resin beads prior to the binding.

5.3.1 In-solution enzymatic digestions

First of all the concentrated rTau solution was diluted from 5.8 mg/mL to 0.6 mg/mL by mixing 149 uL of rTau (c = 5.8 ug/uL) with 1251 uL of PBS buffer.

Reaction mixture of molar ratio 1Lys-C:80rTau was prepared by addition of 11.7 uL of Lys C solution B (c = 0.25 ug/uL) to 600 uL rTau (c = 0.6 ug/uL).

Reaction mixture of molar ratio 1Asp-N:95rTau was prepared by addition of 10 uL of Asp-N solution B (c = 0.2 ug/uL) to 600 uL rTau (c = 0.6 ug/uL).

The digestion reaction was carried out at the temperature of 37˚C on a heating block and proceeded overnight (~15 hrs). To inhibit the enzyme and terminate the digestion, the sample mixture was incubated at 60˚C for 30 minutes which should lead to denaturation and deactivation of Lys-C.

5.3.2 SDS PAGE

The same procedure as in experimental approach A was used (see the chapter 5.1.3). Detailed sample preparation is shown in the Table 12 below.

Table 12: Summary of the sample preparation for SDS PAGE

#	Sample name	[Sample] (ug/uL)	Load (ug/lane)	Incubation (10 min)	Sample load (uL)	H2O (uL)	LDS buffer 4x (uL)
1	Sample Buffer	NA	NA	NA	0	0	10
2	Sample Buffer	NA	NA	NA	0	0	10
3	Marker Standard	NA	NA	NA	10	0	0
4	Tau fresh - Control	0.6	6	98 °C	10.0	5.0	5
5	Lys-C Digest	0.6	6	98 °C	10.0	5.0	5
6	Tau inc. at 37 °C - Control	0.6	6	98 °C	10.0	5.0	5
7	Asp-N Digest	0.6	6	98 °C	10.0	5.0	5
8	Marker Standard	NA	NA	NA	10	0	0
9	Sample Buffer	NA	NA	NA	0	0	10
10	Sample Buffer	NA	NA	NA	0	0	10

5.3.3 Immobilization of antibodies

50 uL of the MabSelect SuRe resin was to be used for the antibody immobilization. The amount of the resin calculated based on the resin capacity (20 mg/mL), the percentage of the resin in the slurry (78%) and the amount of the antibody to be immobilized (~ 273 ug), was 20 uL. For practical reasons, mainly to avoid errors due to low volume handling, higher amount of resin was used.

The immobilization of antibodies was performed in three basic steps:

Washing the resin:

Resin slurry was calculated to be ~ 78% (in 20% EtOH). 100 uL of the resin were pipetted into 1.5 mL Eppendorf test tube, 3 x as much of 1PBS:1MQ was added and the mixture was mixed and consequently centrifuged for 5 minutes at 1500 g. The supernatant was carefully discarded and the resin was washed twice more using the same procedure. Finally, four times the volume of 1PBS:1MQ (in respect to the resin slurry = 200 uL) was added to the resin.

Six test tubes each with 50 uL of resin were prepared in order to perform six separate binding experiments

Antibody immobilization:

33.3 uL of CB tau 28.1 (Sample 1), 32.9 uL of CB tau 27.1 (Sample 2) and 29.0 uL of CB tau 24.1 (Sample 3) - each corresponding to ~273 ug of the material - were added to the individual test tubes containing the washed resin. For each of the antibody samples, two separate test tubes were prepared. All the solutions were incubated overnight at 4 °C, constantly shaken on a roller shaker.

Removal of unbound material:

After the incubation, the mixtures were centrifuged at 1500 g for 5 minutes, the supernatant was removed and the resin in each of the test tubes was resuspended with 250 uL of 1PBS:1MQ (5 x the resin volume). This procedure was repeated once more. In the final step, as much of supernatant as possible was removed, yielding the washed resin with immobilized antibody.

5.3.4 Binding of antibodies with peptides

The amounts of individual binding partners were calculated based on the molar ratio 1rTau:1mAb. To each of the test tubes containing individual antibodies immobilized on resin, 140 uL of the digested rTau solution were added, yielding the following samples:

Table 13: Approach B - Overview of samples

Antibody	#	rTau Digest
mCB tau 28.1	1A	Lys-C
mCB tau 28.1	1B	Asp-N
mCB tau 27.1	2A	Lys-C
mCB tau 27.1	2B	Asp-N
mCB tau 24.1	3A	Lys-C
mCB tau 24.1	3B	Asp-N

The solutions were incubated for 4 hrs constantly shaken at 4 ˚C.

5.3.5 Fraction collection

For the separation purposes illustra microspin columns were used. Prior to the filtration, the filters were twice washed/equilibrated with 1PBS:1MQ (400 uL).

Various fractions were collected at different stages of the experiment:

Fractions i - collecting the supernatant:

After the incubation, the mixtures were centrifuged for 5 minutes at 1500 g and the supernatants were collected as fractions 1Ai, 2Ai, 3Ai and 1Bi, 2Bi, 3Bi. All the fractions were then filtrated on the illustra microspin columns prior to the MS experiments to separate the liquid and the resin which potentially remained in the solution. Fractions i should contain unbound peptide species.

Fractions ii and iii - washing the resin:

To wash the resin, 400 uL of 1PBS:1H_2O (4 x the resin volume) were added to each test tube, gently mixed and centrifuged at 1500 g for 5 minutes. The supernatants were collected (1Aii, 2Aii, 3Aii and 1Bii, 2Bii, 3Bii). The resin was washed once more using

the same procedure. Fractions were collected (1Aiii, 2Aiii, 3Aiii and 1Biii, 2Biii, 3Biii). Fractions ii and iii should contain low amounts of unbound or loosely bound peptides.

The fractions ii and iii contain very low amount of peptides. First of all they both are the 'wash' fractions (only containing the residual species) and second of all higher volumes of buffer were used during this part of the procedure.

<u>Fractions iv and v - Eluting the antibody/peptide complex from resin:</u>

To elute the antibody/peptide complex from the resin beads, 0.1% formic acid in H_2O was used (pH ~ 3.0). To each of the microtubes, 140 uL of 0.1% FA were added and the mixtures were incubated for 30 minutes at room temperature. After the incubation, the solutions were centrifuged at 1500 g for 5 minutes and the supernatants were collected as 1Aiv, 2Aiv, 3Aiv and 1Biv, 2Biv, 3Biv. The last step was repeated once more with shorter incubation time (15 minutes) and the supernatants were collected as 1Av, 2Av, 3Av and 1Bv, 2Bv, 3Bv.

5.3.6 LC-MS

Synapt G2 Si MS instrument connected to Aquity UPLC was used for the analysis. Reverse phase C4 analytical column was used for the separation of peptides.

All the collected fractions were injected and analyzed with ESI-MS. All together 30 samples were to be analyzed each with total volume of 130 uL. 10 uL of each sample were to be injected. As controls, the digested rTau samples were injected. 10 uL of each sample corresponding to ~ 130 pmoles of material in each digest (1 x Lys-C/rTau, 1 x Asp-N/rTau) were injected. In between each of the sample run a blank injection was performed. 10 uL of the 0.1 % FA in H_2O (mobile phase A) was used for that purpose.

Flow was set to 0.3 ml/min. Run time for the sample injections was 30 minutes and for the blank injections 18 minutes. Column temperature was set to 40 °C.

Gradient used in UPLC Separation and the operation parameters of the MS instrument are identical to the ones in the previous 'proof of concept' experiment (see the chapter 5.2.5).

Each of the spectra was analyzed using PLGS software to check on the presence of peptides in individual fractions. The data preparation parameters for the identification of the peptides are identical to the once used in Approach A (see the chapter 5.1.6., Table 8) Individual ion peaks for relevant peptides were found using the MassLynx software to confirm the presence of the peptides.

6. Results and Discussion

6.1 Approach A

6.1.1 In-solution enzymatic digestion/SDS PAGE

SDS PAGE of the enzymatically cleaved recombinant tau was performed to check on the overall efficiency of the digestion.

The separation range of the gel only allows resolution of protein fragments of sizes between ~ 3 kDa to ~ 190 kDa. In theory, most of the peptides created via the in-solution digestion of tau with Lys-C are smaller than 3 kDa which means that the resulting bands do not provide information about the digest products themselves (for information about theoretical fragmentation see Appendix, chapter 9.3). We can, however, assume whether the digestion was successful or not based on the presence of higher molecular weight fragments (all fragments < 6 kDa).

Table 14: Overview of the SDS PAGE samples loaded in individual lanes

1Lys-C:50rTau	
#	Sample
1	Marker Standard
2	rTau 'Fresh' – control
3	rTau/Lys-C Digest
4	rTau 'Incubated' – control
5	Marker Standard
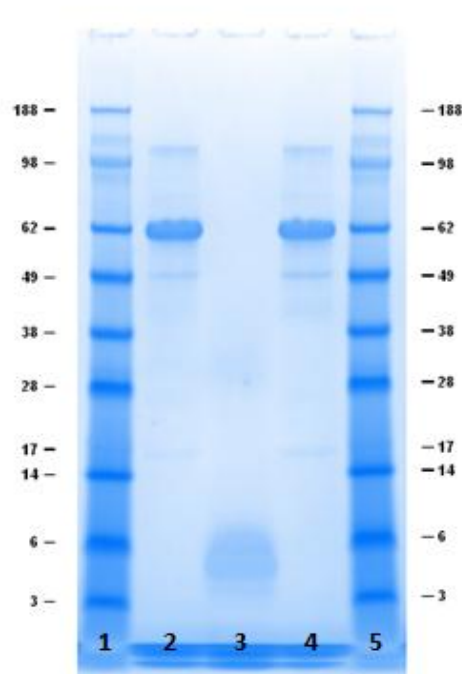
Figure 12: SDS PAGE gel of Lys-C/rTau fragments separated on 4-12% BIS-TRIS gel; MES running buffer; NR conditions.

Intact recombinant tau appears as a band at around 60 kDa as can be seen in lanes 2 and 4 which contain the control samples. The overnight incubation of tau seems to have no effect on the protein. The digestion of rTau can be considered as effective. Comparing the relative intensities of the bands in line 3, we can conclude that most of the digestion products are smaller than 6 kDa as expected in case of complete cleavage of tau protein. Hardly visible trace at around 30 kDa can be neglected. (the Mw standard ladder can is shown in Appendix, chapter 9.1).

6.1.2 SEC-MALS

SEC-MALS analysis is performed in order to obtain fractions containing different peptide species. In case that a peptide interacts with the antibody hCB tau 28.1, its elution time significantly shifts to the left in comparison to the same species in the control sample (sample B, peptide pool created via the Lys-C/rTau digestion). SEC-MALS also serves as a quality check for both binding partners and provides the first glance at the results of the binding experiment.

UV trace at 280 nm for the samples A (blue trace), B (red trace) and C (green trace) along with the molecular masses of the analyzed samples is shown in the Figure 13. The summary of the SEC-MALS results can be seen in the Table 15. The G3000 SEC column is designed to effectively separate polypeptide chains in the mass range between 10 kDa and 500 kDa. Since the digest products do not fall within this range, the majority of the peptides elute in the end of gradient at around 12 minutes, together with other lower molecular species (e.g. buffer salts). We are, however, mainly interested in possible shifts in the elution profile of the peak representing the antibody in complex with peptides (sample A in blue). In case some of the peptides bind to the hCB tau 28.1, the main peak representing the complex of the antibody with peptide should be slightly shifted to the left in comparison to the control sample, hCB tau 28.1 by itself (Sample C in green). The shift to the shorter retention time would not, however, be very significant. The mass of the binding peptide is very low in comparison to the antibody molecule and therefore the molecular weight difference between the antibody in complex and the antibody molecule alone is quite small. Additionally, it´s quite probable that only a fraction of the mAb´s forms a complex with the peptide, leaving the rest of the antibody molecules unbound. The increase in the mass is even less significant because the measured value represents the average of masses of all molecules present in the sample (mAb-peptide complex, mAb).
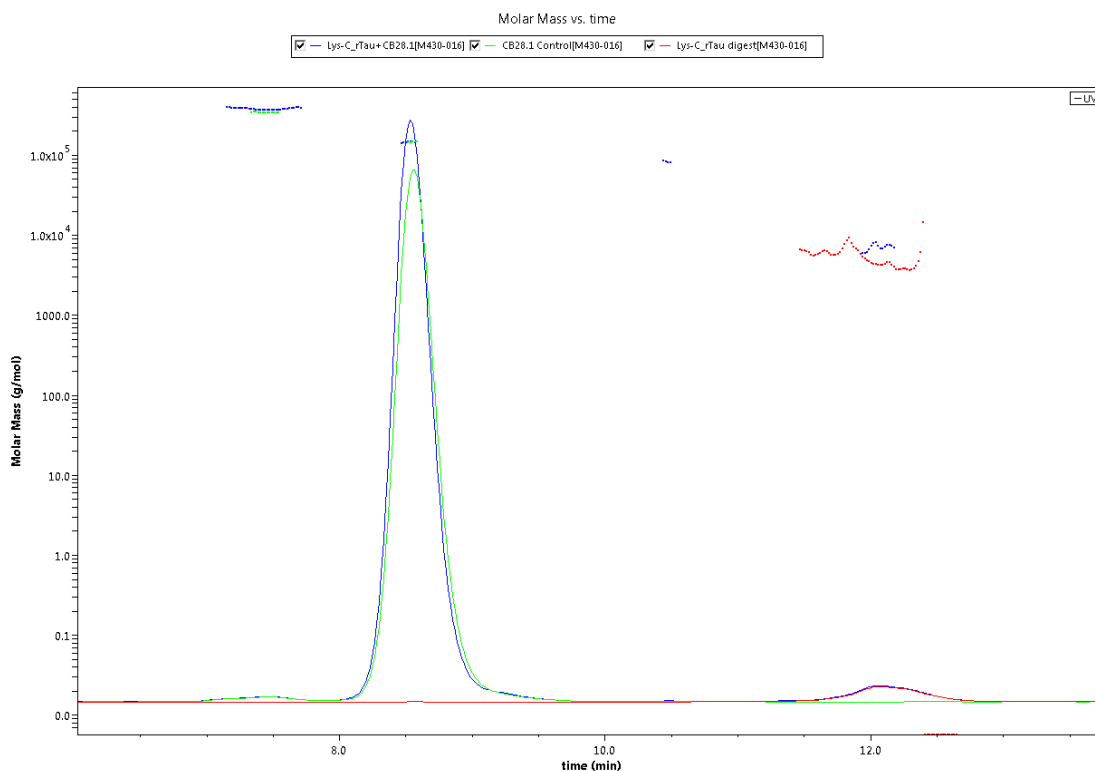
Figure 13: UV absorbance trace measured at 280 nm; rTau/Lys-C Digest + hCB tau 28.1 (sample A, blue trace), rTau/Lys-C Digest (sample B, red trace) hCB tau 28.1 (sample C, green trace). Distributions of the molecular masses of the analyzed samples are represented by dotted lines.

There are couple of conclusions that can be drawn based on the UV absorbance traces (both at 280 nm and 214 nm). It must be mentioned that the measured molecular weights are rather approximate. Conclusions based merely on calculated masses are therefore not reliable.

The main peak representing the sample A shows a small shift to the left in comparison to the antibody control sample C (8.5 min vs. 8.6 min) which would suggest possible binding of peptides to the antibody. Even though the amount of injected material for both samples A and C should be the same, a difference between the intensities of the two peaks can be clearly seen. Difference in intensities is most probably caused by errors in dilution during the sample preparation. This fact could possibly also have an influence on the retention times.

It can be safely deduced that the inhibition of the Lys-C with heat was successful. The antibody hCB tau 28.1 remained intact upon the mixing with the digested rTau sample

yielding signal at molecular weight of about 150 kDa. This was not the case in previous experiments where Lys-C was not effectively deactivated which led to fragmentation of the antibody (see Appendix, chapter 9.4).

Peaks observed at around 7.5 minutes correspond to commonly observed signals in SEC-MALS spectra of antibodies and most often represent oligomeric species/aggregates.

Table 15: Overview of SEC-MALS results

SAMPLE		Elution Time [min]		
		Peak 1	Peak 2	Peak 3
A	rTau/Lys-C Digest + hCB tau 28.1	7.5	8.5	12.2
B	rTau/Lys-C Digest	-	-	12.2
C	hCB tau 28.1	7.5	8.6	-
SAMPLE		Molecular Weight [kDa]*		
		Peak 1	Peak 2	Peak 3
A	rTau/Lys-C Digest + hCB tau 28.1	367	146	7
C	hCB tau 28.1	342	147	-
SAMPLE		Mass Recovery [%]*			
		Peak 1	Peak 2	Peak 3	Total
A	rTau/Lys-C Digest + hCB tau 28.1	1.1	78.0	2.5	82.2
C	hCB tau 28.1	1.4	93.7	-	95.4

*dRI concentration source was used for determination of molecular masses as well as the mass recoveries. The assigned molecular weights represent the detected mass at the apex of the peak (m_p). In case of the rTau/Lys-C digest, the dRI signal overlaps with the signal for eluting buffer salts, the calculated value is therefore not reliable. For the same reason, the mass recoveries are also approximate.

6.1.3 Fraction collection/Concentration measurements

In case that the peptide forms a complex with the antibody, it is expected to elute at around the same time as the antibody control sample. We can assume that this is not going to happen if the interaction between the antibody and a peptide is not strong. It is quite possible that the weakly bound complex dissociates somewhere along the way while traveling through the SEC column. If that is the case, peptides are not be found together with the antibody, however, their elution times will be more or less significantly shifted to the left, depending on the strength of interaction between the two binding partners. For this reason, fractions are collected during the course of the whole run instead of only around the elution time of the antibody.

The content of amino acids which are able to absorb ultraviolet light at 280 nm (F, W, Y) is very low in tau protein. Majority of the peptides will therefore not absorb light at this wavelength and the measured absorbance can be assigned predominantly to the antibody hCB tau 28.1. Fractions containing peptides can be detected based on the absorbance of light with wavelength of 214 nm. Peptide bonds absorb UV light at this wavelength.

6.1.3.1 Fractions A
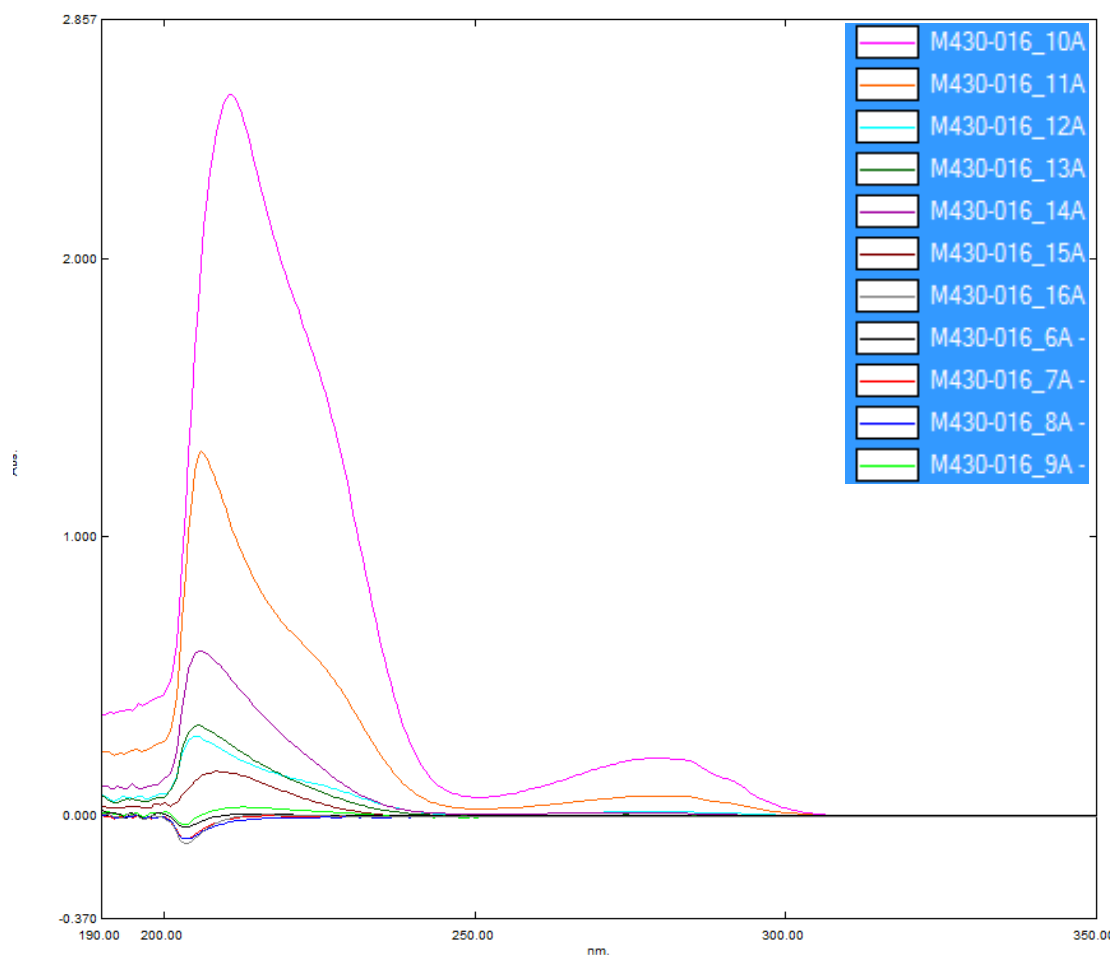
Figure 14: The absorption spectra of the fractions 6A-16A measured in range 180 nm - 350 nm

Table 16: Absorbance values for fractions 6A-16A measured at 214 nm, 280 nm and 350 nm.

#	A at 280nm [A.U.]	A at 214 nm [A.U.]	A at 350 nm [A.U.]
6A	0.000	0.007	-0.001
7A	0.000	0.000	-0.004
8A	0.000	-0.009	-0.005
9A	0.001	0.034	-0.006
10A	0.208	2.403	-0.003
11A	0.072	0.876	-0.002
12A	0.014	0.184	-0.001

13A	0.005	0.209	-0.003
14A	0.010	0.408	-0.001
15A	-0.001	0.126	0.006
16A	0.005	0.000	-0.004
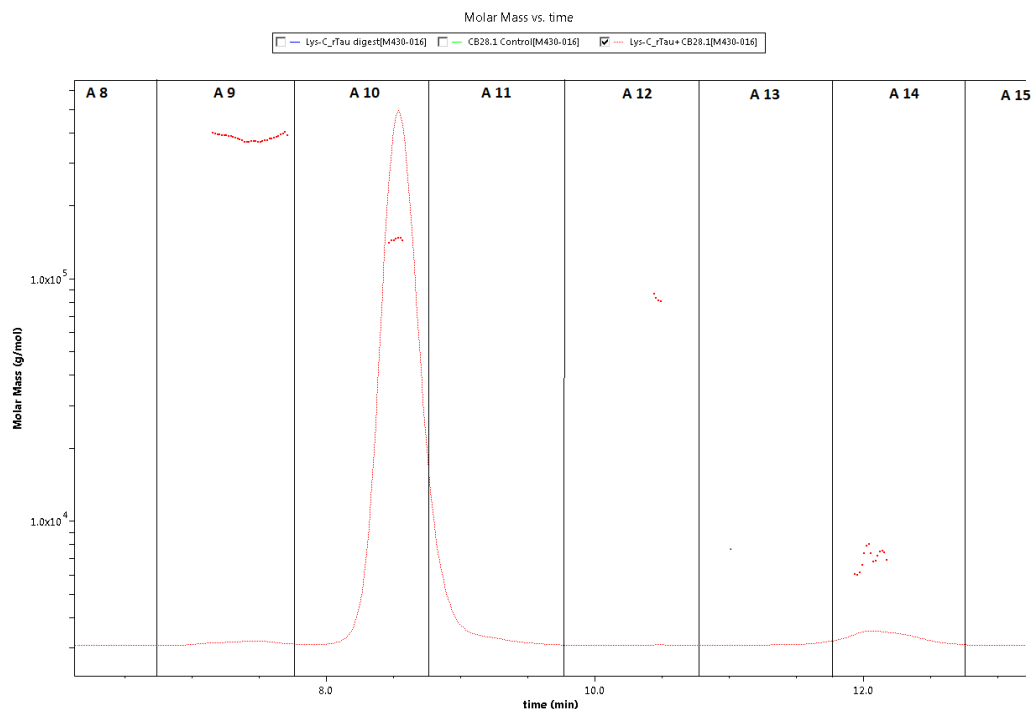
Figure 15: Graphical representation of fractions A collected during the SEC-MALS run. The delay volume of the instrument is based on an approximation. The time of the collection of the fractions was assumed according to the measured UV absorbance (see the Table 16 above) and the knowledge of the instrumental parameters.

Fractions A9, A10, A11, A12, A13, A14 and A15 which showed absorbance (even if low) were selected for LC-MS experiments.

6.1.3.2 Fractions B
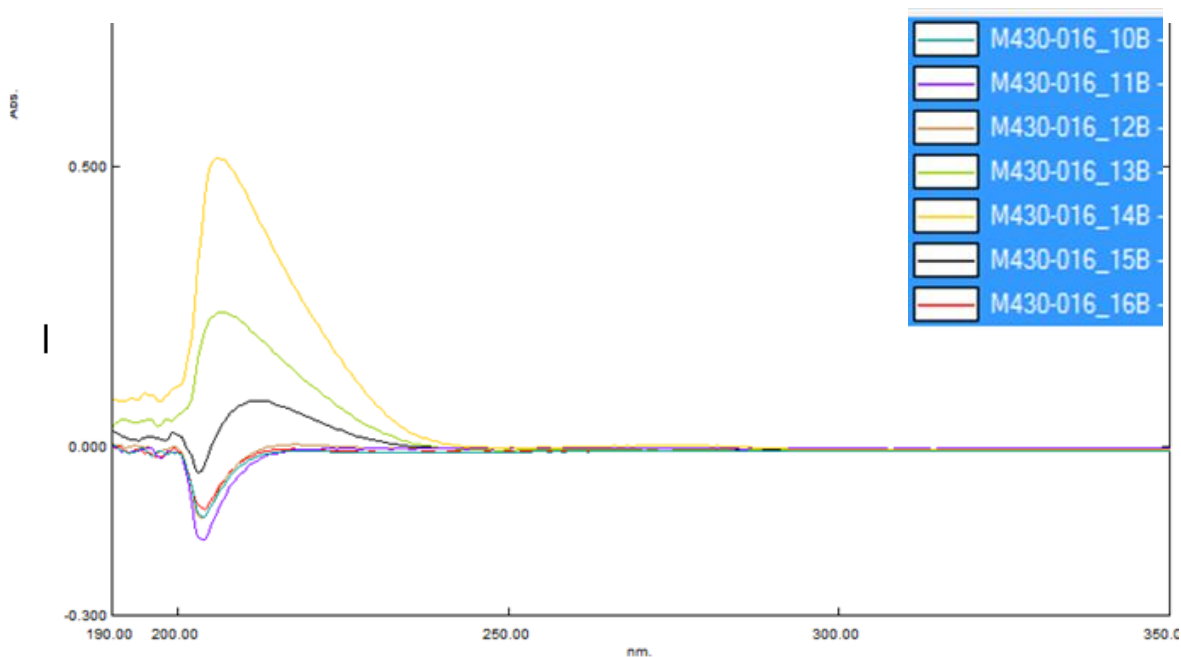
Figure 16: The absorption spectra of the fractions 6B-16B measured in range 180 nm-350 nm

Table 17: Absorbance values for fractions 6B-16B measured at 214 nm, 280 nm and 350 nm.

#	A at 280nm [A.U.]	A at 214 nm [A.U.]	A at 350 nm [A.U.]
10B	-0.001	-0.005	-0.006
11B	0.001	-0.010	-0.003
12B	0.001	0.005	-0.004
13B	0.002	0.182	-0.006
14B	0.007	0.371	-0.005
15B	0.000	0.083	-0.003
16B	-0.001	0.000	-0.006

Sample B is a control sample containing the peptide pool generated via the digestion of rTau with Lys-C. As expected, the absorbance at 280 nm is not relevant. Fractions only absorb at 214 nm.

The UV absorbance of the B fractions is measured mainly for control purposes. Fractions to be analyzed with LC-MS are chosen based on the results of the MS analysis of the fractions A. B serves as a control sample for the measurement.

6.1.4 LC-MS

Using the PLGS software, peptides in each of the fractions were identified.

If a strong complex was formed between the hCB tau 28.1 and a peptide, we would expect the peptides to elute within the fraction A10. In case of a weaker interaction, peptides should be found within the elution times that fall in between the elution of the antibody itself (~ 8.5 min) and that of the control sample B (~ 12.2 min). Sample B is used as a control sample to make sure that peptides found in fractions A are present due to an interaction with the antibody and not for other reasons.

Fractions A9-A15 were first injected on LC-MS. Out of the seven samples three contained peptides. These were fractions A10, A12 and A14. Based on this result, corresponding control fractions B10, B12 and B14 were analyzed. Fractions A10 (~ 7.8-8.8 min) and A12 (~ 9.8-10.8 min) are the main focus of this experiment. As previously mentioned, the peptide species interacting with the antibody CB tau 28.1 should elute within the two samples.

In theory, there are three different peptides created via the enzymatic digestion of tau protein with endoproteinase Lys-C, that cover the epitope sequence of the CB tau 28.1 (amino acids E058-T115 shown in red). These are fragments of rTau which cover the protein sequence at E058-K080, S081-K100 and Q101-K143. In case of the S081-K100 peptide, residues V088-D094 (highlighted in blue) are believed to not directly participate in the binding (based on previous HDX-MS experiments). The sequences of the three mentioned peptides are:

E058-K080: ESPLQTPTEDGSEEPGSETSDAK

S081-K100: STPTAEDVTAPLVDEGAPGK

Q101-K143: QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK

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There are five different peptides detected within the fraction A10. Two of them are peptides originating from the area of the epitope of CB tau 28.1. These are peptides E058-K080 and S081-K100. Both peptides were however detected at very low intensities (order of magnitude of +E03). Additionally the MH+ error in case of the E058-K080 is very high which does not make the result very reliable. However the fact, that the peptides elute at around the same time as the antibody suggest quite a strong interaction between them and the CB tau 28.1

Three other peptides which were found in the fraction A10 originate from the part of the tau sequence which is not known to participate in the interaction with the antibody (I273-K294, S354-K366, S354-382). The most probable explanation is that these peptides are present due to carryover from previous runs (in spite of the blank injections used in between each of the runs). Their presence within the fraction A10 due to an interaction with the CB tau 28.1 cannot be excluded solely based on this result but is highly unlikely. There were various experiments of similar sort performed prior to and following the described one. Unlike the peptides that are known to be a part of the epitope for CB tau 28.1, the three mentioned fragments didn´t show interactions in any other experiments. The peptides are probably present due to carryover or other contamination.

Fraction A12 contains two peptides only. Both of the fragments partially cover the sequence of the CB tau 28.1 epitope. The peptides E058-K080 and Q101-143 are detected with very high intensities. It seems that both peptides form a complex with the antibody, however, due to weaker interactions or harsh experimental conditions, the complex dissociates during the separation on the column. For this reason the peptides elute at higher retention times than would be expected for the elution of the peptide in complex with the antibody.

Fraction A14 is collected at around the time of the elution of the peptide pool and therefore contains various peptides that do not interact with the antibody. In the ideal case, the list of peptides should be a good match with the species contained within the fraction B14 and both controls with the exception of the interacting peptides that should not be present within the sample (or should appear at very low intensities).

Looking at the ESI-MS results, this is partially true. All four samples contain various digestion products and are on some levels comparable. There is, however, a difference between the incubated and non-incubated controls. It seems that the incubation of the rTau digest might have an effect on the generated peptides. Peptides are detected with lower intensities or not at all in comparison to the non-incubated control sample. Considering the peptides found within the fraction A14, it is not true, that peptide species interacting with the antibodies are not present. They all are detected with quite high intensities.

In tables 18 and 19 below, the summary of the ESI-MS data for the six most relevant peptides which were detected within the fractions A10, A12 and A14 together with the corresponding control fractions B and the control digest samples are shown. The list of all peptides identified within each of the fraction can be found in the Appendix, chapter 9.5.

Table 18: Summary of peptides covering the tau sequence at the CB tau 28.1 epitope area, their corresponding intensities and the MH+ Error

	Peptide AA Sequence					
Fraction	**E058-K080**		**S081-K100**		**Q101-K143**	
	Intensity	**MH+ Error [ppm]**	**Intensity**	**MH+ Error [ppm]**	**Intensity**	**MH+ Error [ppm]**
A10	6.54+E03	2.88	0.82+E03	24.37	-	-
B10	-	-	-	-	-	-
A12	2.17+E04	2.29	-	-	1.47+E05	3.38
B12	-	-	-	-	-	-
A14	1.07+E04	-4.71	2.01+E05	4.92	2.20+E04	2.44
B14	-	-	1.02+E05	1.52	2.84+E04	3.66
Digest non-incubated	1.42+E04	4.38	1.36+E05	1.76	1.12+E04	-1.67
Digest incubated at 60˚C	3.32+E	1.38	1.09+E03	2.18	9.76+E03	3.77

Table 19: Summary of peptides found within the relevant fraction A12, their corresponding intensities and the MH+ Error

Fraction	Peptide AA Sequence					
	I273-K294		S354-K366		S354-K382	
	Intensity	MH+ Error [ppm]	Intensity	MH+ Error [ppm]	Intensity	MH+ Error [ppm]
A10	4.69+E03	7.61	2.24+E03	-6.30	6.23+E03	-7.66
B10	-	-	-	-	-	-
A12	-	-	-	-	-	-
B12	-	-	-	-	-	-
A14	-	-	-	-	-	-
B14	-	-	-	-	3.93+E03	2.84
Digest non-incubated	-	-	-	-	-	-
Digest incubated at 60˚C	-	-	-	-	-	-

It must be noted that there were some technical issues observed during the experiment which had an effect on the ESI-MS measurements. High content of salts in the solvent caused damage to the MS instrument which lead to several problems. The sensitivity was most probably varying with each of the samples. Technical problems with the HPLC instrument used for separation of the peptides were also experienced. These facts must be taking into consideration.

Summary

In general, the experimental results are promising. Several peptides belonging to the region of the epitope of CB tau 28.1 eluted within the fractions A10 and A12. The shifts in the elution times suggest possible interactions of the peptides with the antibody. The detected intensities of ions are however very low in all cases. Even thought the

approach seems to be working and many parameters could be optimized to improve the results, there are too many drawbacks to the method.

First of all, the method is very time consuming and demanding when it comes to both, the instrumentation and the data interpretation. Further, the experimental conditions are not very flexible and cannot be easily changed. Lastly the mobile phase used in the SEC-MALS experiments contains salts which are not compatible with MS instrumentation. An extra desalting step could be employed (e.g. using ZipTips), however, along with additional procedures, the error in the experiment would be further increased. A new approach must be developed. Since most of the issues are linked to the SEC-MALS, this procedure must be substituted by a different step.

6.2 Approach B/Proof of concept experiment

6.2.1 LC-MS

MassLynx software was used to generate a list of theoretical masses of ionized peptide species. Each of the fractions was then investigated for the presence of individual peptides of the synthetic pool using the same program. The BPI chromatograms of all the fractions of each antibody sample along with the overview of identified peptides for the fractions A and D are summarized in the figures and tables below. Fractions A and D are the samples of interest. By comparing the experimental results with theoretical predictions, it is possible to validate the approach. For clarity about the fraction designation, see the Figure 18.

The table with theoretical masses of ionized peptides and summarized ESI-MS results for fractions B, C, D can be found in the Appendix, chapter 9.6.

6.2.1.1 ESI-MS result overview:

A. Control Sample – Synthetic peptide pool
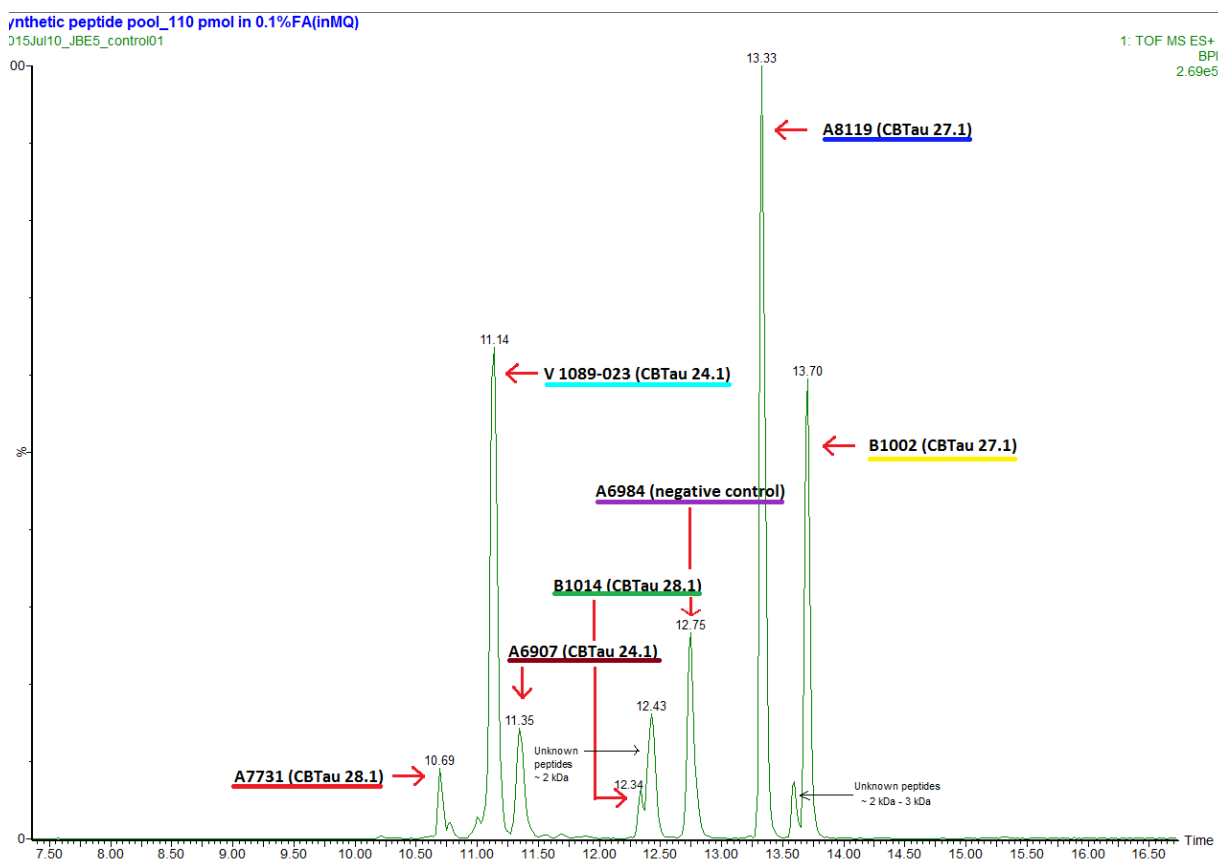
Figure 17: BPI chromatogram of the positive control sample/peptide pool corresponding to ~ 120 pmoles of each peptide.

All seven peptides of the synthetic pool can be identified on the BPI chromatogram of the control sample (Figure 17). The peaks for individual peptides are clearly visible, well separated and appearing with different intensities. Several other peaks also show up on the chromatogram. Based on the MS spectra analysis, they can be assigned to unknown peptide species of mass of approximately 2-3 kDa. It is quite probable that these are the fragments of some of the synthetic peptides used in the experiment. Contamination of the original samples or one introduced during the sample preparation also cannot be excluded.

The ESI-MS data for the individual peptides, listed according to the increasing elution times, is summarized in the Table 20 below. All the peptides were found with quite high

intensities. There is a variation among the individual signal intensities. The ionization of the peptide molecules and the way they travel through the MS instrument till they reach the detector, is highly dependent on the amino acid sequence hence the properties of the peptides. The control sample is used as a reference for the remaining samples.

Table 20: Overview of peptides/charged species identified in the control sample

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	10.69	A7731	788.67	3	788.67	2363.0	7.06E+04
	10.69	A7731	1182.51	2	1182.51	2363.0	1.81E+04
CB tau 24.1	11.14	V1089-023	590.32	4	590.32	2357.22	1.01E+06
	11.14	V1089-023	786.76	3	786.75	2357.22	5.56E+05
CB tau 24.1	11.35	A6907	918.36	8	918.36	7338.83	2.52E+05
	11.35	A6907	816.44	9	816.43	7338.83	2.51E+05
CB tau 28.1	12.34	B1014	848.13	4	848.13	3388.50	8.01E+04
	12.34	B1014	1130.51	3	1130.51	3388.50	3.97E+04
None - NC	12.75	A6984	1092.66	6	1092.65	6549.86	3.36E+05
	12.75	A6984	1310.99	5	1310.98	6549.86	5.89E+04
CB tau 27.1	13.33	A8119	605.34	4	605.34	2417.31	5.11E+05
	13.33	A8119	806.78	3	806.78	2417.31	4.41E+05
CB tau 27.1	13.70	B1002	845.45	4	845.45	3377.78	6.30E+05
	13.70	B1002	676.56	5	676.56	3377.78	1.31E+05

B. Antibody Samples

Peptides detected within each of the collected fraction provide us with different pieces of information. Since the peptides and their sequences as well as the epitope regions for the selected anti-tau antibodies are known, certain predictions can be made. In theory, peptides A7731 and B1014 bind to the CB tau 28.1, peptides A8119 and B1002 should

form a complex with CB tau 27.1 and peptides V1089-023 and A6907 should interact with CB tau 24.1. Peptide A6984 originates from the region of tau sequence that does not contain epitopes for any of the used antibodies and therefore is used as a negative control in the experiment. Peptides which don´t bind with the immobilized antibody, should be detected within the fractions A. Peptides which do form a complex with the mAb should elute within the fractions D. Fractions E might contain the same peptide species as fractions D, however in lower amounts. Fractions B and C are the wash fractions and might contain the same species as fractions A, however, with significantly lower intensities. The main fractions of interest are understandably the fractions A and D. Comparison of the experimental results with the hypothesis provide us with a tool for validation of the new approach to epitope mapping.

		SAMPLE 1 CB tau 28.1 + syn. peptide pool	SAMPLE 2 CB tau 27.1 + syn. peptide pool	SAMPLE 3 CB tau 24.1 + syn. peptide pool	SAMPLE 4 CR8043 + syn. peptide pool
Step A	• Removal of supernatant → UNBOUND PEPTIDES	fr. 1A	fr. 2A	fr. 3A	fr. 4A
Step B	• Wash step nr. 1 → RESIDUAL UNBOUND PEPTIDES	fr. 1B	fr. 2B	fr. 3B	fr. 4B
Step C	• Wash step nr. 2 → RESIDUAL UNBOUND PEPTIDES	fr. 1C	fr. 2C	fr. 3C	fr. 4C
Step D	• Elution step nr. 1 → BOUND PEPTIDES	fr. 1D	fr. 2D	fr. 3D	fr. 4D
Step E	• Elution step nr. 2 → RESIDUAL UNBOUND PEPTIDES	fr. 1E	fr. 2E	fr. 3E	fr. 4E

Figure 18: Over view of the fractions collected at individual steps and their corresponding designation

I. Sample 1: CB tau 28.1

FRACTIONS 1A and 1D
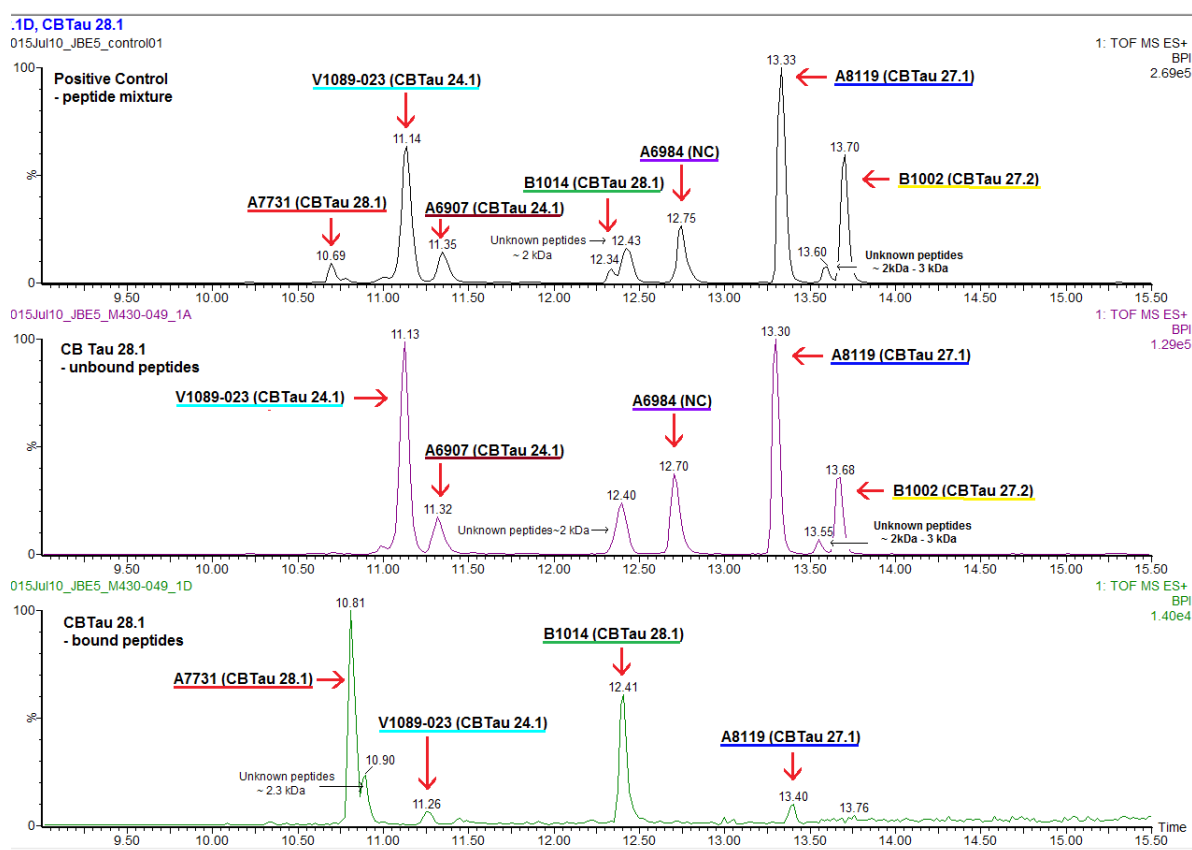
Figure 19: BPI chromatograms of the control sample and the CB tau 28.1 sample: fractions 1A and 1D

The summary of peptide species detected within the fraction 1A is shown in the Table 21. As expected, the peptides which are not supposed to bind to the CB tau 28.1 are all detected within the fraction 1A with high intensities. The signal intensities are very comparable to the control sample. Peptide A7731 and B1014 were also detected, however, with very low intensities.

Table 21: Overview of peptides/charged species identified in the fraction 1A

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	10.70	A7731	788.67	3	788.67	2363.0	2.24E+03
	-	A7731	-	2	1182.51	2363.0	-

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Antibody	RT[min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 24.1	11.13	V1089-023	590.32	4	590.32	2357.22	7.53E+05
	11.13	V1089-023	786.76	3	786.75	2357.22	3.41E+05
CB tau 24.1	11.32	A6907	918.37	8	918.36	7338.83	1.33E+05
	11.32	A6907	816.44	9	816.43	7338.83	1.41E+05
CB tau 28.1	12.29	B1014	848.13	4	848.13	3388.50	2.58E+03
	-	B1014	-	3	1130.51	3388.50	-
None - NC	12.70	A6984	1092.66	6	1092.65	6549.86	2.36E+05
	12.70	A6984	1310.99	5	1310.98	6549.86	3.84E+04
CB tau 27.1	13.30	A8119	605.33	4	605.34	2417.31	3.43E+05
	13.30	A8119	806.78	3	806.78	2417.31	2.46E+05
CB tau 27.1	13.68	B1002	845.45	4	845.45	3377.78	2.06E+05
	13.66	B1002	676.56	5	676.56	3377.78	5.51E+04

As summarized in the Table 22 below, all peptides except the A6984 (NC) are detected in the elution fraction 1D. It is, however, important to take a look at the intensities of the signals. Peptides A7731 and B1014, which cover the epitope region of CB tau 28.1, are found with signal intensities of one order of magnitude higher than the remaining species. Based on this result, we can assume that the two peptides form a complex with the CB tau 28.1. The other peptides detected within the fraction 1D are most probably present because they were not completely removed from the sample during the two washing steps (see the chromatograms of fractions i 1B and 1C, E figure 20).

Table 22: Overview of peptides/charged species identified in the fraction 1D

Antibody	RT[min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	10.81	A7731	788.67	3	788.67	2363.0	4.05E+04
	10.81	A7731	1182.52	2	1182.51	2363.0	5.16E+03
CB tau 24.1	11.27	V1089-023	590.32	4	590.32	2357.22	7.99E+03
	11.26	V1089-023	786.76	3	786.75	2357.22	1.52E+03

CB tau 24.1	11.45	A6907	918.36	8	918.36	7338.83	1.60E+03
	11.45	A6907	816.44	9	816.43	7338.83	2.24E+03
CB tau 28.1	12.41	B1014	848.13	4	848.13	3388.50	3.86E+04
	12.41	B1014	1130.51	3	1130.51	3388.50	1.75E+04
None - NC	-	A6984	-	6	1092.65	6549.86	-
	-	A6984	-	5	1310.98	6549.86	-
CB tau 27.1	13.39	A8119	605.34	4	605.34	2417.31	7.75E+03
	13.39	A8119	806.78	3	806.78	2417.31	3.74E+03
CB tau 27.1	13.76	B1002	845.45	4	845.45	3377.78	2.68E+03
	13.76	B1002	676.56	5	676.56	3377.78	1.25E+03

FRACTIONS 1B, 1C and 1E
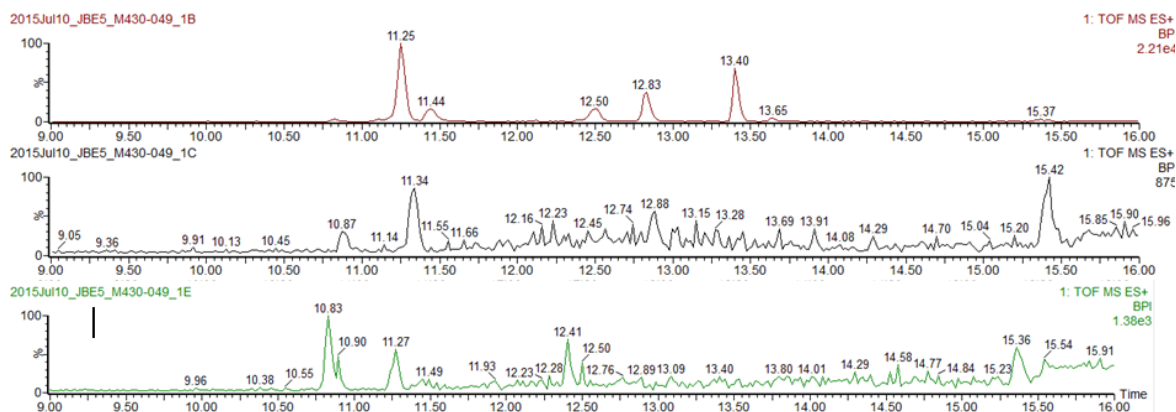
Figure 20: BPI chromatograms of the fractions 1B, 1C and 1E.
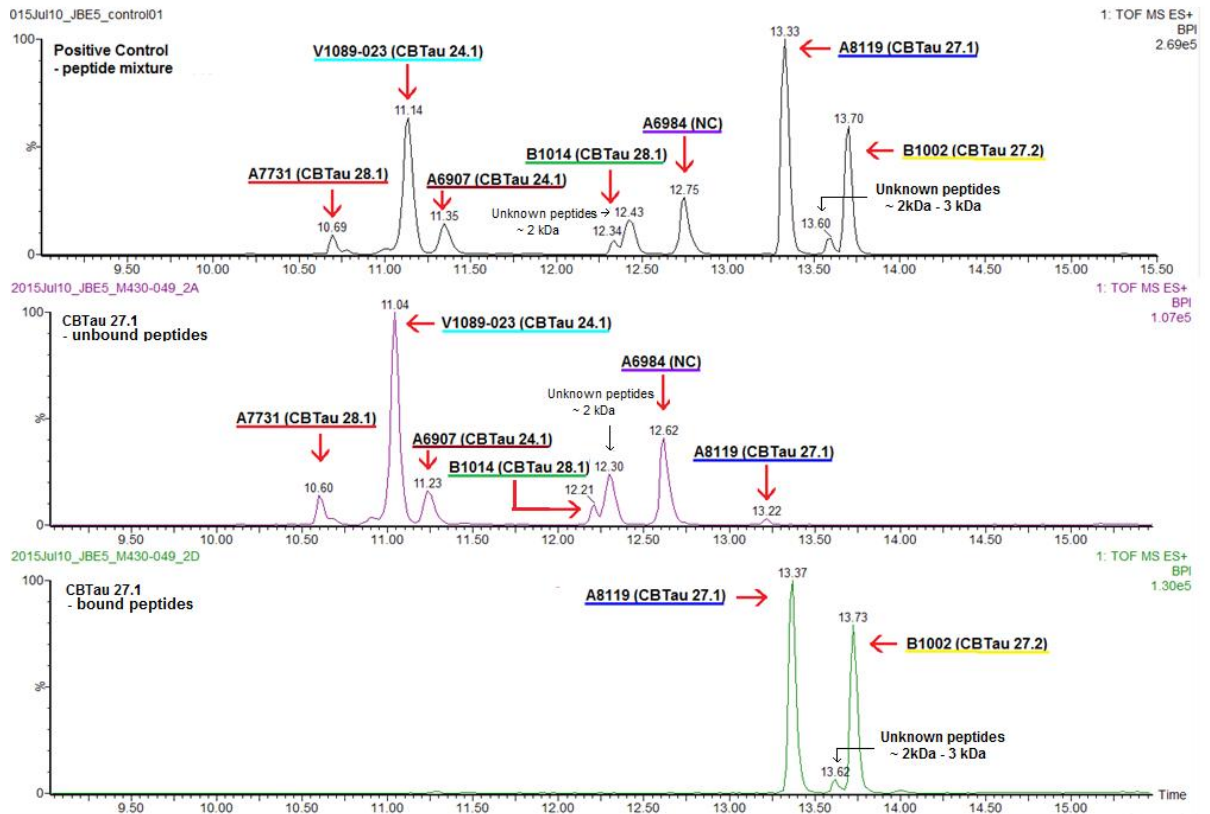
FRACTIONS 2A and 2D

Figure 21: BPI chromatograms of the control sample and the CB tau 27.1 sample: fractions 2A and 2D

Summary of the peptides detected within the fraction 2A can be found in the Table 23. All the peptides, except the B1002, are detected in the fraction 2A with quite high intensities, supporting the theory that they do not bind to the CB tau 27.1. Among them is also the peptide A8119 which contains part of the epitope sequence for CB tau 27.1. The intensity of the signal for peptide A8119 seems similar to the intensities of the unbound peptides. It is, however, important to put this result into perspective and consider the signal intensities for this peptide within the control sample and the fraction 2D. A8119 is detected with significantly higher intensity than the other peptides in the pool. This also means that in comparison to the other species, the traces of this peptide will appear with higher intensities in all the analyzed fractions. The peptide B1002, which binds to the CB tau 27.1, is not found in the 2A fraction.

Table 23: Overview of the peptides/charged species found within the fraction 2A

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	10.60	A7731	788.67	3	788.67	2363.0	4.51E+04
	10.60	A7731	1182.51	2	1182.51	2363.0	7.33E+03
CB tau 24.1	11.04	V1089-023	590.32	4	590.32	2357.22	6.70E+05
	11.04	V1089-023	786.76	3	786.75	2357.22	2.75E+05
CB tau 24.1	11.23	A6907	918.36	8	918.36	7338.83	8.89E+04
	11.23	A6907	816.44	9	816.43	7338.83	1.06E+05
CB tau 28.1	12.34	B1014	848.13	4	848.13	3388.50	4.21E+04
	12.34	B1014	1130.51	3	1130.51	3388.50	2.30E+04
None - NC	12.62	A6984	1092.66	6	1092.65	6549.86	1.71E+05
	12.62	A6984	1310.99	5	1310.98	6549.86	2.96E+04
CB tau 27.1	13.22	A8119	605.34	4	605.34	2417.31	1.89E+04
	13.22	A8119	806.78	3	806.78	2417.31	7.74E+03
CB tau 27.1	-	B1002	-	4	845.45	3377.78	-
	-	B1002	-	5	676.56	3377.78	-

Summary of the peptides detected within the fraction 2D can be found in the Table 24. Peptides A8119 and B1002 are detected in the fraction 2D with very high intensities. Two other species are found in the fraction (V1089-023 and A6907), the signal intensities are however very low. As in the other cases, unbound peptides were probably not washed off.

Table 24: Overview of the peptides/charged species found within the fraction 2D

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	-	A7731	-	3	788.67	2363.0	-
	-	A7731	-	2	1182.51	2363.0	-
CB tau	11.28	V1089-023	590.32	4	590.32	2357.22	9.38E+03

24.1	11.28	V1089-023	786.76	3	786.75	2357.22	2.37E+03
CB tau 24.1	11.45	A6907	918.37	8	918.36	7338.83	2.66e+03
	11.49	A6907	816.43	9	816.43	7338.83	3.30e+03
CB tau 28.1	-	B1014	-	4	848.13	3388.50	-
	-	B1014	-	3	1130.51	3388.50	-
None - NC	-	A6984	-	6	1092.65	6549.86	-
	-	A6984	-	5	1310.98	6549.86	-
CB tau 27.1	13.37	A8119	605.34	4	605.34	2417.31	3.64E+05
	13.37	A8119	806.78	3	806.78	2417.31	2.53E+05
CB tau 27.1	13.73	B1002	845.46	4	845.45	3377.78	2.49E+05
	13.73	B1002	676.56	5	676.56	3377.78	1.03E+05

FRACTIONS 2B, 2C, and 2E
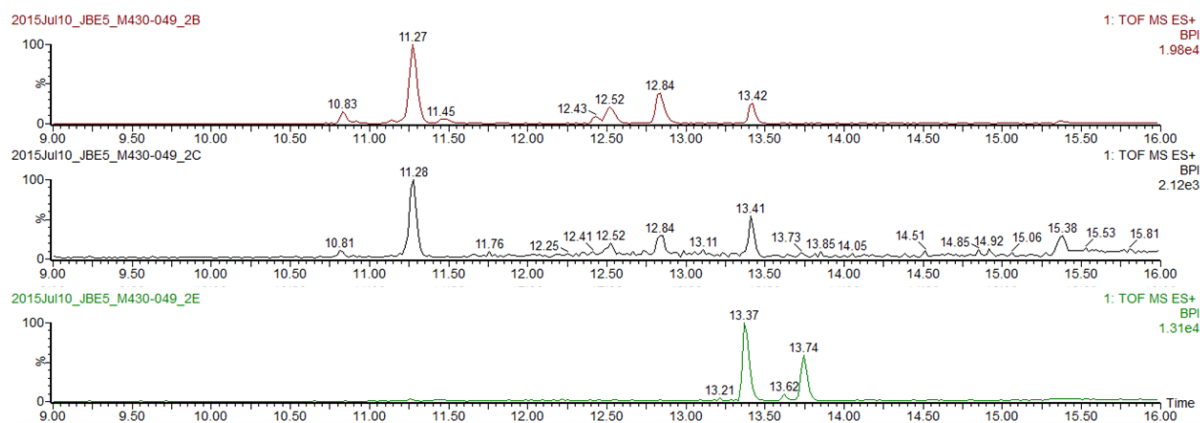
Figure 22: BPI chromatograms of the fractions 2B, 2C and 2E.

III.   Sample 3: CB tau 24.1

FRACTIONS 3A and 3D
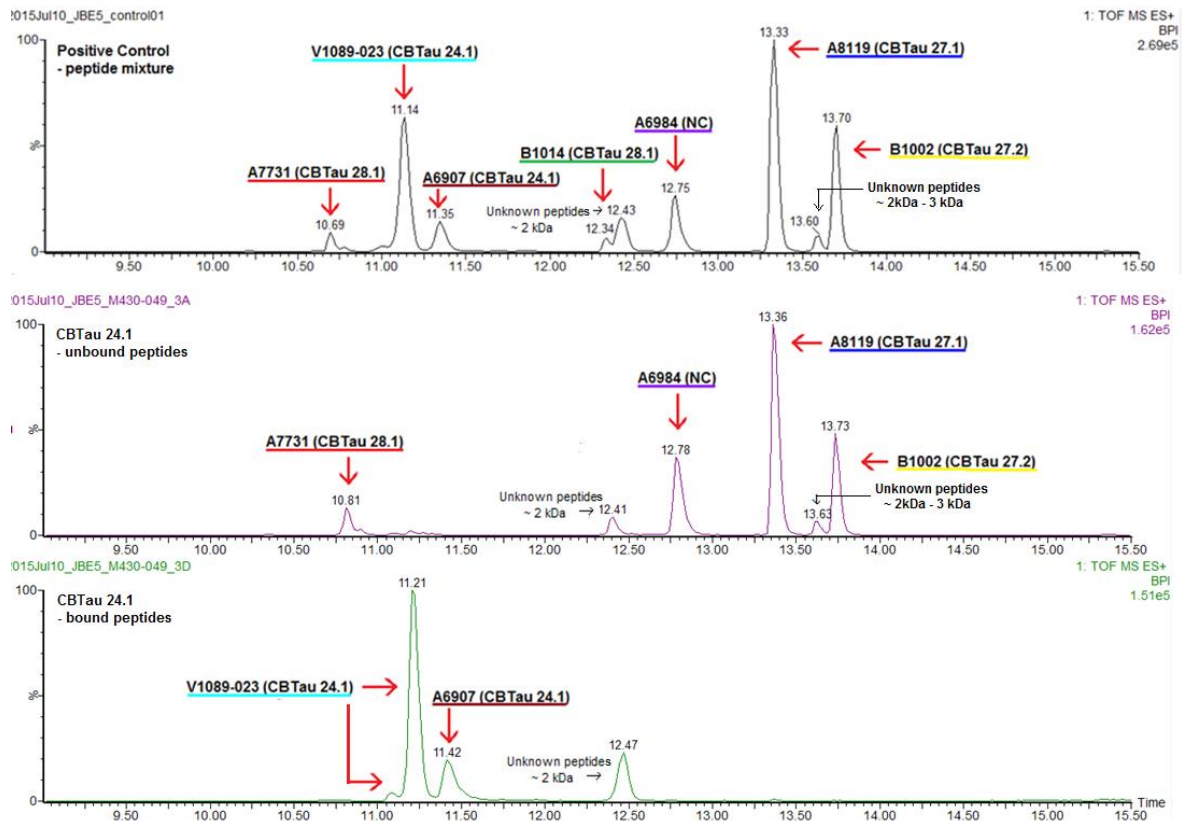
Figure 23: BPI chromatograms of the control sample and the CB tau 24.1 sample: fractions 3A and 3D

The peptides detected within the fraction 3A are summarized in the Table 25. All the species with the exception of the peptide A6907 are detected in the fraction 3A with high intensities. Peptide V1089-023 is detected with quite high intensity in respect to the unbound peptides. However, as in case of the peptide A8119 binding to the CB tau 27.1, we have to consider the high intensity of the signal for the peptide in other fractions and the control sample. Its presence within the fraction 3A does not necessarily mean that the peptide is not binding to the mAb. The peptide A6907, which binds to the CB tau 24.1, is not found in the fraction A3.

Table 25: Overview of peptides/charged species found in the fraction 3A

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	10.81	A7731	788.67	3	788.67	2363.0	6.03E+04
	10.81	A7731	1182.51	2	1182.51	2363.0	1.17E+04
CB tau 24.1	11.20	V1089-023	590.32	4	590.32	2357.22	2.51E+04
	11.20	V1089-023	786.76	3	786.75	2357.22	4.76E+03
CB tau 24.1	-	A6907	-	8	918.36	7338.83	-
	-	A6907	-	9	816.43	7338.83	-
CB tau 28.1	12.41	B1014	848.13	4	848.13	3388.50	4.51E+04
	12.41	B1014	1130.51	3	1130.51	3388.50	3.48E+04
None - NC	12.80	A6984	1092.66	6	1092.65	6549.86	1.90E+05
	12.78	A6984	1310.99	5	1310.98	6549.86	4.47E+04
CB tau 27.1	13.36	A8119	605.34	4	605.34	2417.31	4.15E+05
	13.36	A8119	806.78	3	806.78	2417.31	3.10E+05
CB tau 27.1	13.73	B1002	845.46	4	845.45	3377.78	2.01E+05
	13.73	B1002	676.57	5	676.56	3377.78	8.15E+04

Summary of peptides found within the fraction 3D is shown in the Table 26. Peptides found in this fraction support the previously discussed hypothesis. V1089-023 and A6907, both containing the tau epitope region for CB tau 24.1, are found with very high intensities. Peptides B1002 and A8119 are detected as well, however, both with negligible signal intensities.

Table 26: Overview of peptides/charged species found within the fraction 3D.

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	-	A7731	-	3	788.67	2363.0	-
	-	A7731	-	2	1182.51	2363.0	-

CB tau 24.1	11.21	V1089-023	590.32	4	590.32	2357.22	9.42E+05
	11.21	V1089-023	786.76	3	786.75	2357.22	4.68E+05
CB tau 24.1	11.42	A6907	918.36	8	918.36	7338.83	1.37E+05
	11.42	A6907	816.43	9	816.43	7338.83	1.71E+05
CB tau 28.1	-	B1014	-	4	848.13	3388.50	-
	-	B1014	-	3	1130.51	3388.50	-
None - NC	-	A6984	-	6	1092.65	6549.86	-
	-	A6984	-	5	1310.98	6549.86	-
CB tau 27.1	13.37	A8119	605.34	4	605.34	2417.31	7.27E+03
	13.37	A8119	806.78	3	806.78	2417.31	3.58E+03
CB tau 27.1	13.73	B1002	845.45	4	845.45	3377.78	1.04E+03
	-	B1002	-	5	676.56	3377.78	-

FRACTIONS 3B, 3C and 3E
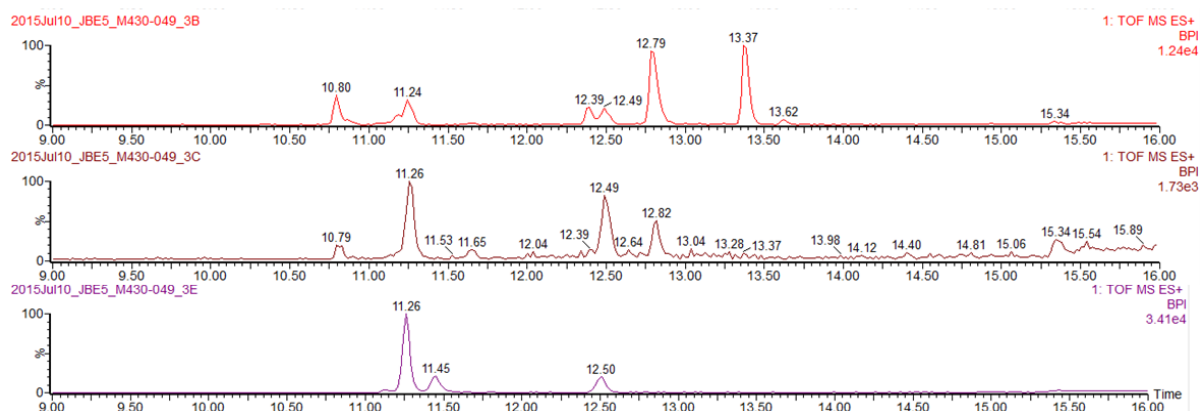
Figure 24: BPI chromatograms of the fractions 3B, 3C and 3E.

IV. Sample 4: CR8043-Negative Control

FRACTIONS 4A and 4D
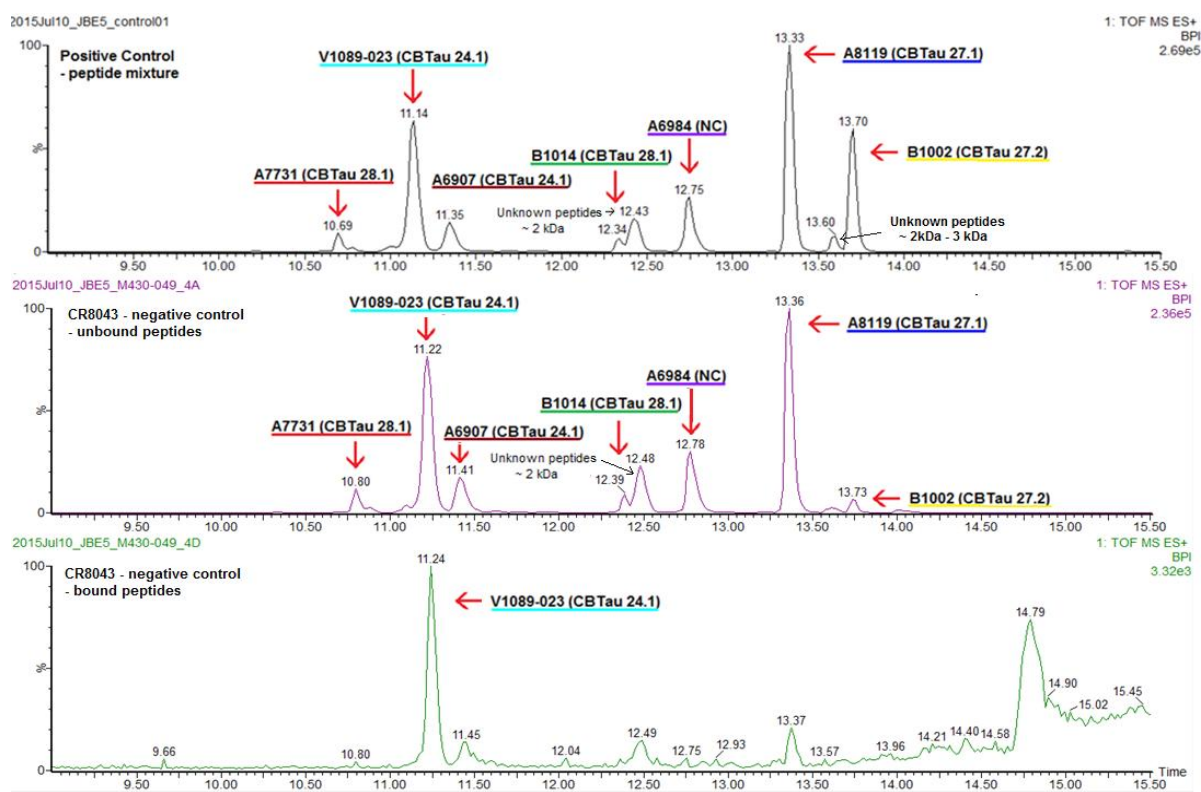
Figure 25: BPI chromatograms of the control sample and the CR8043 sample: fractions 4A and 4D

Peptide species found in the fraction 4A are summarized in the Table 27. As expected all the peptides of the synthetic peptide pool elute within this with high intensities which are very comparable to the control sample. Exception is the peptide B1002, which is detected with much lower intensity in comparison to the control. It is difficult to assume a reason for that. The peptide is not found in any other fractions of the sample 4, therefore the peptide is not present in lower amount because of binding to the antibody.

The antibody CR8043 is used as a negative control. This "flu" antibody is not specific for the tau sequence and therefore should not interact with the protein/synthetic peptides. The assumption therefore is that the synthetic peptides would only be detected within the fraction A (and possibly the "wash" fractions B, C). The ESI-MS analysis shows, however, different results.

As shown in the Figures 25 and 26, there is a peptide V1089-023 eluting within fractions D and E. It can only be hypothesized why is that so. The peptide is measured with quite low intensities, however, its presence due to contamination is quite improbable. As seen from the BPI chromatograms and MS data, the peptide is not present within the wash fractions. It is however detected upon elution with formic acid. This fact suggests that there is an interaction between the antibody and the peptide. Containing phosphoserine within its sequence, peptide V1089-023 is the only phosphorylated peptide present in the peptide pool. A possible explanation is unspecific binding between the molecule and the antibody due to electrostatic interactions. It is also possible, that by a coincidence, the antibody is specific to part of the peptide sequence. The negative control CR8043 was chosen by chance, assuming that it is not specific to tau protein (CR8043 antibody is targeted against flu viral proteins. No experimental data on its interaction with tau are available). New negative control has to be established in order to have more reliable experimental results.

Table 27: Overview of peptides/charged species present in the fraction 4A

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	10.80	A7731	788.67	3	788.67	2363.0	8.19E+04
	10.80	A7731	1182.51	2	1182.51	2363.0	2.24E+04
CB tau 24.1	11.22	V1089-023	590.32	4	590.32	2357.22	1.06E+06
	11.22	V1089-023	786.76	3	786.75	2357.22	5.60E+05
CB tau 24.1	11.41	A6907	918.37	8	918.36	7338.83	1.87E+05
	11.41	A6907	816.44	9	816.43	7338.83	2.16E+05
CB tau 28.1	12.39	B1014	848.13	4	848.13	3388.50	5.65E+04
	12.39	B1014	1130.51	3	1130.51	3388.50	4.96E+04
None - NC	12.78	A6984	1092.66	6	1092.65	6549.86	2.09E+05
	12.78	A6984	1310.99	5	1310.98	6549.86	5.66E+04
CB tau 27.1	13.36	A8119	605.34	4	605.34	2417.31	5.02E+05
	13.36	A8119	806.78	3	806.78	2417.31	3.93E+05

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 27.1	13.73	B1002	845.46	4	845.45	3377.78	4.94E+04
	13.73	B1002	676.57	5	676.56	3377.78	2.13E+04

Table 28: Overview of peptides/charged species present in the fraction 4D

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	-	A7731	-	3	788.67	2363.0	-
	-	A7731	-	2	1182.51	2363.0	-
CB tau 24.1	11.24	V1089-023	590.32	4	590.32	2357.22	2.60E+04
	11.24	V1089-023	786.76	3	786.75	2357.22	5.34E+03
CB tau 24.1	11.43	A6907	918.37	8	918.36	7338.83	1.81E+03
	11.43	A6907	816.44	9	816.43	7338.83	2.43E+03
CB tau 28.1	-	B1014	-	4	848.13	3388.50	-
	-	B1014	-	3	1130.51	3388.50	-
None - NC	-	A6984	-	6	1092.65	6549.86	-
	-	A6984	-	5	1310.98	6549.86	-
CB tau 27.1	13.37	A8119	605.34	4	605.34	2417.31	3.89E+03
	13.37	A8119	806.78	3	806.78	2417.31	1.96E+03
CB tau 27.1	-	B1002	-	4	845.45	3377.78	-
	-	B1002	-	5	676.56	3377.78	-

Fractions 4B, 4C and 4D
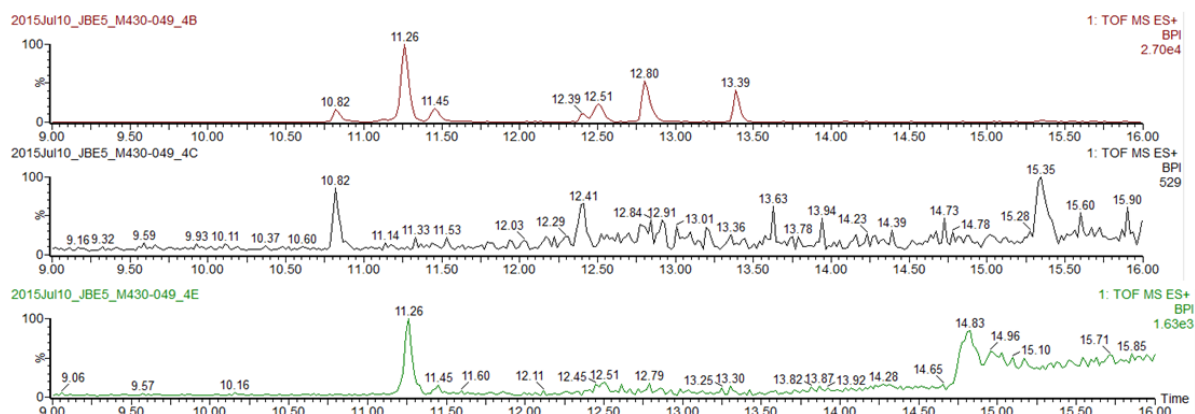
Figure 26: BPI chromatograms of the fractions 4B, 4C and 4E.

Summary:

The overall result is quite satisfying. We can conclude that the synthetic peptides are indeed specifically binding to the rTau antibodies as expected, proving that the epitope mapping approach is working. Further optimizations of the experiment are required, especially considering the choice of the negative control.

6.3 Approach B/Tau epitope mapping experiment

6.3.1 In-solution enzymatic digestion/SDS PAGE

SDS PAGE of the recombinant tau after digestion with Lys-C and Asp-N was performed to check on the overall efficiency of the digestion.

1Lys-C:80rTau / 1Asp-N:95rTau	
#	Sample
1	Marker Standard
2	rTau 'Fresh' – control
3	rTau/Lys-C Digest (1:80)
4	rTau 'Incubated' – control
5	rTau/Asp-N Digest (1:95) 'Fresh' -control
6	Marker Standard
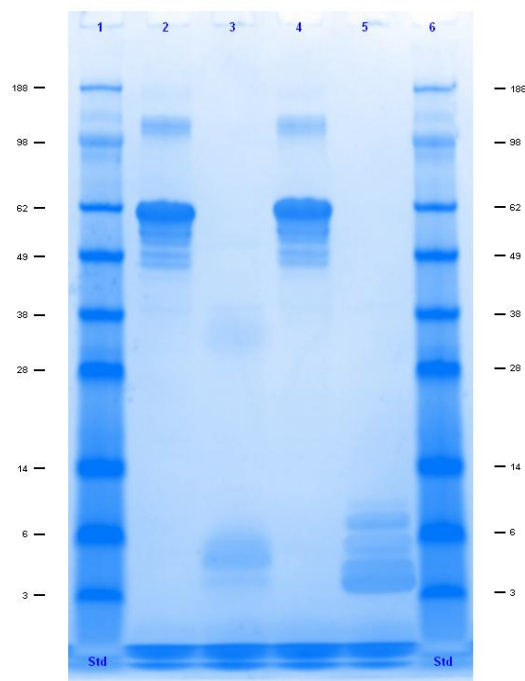
Figure 27: Picture of Lys-C/rTau and Asp-N/rTau fragments spearated on 4-12% BIS-TRIS gel; MES running buffer; NR conditions.

Intact recombinant tau appears as a band at around 60 kDa as can be seen in lanes 2 and 4 which contain both control samples. The two samples run identically on the gel

suggesting that the overnight incubation of tau seems to have no effect on the protein (we however cannot exclude aggregation since SDS is used). The digestion of rTau can be considered as effective. Comparing the relative intensities of the bands, we can conclude that most of the digestion products are smaller than 6 kDa as expected in case of complete cleavage of tau protein with both Lys-C and Asp-N (see Appendix, chapter 9.2 for the theoretical fragmentation of rTau with the enzymes). The bands present at around 95 kDa probably represent disulfide cross-linked tau dimers. Hardly visible trace at around 30 kDa appears in the lane three (Lys-C/rTau digestion product). The band might represent partially digested or fragmented rTau. Its intensity is, however, very low and can therefore be neglected.

6.3.2 LC-MS

As mentioned in the previous sections, endoproteinase Lys-C cleaves at the C-terminal site of lysine residues and endoproteinase Asp-N at the N-terminal site of the aspartic acid residues. Proline residue at the direct proximity of the cleavage site of Lys-C often leads to miscleavages. Shown below is the theoretical cleavage of tau protein with both enzymes. The so far identified epitopes for the CB tau 28.1, CB tau 27.1 and CB tau 24.1 are highlighted in different colors.

Theoretical peptide pool/rTau Digestions with:

<u>Lys-C:</u>

MAHHHHHHDDDDK/MAEPRQEFEVMEDHAGTYGLGDRK/DQGGYTMHQDQE GDTDAGLK/ESPLQTPTEDGSEEPGSETSDAK/STPTAEDVTAPLVDEGAPGK/QA AAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK/SK/DGTGSDDK/K/ AK/GADGK/TK/IATPRGAAPPGQK/GQANATRIPAK/TPPAPK/TPPSSGEPPK/SG DRSGYSSPGSPGTPGSRSRTPSLPTPPTREPK/K/VAVVRTPPK/SPSSAK/SRLQTA PVPMPDLK/NVK/SK/IGSTENLK/HQPGGGK/VQIINK/K/LDLSNVQSK/CGSK/DN IK/HVPGGGSVQIVYK/PVDLSK/VTSK/CGSLGNIHHK/PGGGQVEVK/SEK/LDFK /DRVQSK/IGSLDNITHVPGGGNK/K/IETHK/LTFRENAK/AK/TDHGAEIVYK/SPV VSGDTSPRHLSNVSSTGSIDMVDSPQLATLADEVSASLAK/QGL

96

Asp-N:

MAHHHHHH/D/D/D/DKMAEPRQEFEVME/DHAGTYGLG/DRK/DQGGYTMHQ/
DQEG/DT/DAGLKESPLQTPTE/DGSEEPGSETS/DAKSTPTAE/DVTAPLV/DEGAP
GKQAAAQPHTEIPEGTTAEEAGIG/DTPSLE/DEAAGHVTQARMVSKSK/DGTGS/
D/DKKAKGA/DGKTKIATPRGAAPPGQKGQANATRIPAKTPPAPKTPPSSGEPPK
SG/DRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKKVAVVRTPPKSPSSAKSRLQT
APVPMP/DLKNVKSKIGSTENLKHQPGGGKVQIINKKL/DLSNVQSKCGSK/DNI
KHVPGGGSVQIVYKPV/DLSKVTSKCGSLGNIHHKPGGGQVEVKSEKL/DFK/DR
VQSKIGSL/DNITHVPGGGNKKIETHKLTFRENAKAKT/DHGAEIVYKSPVVSG/D
TSPRHLSNVSSTGSI/DMV/DSPQLATLA/DEVSASLAKQGL

Legend:

CB tau 28.1 epitope sequence E058-T115*

*(according to HDX MS results, sequence of amino acids highlighted in blue seems to not take a direct part in the interaction)

CB tau 27.1 epitope sequence Y323-V331

CB tau 24.1 epitope sequence P249-L256

/ The cleavage site highlighted in red represents the miscleaved sites (due to proline residue following after lysine)

The epitopes highlighted within the sequence are approximate. Their localization is based on previous experiments (X-ray crystallography, HDX MS). The exact epitopes for the mentioned antibodies are still to be determined.

6.3.2.1 ESI-MS results
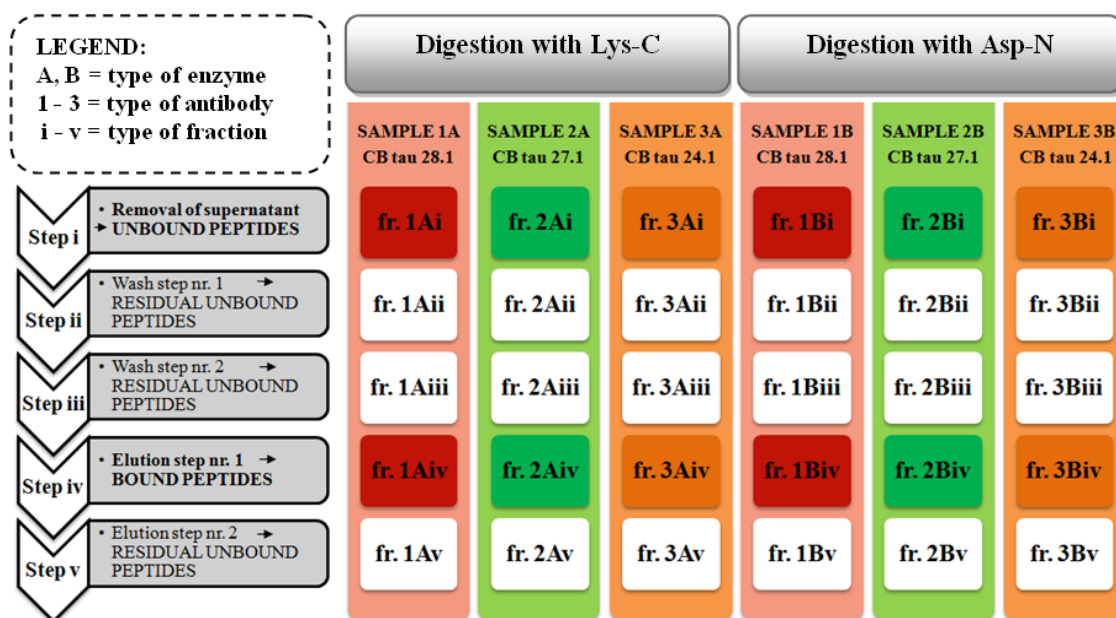
Figure 28: Overview of the analyzed samples, their fractions collected during the individual steps and the corresponding designations.

Using the PLGS software, peptides in each of the fractions were identified. In theory, it should be possible to predict whether the peptide species interact with the antibody of interest based on their elution within different fractions. The fractions i should contain peptides which do not bind to the antibody and therefore remain within the solution after the incubation. The peptides which interact with the antibody should not be present in this fraction at all or with low intensities. This depends on the strength of the interaction as well as the sensitivity of the MS instrument. Fractions ii and iii represent the liquid collected after the washing steps 1 and 2 respectively. Within the samples ii, residual peptides should be found (especially the ones appearing in the fractions i with very high intensities). The fractions ii should preferably not contain any peptide species since the purpose of the second wash is to make sure no unbound peptides will be present in fractions iv and v.

The fractions iv collected after the 1st elution of the complex from the resin with 0.1% FA are the essential samples in this experiment (the remaining fractions serve mostly as controls). Within these samples, peptides which interact with the antibodies of interest and therefore represent a part of the epitope sequence, should be found.

Fractions v which are collected after the 2nd elution of the complex from the resin with 0.1% FA should contain either lower amounts of the same peptide species which were found in corresponding fractions iv or no peptides at all.

Note: For each of the samples, a summary of ESI-MS data for the relevant peptides which theoretically cover the epitope sequence for given antibody are shown below. Summary of all identified peptides in individual fractions along with the list of theoretical masses of the ionized species can be found in the Appendix, chapter 9.7. The complete list of peptides (generated in PLGS) detected in the remaining fractions for all antibody samples is also shown in the appendix.

I. Samples 1: Antibody CB tau 28.1

a) Sample 1A: Lys-C + CB tau 28.1

Theoretically, there are three tau peptides produced via Lys-C digestion that cover the tau epitope sequence for the antibody CB tau 28.1:

peptides:

58-80: ESPLQTPTEDGSEEPGSETSDAK

81-100: STPTAEDVTAPLVDEGAPGK

101-143: QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK

The only peptide detected and identified within the fraction 1Aiv is a peptide covering the tau sequence residues 58-80. The peptide is only found in the elution fraction and not in the sample containing the unbound peptides, which suggests a strong interaction with the antibody CB tau 28.1.

Summary of the intensities and errors for the relevant peptides detected within each of the 1A fractions are shown in the Table 30. Comparing the intensities of the signals between the sample 1Aiv and the control sample containing the digested tau, strong binding is suggested. No peptides were detected within the fractions 1Aiii and 1Av.

Table 30: Summary of the intensities and MH+ errors of the three tau peptides which cover the epitope sequence of CB tau 28.1. Data for all the fractions 1A are shown.

Fraction	Peptide AA Sequence					
	58-80		81-100		101-143	
	Intensity	MH+ Error [ppm]	Intensity	MH+ Error [ppm]	Intensity	MH+ Error [ppm]
1Ai	-	-	2.05E+05	6.73	3.66E+05	1.41
1Aii	-	-	1.39E+04	-1.38	3.02E+04	1.33
1Aiii	-	-	-	-	-	-
1Aiv	8.4E+04	0.35	-	-	-	-
1Av	-	-	-	-	-	-
Control	1.32E+05	1.97	2.49E+05	4.08	4.66E+05	1.64

b) Sample 1B: Asp-N + CB tau 28.1

There are five tau peptides produced via Asp-N digestion of tau that cover the tau epitope sequence for the antibody CB tau 28.1. The fragments covering the sequence are quite small which decreases the probability of interaction of peptides with antibody.

peptides:

53-66: DAGLKESPLQTPTE

67-77: DGSEEPGSETS (not found in the control sample!!)

78-86: DAKSTPTAE

87-93: DVTAPLV

94-122: DEGAPGKQAAAQPHTEIPEGTTAEEAGIG

No peptides were found within the relevant fraction 1Biv or the other fractions 1Biii and 1Bv. The results suggest that there is no interaction among the Asp-N/rTau

digestion products and the antibody CB tau 28.1. The most probable reason, as previously mentioned, is the multiple cleavages within the epitope which result into short fragments. Short peptides are not able to take a part in the interaction

II. Samples 2: Antibody CB tau 27.1:

a) Sample 2A: Lys-C + CB tau 27.1

There are two tau peptides produced via Lys-C digestion of tau that cover the tau epitope sequence for the antibody CB tau 27.1. One of them, containing the longer part of the epitope sequence, is a peptide generated via miscleavage due to the presence of proline residue which is located C-terminally from the lysine as shown below.

peptides:

312-330: HVPGGGSVQIVYK PVDLSK

331-334: VTSK (not found in the control sample!!)

The only peptide detected and identified within the fraction 2Aiv is a peptide covering the tau sequence at residues 312-330. This peptide is found in the elution fraction 2Aiv as well as in sample 2Ai containing the unbound peptides. The peptide is also detected within the 'wash' fraction 2Aii. In all three cases, the peptide is found with comparable intensities, suggesting that the peptide is binding to the antibody CB 27.1, however, with lower affinity. The interaction of the peptide 312-330 was expected since the molecule contains the whole epitope except one amino acid, which is the valine residue at the C-termius.

Peptide 331-334 was not detected at all, even within the control sample. Therefore we cannot assume anything about its binding with the mAb solely based on the results. However, since the peptide only contains one amino acid which is thought to be a part of the epitope, its interaction with the antibody is quite unlikely.

It can be concluded that the C-terminal valine is not essential for the interaction between the tau protein and the antibody CB tau 27.1. It might, however, possibly have

an effect on the affinity of the antibody for the epitope. No peptides were detected within the fractions 1Aiii and 1Av.

Table 31: Summary of the intensities and MH+ errors of the two tau peptides which cover the epitope sequence of CB tau 27.1. Data for all the fractions 2A are shown.

| Fraction | Peptide AA Sequence | | | |
| | 312-330 | | 331-334 | |
	Intensity	MH+ Error [ppm]	Intensity	MH+ Error [ppm]
2Ai	1.95E+05	-4.53	-	-
2Aii	2.75E+04	-2.76	-	-
2Aiii	-	-	-	-
2Aiv	1.31E+04	-2.20	-	-
2Av	-	-	-	-
Control	2.62E+05	-2.19	-	-

b) Sample 2B: Asp-N epitope + CB tau 27.1

There are two tau peptides produced via Asp-N digestion that cover the tau epitope sequence for the antibody CB tau 27.1. The enzyme cleaves the already quite short tau epitope sequence right in the middle.

Peptides:

308-326: DNIKHVPGGGSVQIVYKPV

327-357: DLSKVTSKCGSLGNIHHKPGGGQVEVKSEKL (not found in the control sample)

No peptides were found within the relevant fraction 2Biv or the fractions 2Biii and 2Bv. The results suggest there is no interaction among the Asp-N/rTau digestion products and the antibody CB tau 27.1. The epitope was cut in the middle. It is quite probable that neither parts of the epitope sequence contained within the two peptides can interact

with the mAb by themselves. Definite conclusion cannot be made for the peptide 327-357 since this fragment was not detected within the control sample.

III. Samples 3: Antibody CB tau 24.1

a) Sample 3A: Lys-C + CB tau 24.1

There are two tau peptides produced via Lys-C digestion of tau protein that cover the tau epitope sequence for the antibody CB tau 24.1.

peptides:

248-253: S**PSSAK** (not found in the control sample)

254-267: **SRL**QTAPVPMPDLK

The results for the binding of Lys-C/rTau digest with antibody CB tau 24.1 are comparable to the results in case of the sample 2B.

No peptides were found within the relevant fraction 3Aiv or the fractions 3Aiii and 3Av. The data suggest there is no interaction among the Lys-C/rTau digestion products and the antibody CB tau 24.1. The epitope is quite short. It is probable that neither part of the epitope sequence contained within the two peptides can interact with the mAb by themselves and that longer AA sequence is required for successful binding. The conclusion cannot be made for the peptide 248-267 since this fragment is not detected within the control sample.

b) Sample 3B: Asp-N + CB tau 24.1

There is only one tau peptide produced via Asp-N digestion of tau protein. This peptide contains the complete tau epitope sequence for the antibody CB tau 24.1.

peptide:

206-264:
DRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKKVAVVRTPPKSPSSAKSRLQT APVPMP

The only peptide detected and identified within the fraction 3Biv is a peptide containing the whole epitope. The peptide is also found in the elution fraction 3Bi containing the unbound species.

The intensity of the signal is very high in case of the elution fraction 3Biv, suggesting quite a strong interaction between the peptide and the CB tau 24.1. The results correspond to the expectations since the peptide sequence contains the whole epitope. No peptides were detected within the fractions 3Bii, 3Biii and 3Bv.

Table 32: Summary of the intensities and MH+ errors of the tau peptide which contains the epitope sequence of CB tau 24.1. Data for all the fractions 3B are shown.

Fraction	Peptide AA Sequence	
	312-330	
	Intensity	MH+ Error [ppm]
3Bi	1.49E+04	-2.17
3Bii	-	-
3Biii	-	-
3Biv	2.41E+05	-0.08
3Bv	-	-
Control	3.00E+05	-1.09

Summary:

The novel approach to epitope mapping seems to be successful. Tau peptides which were found back in the fractions correspond to the parts of the tau epitope sequence for the anti-tau.

There are certain issues to be tackled. Most importantly, some of the peptides were not found within the control sample which understandably represents a major inconvenience when it comes to the reliability of the experiment. The peptide species

that were not detected are mainly short, highly polar fragments. Poor ionization and possibly also the separation on the column prior to the MS might play a role.

This issue can partially overcome by optimization of the experimental conditions such is for example the separation gradient. Otherwise a combination of peptide pools created via different enzymes should ensure the reliability and help to elucidate the primary structures involved in binding.

7. Conclusion

Both approaches to linear epitope mapping can be, to a certain extent, considered successful. Out of the two experimental designs, the approach B is definitely the more promising one. It is quite fast, requires low amounts of materials and the experimental conditions are flexible. There are still steps that require optimization in order to establish a robust method.

The method might be mainly useful for initial, rough epitope mapping of antigen molecules. Upon adjustment of experimental conditions, the approach B can be applied to any antibody-antigen systems.

8. Publication bibliography

Aguilar, Marie-Isabel (2000): Reversed-Phase High-Performance Liquid Chromatography in Peptide and Protein Analysis. In Robert A. Meyers (Ed.): Encyclopedia of analytical chemistry. Applications, theory and instrumentation / edited by R.A. Meyers. Chichester: John Wiley.

Alberts, Bruce (2002): Molecular biology of the cell. 4th ed / Bruce Alberts [et al.]. New York: Garland Science.

Aybay, Cemalettin (2003): Differential binding characteristics of protein G and protein A for Fc fragments of papain-digested mouse IgG. In *Immunology Letters* 85 (3), pp. 231–235. DOI: 10.1016/S0165-2478(02)00262-6.

Beck, Alain; Wurch, Thierry; Bailly, Christian; Corvaia, Nathalie (2010): Strategies and challenges for the next generation of therapeutic antibodies. In *Nature reviews. Immunology* 10 (5), pp. 345–352. DOI: 10.1038/nri2747.

Berg, Jeremy M.; Tymoczko, John L.; Stryer, Lubert (2001): Biochemistry. 5th ed. New York: W. H. Freeman and CO.

Bergen, M. von; Friedhoff, P.; Biernat, J.; Heberle, J.; Mandelkow, E.-M.; Mandelkow, E. (2000): Assembly of tau protein into Alzheimer paired helical filaments depends on a local sequence motif (306VQIVYK311) forming beta structure. In *Proceedings of the National Academy of Sciences* 97 (10), pp. 5129–5134. DOI: 10.1073/pnas.97.10.5129.

Boysen, R. I.; Hearn, M. T. (2001): HPLC of peptides and proteins: preparation and system set-up. In *Current protocols in molecular biology / edited by Frederick M. Ausubel … [et al.]* Chapter 10, pp. Unit 10.12. DOI: 10.1002/0471142727.mb1012s54.

Buée, Luc; Bussière, Thierry; Buée-Scherrer, Valérie; Delacourte, André; Hof, Patrick R. (2000): Tau protein isoforms, phosphorylation and role in neurodegenerative disorders11These authors contributed equally to this work. In *Brain Research Reviews* 33 (1), pp. 95–130. DOI: 10.1016/S0165-0173(00)00019-9.

Citron, Martin (2010): Alzheimer's disease: strategies for disease modification. In *Nature reviews. Drug discovery* 9 (5), pp. 387–398. DOI: 10.1038/nrd2896.

Ekici, Ozlem Doğan; Paetzel, Mark; Dalbey, Ross E. (2008): Unconventional serine proteases: variations on the catalytic Ser/His/Asp triad configuration. In *Protein science : a publication of the Protein Society* 17 (12), pp. 2023–2037. DOI: 10.1110/ps.035436.108.

Elie, Auréliane; Prezel, Elea; Guérin, Christophe; Denarier, Eric; Ramirez-Rios, Sacnicte; Serre, Laurence et al. (2015): Tau co-organizes dynamic microtubule and actin networks. In *Scientific reports* 5, p. 9964. DOI: 10.1038/srep09964.

Finehout, Erin J.; Lee, Kelvin H. (2004): An introduction to mass spectrometry applications in biological research. In *Biochemistry and molecular biology education : a bimonthly publication of the International Union of Biochemistry and Molecular Biology* 32 (2), pp. 93–100. DOI: 10.1002/bmb.2004.494032020331.

Fukasawa, Kayoko M.; Hata, Toshiyuki; Ono, Yukio; Hirose, Junzo (2011): Metal preferences of zinc-binding motif on metalloproteases. In *Journal of amino acids* 2011, p. 574816. DOI: 10.4061/2011/574816.

Garcia-Carreon, Fernando Luis (1997): Classification of Proteases without tears. In *Biochemical Education* 25 (3), pp. 161–167. DOI: 10.1016/S0307-4412(97)00005-8.

Janeway, Charles A. (2001): Immunobiology 5. The immune system in health and disease / Charles A. Janeway … [et al.]. 5th ed. New York: Garland; Edinburgh : Churchill Livingstone.

Kolarova, Michala; García-Sierra, Francisco; Bartos, Ales; Ricny, Jan; Ripova, Daniela (2012): Structure and pathology of tau protein in Alzheimer disease. In *International journal of Alzheimer's disease* 2012, p. 731526. DOI: 10.1155/2012/731526.

Kolsrud, Hanne; Malerod, Helle; Ray, Steven; Reubsaet, Leon; Lundanes, Elsa; Greibrokk, Tyge (2012): A Critical Review of Trypsin Digestion for LC-MS Based Proteomics. In Min Jia, Kah Wai Lin, Serhiy Souchelnytskyi (Eds.): Phosphoproteomics: Detection, Identification and Importance of Protein Phosphorylation: INTECH Open Access Publisher.

Lu, Xiaojun; DeFelippis, Michael R.; Huang, Lihua (2009): Linear epitope mapping by native mass spectrometry. In *Analytical biochemistry* 395 (1), pp. 100–107. DOI: 10.1016/j.ab.2009.08.018.

Luna-Munoz, Jose; R., Charles; M., Claude; Flores-Rodriguez, Paola; Avila, Jesus; R., Sergio et al. (2013): Phosphorylation of Tau Protein Associated as a Protective Mechanism in the Presence of Toxic, C-Terminally Truncated Tau in Alzheimer's Disease. In Armand Perret-

Liaudet, Aline Dorey, Benoit Dumont, Isabelle Quadrio, Yannick Tholance (Eds.): Pre-Analytical and Analytical Critical Factors Influencing the High Variability of the Concentrations Levels of Alzheimer Disease Biomarkers in Cerebral Spinal Fluid: INTECH Open Access Publisher.

Maleknia, Simin D.; Johnson, Richard (2009-): Mass Spectrometry of Amino Acids and Proteins. In Andrew B. Hughes (Ed.): Amino acids, peptides and proteins in organic chemistry. Weinheim: Wiley-VCH, pp. 1–50.

Mann, M.; Hendrickson, R. C.; Pandey, A. (2001): Analysis of proteins and proteomes by mass spectrometry. In *Annual review of biochemistry* 70, pp. 437–473. DOI: 10.1146/annurev.biochem.70.1.437.

Oliva, Alexis; Llabres, Matias; Farina, Jose (2004): Applications of Multi-Angle Laser Light-Scattering Detection in the Analysis of Peptides and Proteins. In *CDDT* 1 (3), pp. 229–242. DOI: 10.2174/1570163043334938.

Price, Nicholas C.; Nairn, Jacqueline (2009): Exploring proteins. A student's guide to experimental skills and methods / Nicholas C. Price and Jacqueline Nairn. Oxford: Oxford University Press.

Rawlings, Neil D.; Barrett, Alan J. (2009): Peptidases. In Katherine E. Cullen (Ed.): Encyclopedia of life science. New York: Facts On File (Facts on File science library).

Rosenmann, Hanna; Meiner, Zeev; Geylis, Valeria; Abramsky, Oded; Steinitz, Michael (2006): Detection of circulating antibodies against tau protein in its unphosphorylated and in its neurofibrillary tangles-related phosphorylated state in Alzheimer's disease and healthy subjects. In *Neuroscience letters* 410 (2), pp. 90–93. DOI: 10.1016/j.neulet.2006.01.072.

Salazar, Carlos; Höfer, Thomas (2009): Multisite protein phosphorylation--from molecular mechanisms to kinetic models. In *The FEBS journal* 276 (12), pp. 3177–3198. DOI: 10.1111/j.1742-4658.2009.07027.x.

Schmid, Franz-Xaver (2005): Biological Macromolecules: UV-visible Spectrophotometry. In : Encyclopedia of life sciences. London, New York: John Wiley & Sons.

Sobott, Frank; Robinson, Carol V. (2004): Characterising electrosprayed biomolecules using tandem-MS—the noncovalent GroEL chaperonin assembly. In *International Journal of Mass Spectrometry* 236 (1-3), pp. 25–32. DOI: 10.1016/j.ijms.2004.05.010.

Tenreiro, Sandra; Eckermann, Katrin; Outeiro, Tiago F. (2014): Protein phosphorylation in neurodegeneration: friend or foe? In *Frontiers in molecular neuroscience* 7, p. 42. DOI: 10.3389/fnmol.2014.00042.

Turk, Boris; Turk, Dušan; Turk, Vito (2012): Protease signalling: the cutting edge. In *The EMBO journal* 31 (7), pp. 1630–1643. DOI: 10.1038/emboj.2012.42.

Ubhi, Kiren; Masliah, Eliezer (2011): Recent advances in the development of immunotherapies for tauopathies. In *Experimental neurology* 230 (2), pp. 157–161. DOI: 10.1016/j.expneurol.2010.10.007.

Wang, Wei; Singh, Satish; Zeng, David L.; King, Kevin; Nema, Sandeep (2007): Antibody structure, instability, and formulation. In *Journal of pharmaceutical sciences* 96 (1), pp. 1–26. DOI: 10.1002/jps.20727.

Wysocki, Vicki H.; Resing, Katheryn A.; Zhang, Qingfen; Cheng, Guilong (2005): Mass spectrometry of peptides and proteins. In *Methods (San Diego, Calif.)* 35 (3), pp. 211–222. DOI: 10.1016/j.ymeth.2004.08.013.

9. Appendix

9.1 SDS PAGE standard Mw ladder
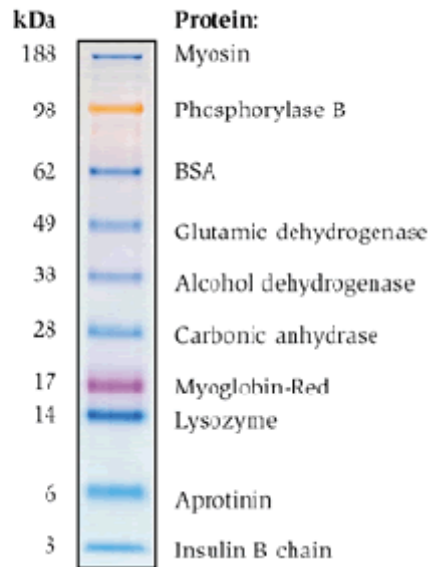
Figure 1: Molecular weights of SeeBlue Plus2 Prestained Standard on NuPAGE 4-12% BIS-TRIS gel in combination with MES running buffer

9.2 rTau sequence

Histag-rTau441-ctag F8W (transformation, E.coli):

MAHHHHHHDDDDKMAEPRQEWEVMEDHAGTYGLGDRKDQGGYTMHQDQE
GDTDAGLKESPLQTPTEDGSEEPGSETSDAKSTPTAEDVTAPLVDEGAPGKQAA
AQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSKSKDGTGSDDKKAK
GADGKTKIATPRGAAPPGQKGQANATRIPAKTPPAPKTPPSSGEPPKSGDRSGY
SSPGSPGTPGSRSRTPSLPTPPTREPKKVAVVRTPPKSPSSAKSRLQTAPVPMPDL
KNVKSKIGSTENLKHQPGGGKVQIINKKLDLSNVQSKCGSKDNIKHVPGGGSV
QIVYKPVDLSKVTSKCGSLGNIHHKPGGGQVEVKSEKLDFKDRVQSKIGSLDNI
THVPGGGNKKIETHKLTFRENAKAKTDHGAEIVYKSPVVSGDTSPRHLSNVSST
GSIDMVDSPQLATLADEVSASLAKQGLGYQDYEPEA

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Histag-rTau441 (transformation, E.coli):

MAHHHHHHDDDDKMAEPRQEFEVMEDHAGTYGLGDRKDQGGYTMHQDQE
GDTDAGLKESPLQTPTEDGSEEPGSETSDAKSTPTAEDVTAPLVDEGAPGKQAA
AQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSKSKDGTGSDDKKAK
GADGKTKIATPRGAAPPGQKGQANATRIPAKTPPAPKTPPSSGEPPKSGDRSGY
SSPGSPGTPGSRSRTPSLPTPPTREPKKVAVVRTPPKSPSSAKSRLQTAPVPMPDL
KNVKSKIGSTENLKHQPGGGKVQIINKKLDLSNVQSKCGSKDNIKHVPGGGSV
QIVYKPVDLSKVTSKCGSLGNIHHKPGGGQVEVKSEKLDFKDRVQSKIGSLDNI
THVPGGGNKKIETHKLTFRENAKAKTDHGAEIVYKSPVVSGDTSPRHLSNVSST
GSIDMVDSPQLATLADEVSASLAKQGL

9.3 CLC theoretical fragmentation with endoproteinases

9.3.1 Endoproteinase Lys-C
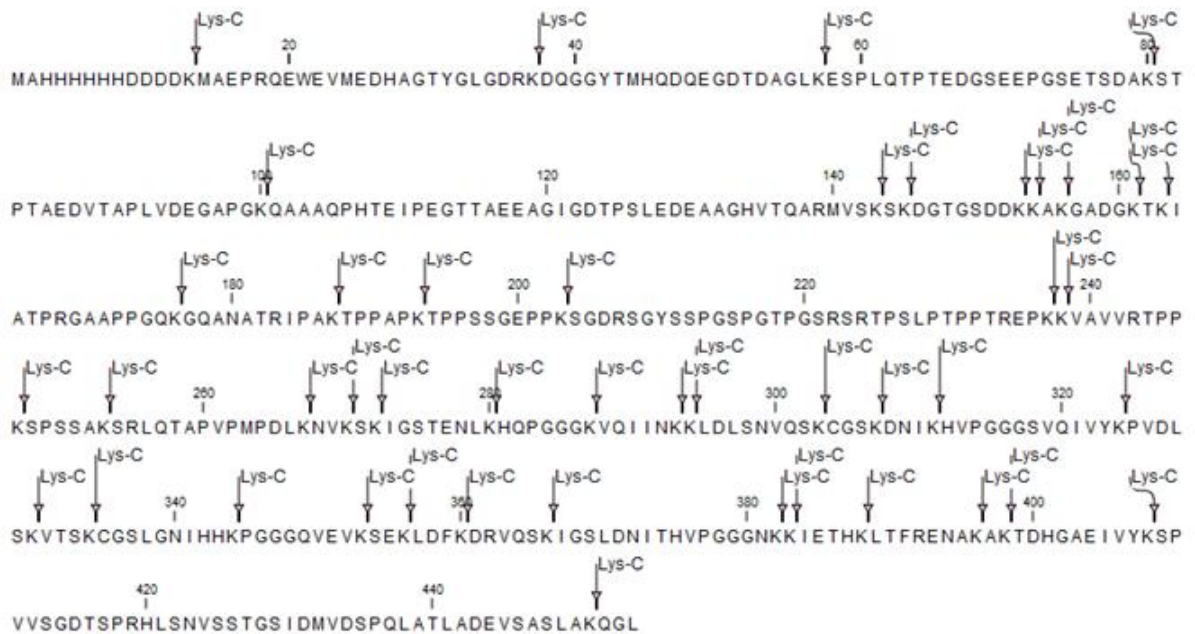
Figure 2: Theoretical fragmentation of rTau with endopeptidase Lys-C. The list is generated with CLC software.

Table 1: Overview of the rTau peptides generated with Lys-C

AA Start	AA End	Mass	pI	Fragment
1	13	1 631.7	6.2	MAHHHHHHDDDDK
14	37	2 767.0	4.9	MAEPRQEFEVMEDHAGTYGLGDRK
38	57	2 166.2	4.3	DQGGYTMHQDQEGDTDAGLK
58	80	2 391.4	3.8	ESPLQTPTEDGSEEPGSETSDAK
81	100	1 955.1	4.1	STPTAEDVTAPLVDEGAPGK
101	143	4 402.7	4.6	QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGH VTQARMVSK
144	145	233.3	9.8	SK
146	153	793.7	4.2	DGTGSDDK
155	156	217.3	10.1	AK
157	161	446.5	7.1	GADGK
162	163	247.3	9.8	TK
164	176	1 263.5	11.3	IATPRGAAPPGQK
177	187	1 126.3	11.3	GQANATRIPAK
188	193	609.7	9.8	TPPAPK
194	203	996.1	6.8	TPPSSGEPPK
204	237	3 454.7	10.9	SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPK
239	247	966.2	11.3	VAVVRTPPK
248	253	575.6	9.8	SPSSAK
254	267	1 552.8	9.8	SRLQTAPVPMPDLK
268	270	359.4	10.1	NVK
271	272	233.3	9.8	SK
273	280	861.0	7.1	IGSTENLK
281	287	679.7	9.8	HQPGGGK
288	293	713.9	10.1	VQIINK
295	303	1 003.1	7.1	LDLSNVQSK
304	307	393.5	9.6	CGSK
308	311	488.5	6.7	DNIK
312	324	1 340.5	9.4	HVPGGGSVQIVYK
325	330	657.8	7.3	PVDLSK
331	334	433.5	10.1	VTSK
335	344	1 065.2	9.6	CGSLGNIHHK
345	353	870.0	7.4	PGGGQVEVK

354	356	362.4	6.9	SEK
357	360	521.6	7.1	LDFK
361	366	731.8	9.6	DRVQSK
367	382	1 578.7	7.9	IGSLDNITHVPGGGNK
384	388	626.7	7.9	IETHK
389	396	978.1	10.1	LTFRENAK
397	398	217.3	10.1	AK
399	408	1 132.2	5.6	TDHGAEIVYK
409	451	4 327.7	4.7	SPVVSGDTSPRHLSNVSSTGSIDMVDSPQLATLA DEVSASLAK
452	454	316.4	5.7	QGL

9.3.2 Endoproteinase Asp-N
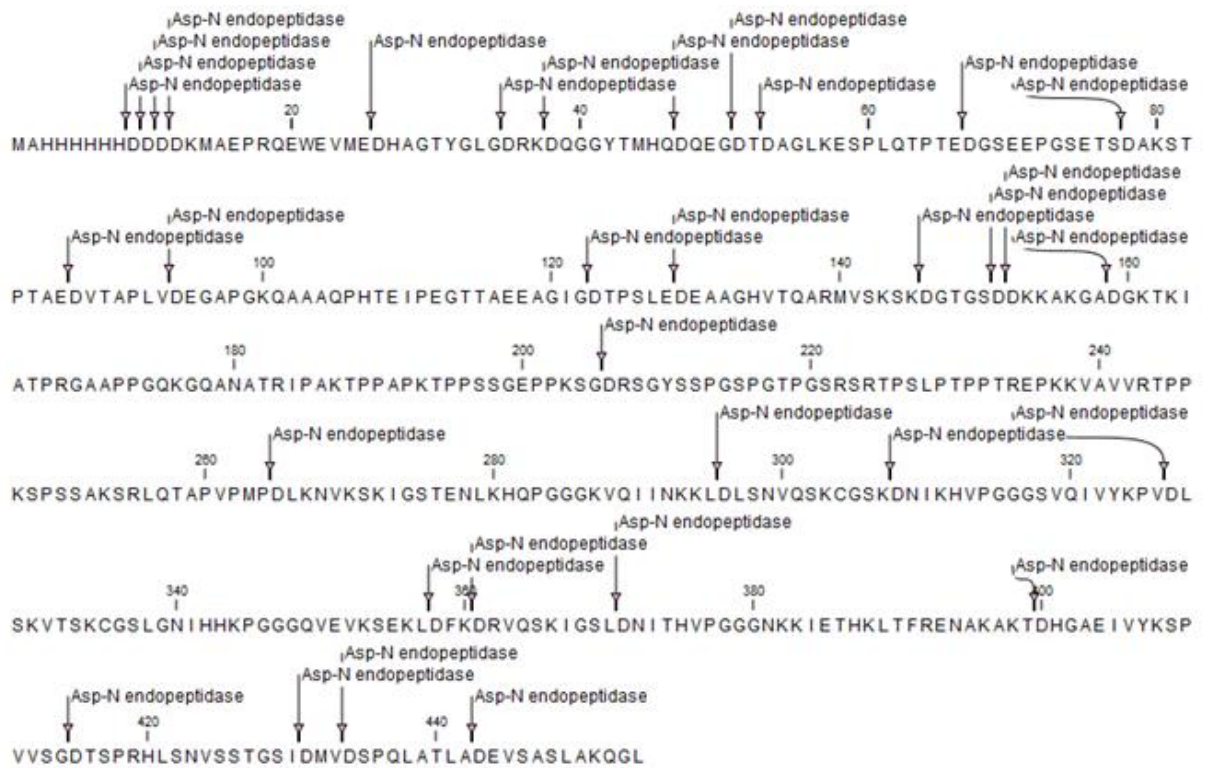
Figure 3: Theoretical fragmentation of rTau with endopeptidase Asp-N. The list was generated with CLC software.

Table 2: Overview of the rTau peptides generated with Asp-N

AA Start	AA End	Mass	pI	Fragment
1	8	1 043.1	8.1	MAHHHHHH
12	25	1 738.9	4.4	DKMAEPRQEFEVME
26	34	889.9	5.4	DHAGTYGLG
35	37	417.5	9.6	DRK
38	46	1 036.1	5.4	DQGGYTMHQ
47	50	447.4	3.3	DQEG
51	52	234.2	3.3	DT
53	66	1 485.6	4.3	DAGLKESPLQTPTE
67	77	1 094.0	3.1	DGSEEPGSETS
78	86	919.0	4,6	DAKSTPTAE
87	93	713.8	3.4	DVTAPLV
94	122	2 832.9	4.3	DEGAPGKQAAAQPHTEIPEGTTAEEAGIG
123	128	660.7	3.2	DTPSLE
129	145	1 815.0	9.4	DEAAGHVTQARMVSKSK
146	150	435.4	3.4	DGTGS
152	158	716.8	10.1	DKKAKGA
159	205	4 615.2	11.0	DGKTKIATPRGAAPPGQKGQANATRIPAKT PPAPKTPPSSGEPPKSG
206	264	6 122.9	11.8	DRSGYSSPGSPGTPGSRSRTPSLPTPPTREPK KVAVVRTPPKSPSSAKSRLQTAPVPMP
265	295	3 372.9	10.5	DLKNVKSKIGSTENLKHQPGGGKVQIINKKL
296	307	1 265.4	9.0	DLSNVQSKCGSK
308	326	2 007.3	9.1	DNIKHVPGGGSVQIVYKPV
327	357	3 233.7	9.5	DLSKVTSKCGSLGNIHHKPGGGQVEVKSEKL
358	360	408.5	6.7	DFK
361	370	1 102.2	9.6	DRVQSKIGSL
371	399	3 205.6	10.2	DNITHVPGGGNKKIETHKLTFRENAKAKT
400	414	1 557.7	5.6	DHGAEIVYKSPVVSG
415	430	1 657.7	7.5	DTSPRHLSNVSSTGSI
431	433	363.4	3.4	DMV
434	442	915.0	3.4	DSPQLATLA
443	454	1 217.3	4.6	DEVSASLAKQGL

9.4 Unsuccessful deactivation of Lys-C with HCl

9.4.1 Experimental setup

Buffer Exchange/Sample Concentration:

CB 28.1 and the rTau were concentrated to 4.25 mg/ml and 3.0 mg/ml respectively, using the amicon ultra centrifugal filters (30K and 10K respectively). Additionally, rTau was transferred from the NaPi buffer (pH = 6.0) into PBS (pH = 7.4) to achieve optimal pH for Lys-C enzymatic activity (pH = 7-9) and therefore to ensure a complete digestion. (UV-vis slope spectroscopy was used to determine the final concentrations of the solutions after the filtration procedure).

Solution Preparation:

The Lys-C stock solution:

Lys-C stock solution (0.25 mg/ml) was prepared by diluting 20 ug of the enzyme in 80 uL of 20 mM acetic acid.

The rTau protein digestion:

Reaction mixture of molar ratio 1Lys-C:80rTau was prepared by addition of 6.6 uL of Lys C (0.25 mg/mL) to 70 uL Tau (3.0 mg/mL).

The reaction proceeded overnight (~ 15 hrs) at 37˚C. To terminate the digestion, 3 uL of 25% HCl were added to the reaction mixture (low pH inhibits the enzymatic activity).

Sample preparation for SEC MALS:

The amounts of samples used for binding experiments were calculated according to 1rTau:1CB 28.1 molar ratio.

a) 40.8 uL of rTau/Lys-C Digest + 89.2 uL of hCB tau 28.1

b) 40.8 uL of rTau/Lys-C Digest + 89.2 uL of PBS

c) 89.2 uL of hCB tau 28.1 + 40.8 uL of PBS

100 uL of each mixture was injected to the SEC MALS system (corresponding to ~ 94 ug of rTau protein).

9.4.2 SEC-MALS results
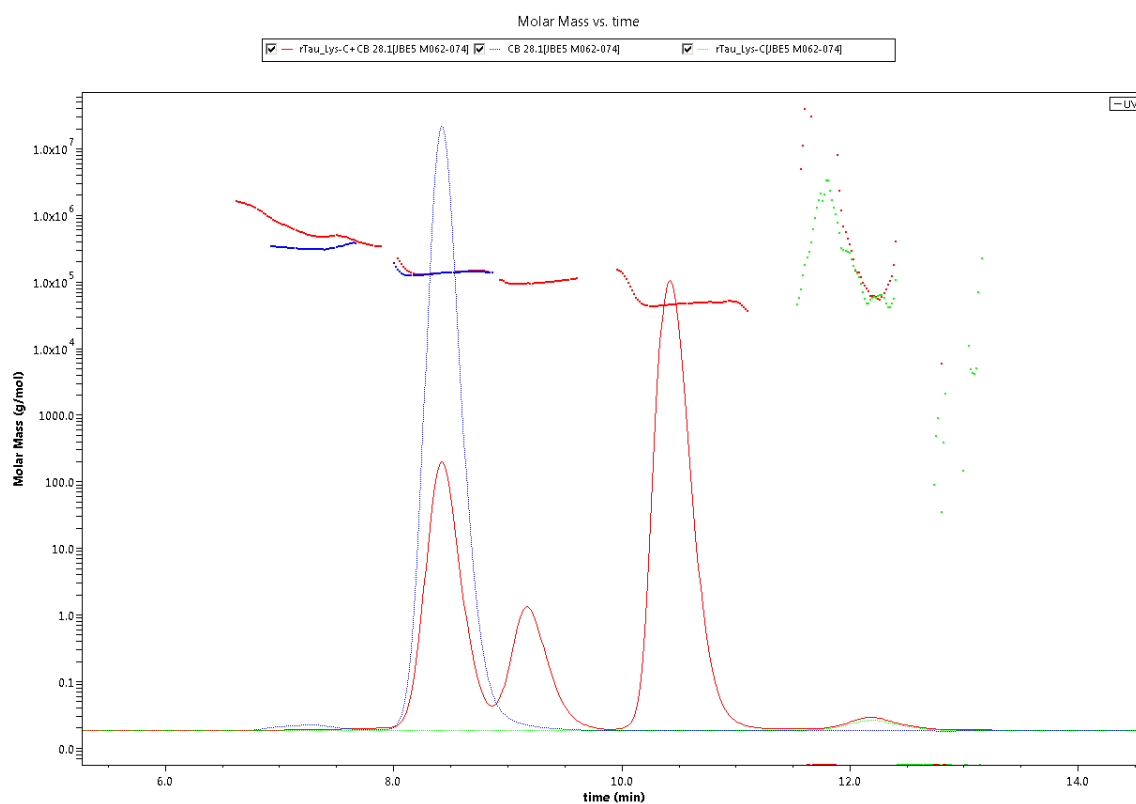
Figure 4: Graph showing the SEC-MALS results. UV absorbance trace measured at 280 nm; rTau/Lys-C Digest + hCB tau 28.1 (red trace), rTau/Lys-C Digest (green trace), hCB tau 28.1 (blue trace). Distributions of the molecular masses of the analyzed samples are represented by dotted lines.

Table 3: Overview of the SEC-MALS results

SAMPLE	Elution Time [min]				
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
rTau/Lys-C Digest + hCB tau 28.1	6.9-7.7	8.0-8.9	8.9-9.6	10.0-11.1	11.6-12.8
hCB tau 28.1	6.9- 7.7	8.0-8.9	-	-	-

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SAMPLE	Molecular Weight [kDa]*				
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
rTau/Lys-C Digest	11.5-12.9	-	-	-	-
rTau/Lys-C Digest + hCB tau 28.1	381.0	135.7	93.5	45.4	58.3
hCB tau 28.1	305.1	134.6	-	-	-
rTau/Lys-C Digest	60.6	-	-	-	-

*dRI concentration source was used for determination of molecular masses. The assigned molecular weights represent the detected mass at the apex of the peak (m_p). The measured masses are only approximate, the calibration of the instrument is required.

The elution profile and calculated masses for individual peaks obtained via SEC-MALS suggest that the Lys-C was not completely deactivated after the digestion and most probably partially cleaved the antibody molecule.

9.5 Approach A - complete PLGS output

The lists below show all the peptides detected during the MS runs (the resulting spectra were analyzed in PLGS). Fractions which are not shown did not contain peptides (10A, 11A, 12B).

Fraction A10

```
  z    MH+(Da)   Error (ppm)  Score   Start  End    Sequence                                RT(min)  Intensity  Type              Products
1.0   1954.9604   24.3697    4.3478   81    100    (K)STPTAEDVTAPLVDEGAPGK(Q)              9.0284    824.0     Pass Two Match    4
2.33  2391.0317    2.8761    4.8776   58    80     (K)ESPLQTPTEDGSEEPGSETSDAK(S)          44.9194   6538.0    Pass One Match    3
3.0   2329.2986   -3.8186    0.0      273   294    (K)IGSTENLKHQPGGGKVQIINKK(L)           10.459    897.0     Neut Loss (NH3)   0
3.0   3121.639     3.7382    0.0      354   382    (K)SEKLDFKDRVQSKIGSLDNITHVPGGGNK(K)    8.2067    3373.0    Neut Loss (H2O)   1
1.0   1561.8333    3.6206    0.0      354   366    (K)SEKLDFKDRVQSK(I)                    7.7935    2026.0    Neut Loss (H2O)   1
1.0   1579.8438   -6.3015    4.1872   354   366    (K)SEKLDFKDRVQSK(I)                    7.2107    2239.0    Missed Cleavage   3
2.63  3139.6494   -7.6545    4.0681   354   382    (K)SEKLDFKDRVQSKIGSLDNITHVPGGGNK(K)    7.9191    6230.0    Missed Cleavage   2
2.0   2346.3252    7.6046    4.6706   273   294    (K)IGSTENLKHQPGGGKVQIINKK(L)           10.5526   4691.0    Missed Cleavage   5
```

Fraction A12

```
  z    MH+ (Da)  Error (ppm)  Score   Start End  Sequence                                               RT(min)  Intensity   Peptide Type    Products
4.76  4401.09     3.3772     8.3335   101   143  (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)     56.8174  146579.0   Pass One Match  58
2.38  2391.0317   2.2909     7.8503   58    80   (K)ESPLQTPTEDGSEEPGSETSDAK(S)                         44.7601  21651.0    Pass One Match  21
4.08  4384.0635   2.6802     0.0      101   143  (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)     57.5131  100467.0   Neut Loss (NH3) 43
```

Fraction A14

```
z      MH+(Da)  Error(ppm) Score Start End      Sequence                                       RT (min) Intensity Type              Products
1.91   1954.9604  4.9195   8.4874  81   100  (K)STPTAEDVTAPLVDEGAPGK(Q)                        53.5251 200588.0 Pass Two Match    42
1.67   1263.7168  4.5895   7.0326 164   176  (K)IATPRGAAPPGQK(G)                               37.8798  23693.0 Pass Two Match     6
1.0    1126.6327  6.0382   6.5467 177   187  (K)GQANATRIPAK(T)                                 33.2216   3000.0 Pass Two Match     6
1.0     861.4676  9.0068   7.328  273   280  (K)IGSTENLK(H)                                    34.7928  34654.0 Pass Two Match     6
2.19   1980.0913  4.5626   7.9564 312   330  (K)HVPGGGSVQIVYKPVDLSK(V)                         54.3825  59886.0 Pass Two Match    20
2.0    2391.0317 -4.7116   7.0932  58    80  (K)ESPLQTPTEDGSEEPGSETSDAK(S)                     44.4404  10742.0 Pass Two Match    10
3.82   4401.09    2.4356   7.5973 101   143  (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S) 56.3694  21954.0 Pass One Match    18
3.69   3453.7104  1.2305   7.6516 204   237  (K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPK(K)          46.6122  49173.0 Pass One Match    18
1.0     966.6095  3.42     7.521  239   247  (K)VAVVRTPPK(S)                                   37.3057  17780.0 Pass One Match    10
1.88   1552.8516  1.9365   8.5448 254   267  (K)SRLQTAPVPMPDLK(N)                              55.7718 235465.0 Pass One Match    26
1.0     714.4509  0.634    7.8995 288   293  (K)VQIINK(K)                                      38.1871  46593.0 Pass One Match     9
1.89   1578.8234  3.1835   8.1379 367   382  (K)IGSLDNITHVPGGGNK(K)                            50.4473 230542.0 Pass One Match    30
1.0    1132.5634 -2.9985   7.6306 399   408  (K)TDHGAEIVYK(S)                                  38.8835  13494.0 Pass One Match     9
2.0    2165.904   3.3138   7.8121  38    57  (K)DQGGYTMHQDQEGDTDAGLK(E)                        41.9039  22945.0 Pass One Match    20
3.85   4384.0635  3.5504   0.0    101   143  (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S) 57.3377  32910.0 Neut.Loss (NH3)9
2.0    1560.8129 -2.0642   0.0    367   382  (K)IGSLDNITHVPGGGNK(K)                            50.4207   4448.0 Neut.Loss (H2O)0
2.0    1245.7062  0.7434   0.0    164   176  (K)IATPRGAAPPGQK(G)                               37.8581   1304.0 Neut.Loss (H2O)0
1.12   1131.6368 -1.6555   7.9565 294   303  (K)KLDLSNVQSK(C)                                  42.4806  34706.0 Missed Clvg.      16
3.95   3581.8054  3.8708   7.0383 204   238  (K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKK(V)         45.1417  10599.0 Missed Clvg.       5
```

Fraction B14

```
z    MH+(Da)   Error(ppm)/Score/Start/End      Sequence                                            RT(min)    Intensity Peptide Type/Product
2.63 1552.8516 6.7518 8.8768  254 267  (K)SRLQTAPVPMPDLK(N)                                        53.8467   6368789.0 Pass Two Match  36
1.0  1132.5634-7.9279 6.4718  399 408  (K)TDHGAEIVYK(S)                                             8.6602     20393.0 Pass Two Match   4
2.95 2165.904  4.5003 7.0305   38  57  (K)DQGGYTMHQDQEGDTDAGLK(E)                                  41.9884     13370.0 Pass Two Match   6
4.76 4401.09   3.6637 7.7443  101 143  (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)            56.6229     28391.0 Pass One Match  23
2.7  1263.7168 2.8293 7.631   164 176  (K)IATPRGAAPPGQK(G)                                         36.6685     55169.0 Pass One Match  11
1.0   610.3559 0.0204 8.0486  188 193  (K)TPPAPK(T)                                                 7.9516      4671.0 Pass One Match   5
5.23 3453.7104 2.6645 7.5355  204 237  (K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPK(K)                     46.1396     19790.0 Pass One Match   7
2.64  966.6095 3.0364 7.9799  239 247  (K)VAVVRTPPK(S)                                             35.6612     80838.0 Pass One Match  13
1.72  714.4509 1.5299 7.8755  288 293  (K)VQIINK(K)                                                35.6872     18023.0 Pass One Match   6
3.29 1980.0913 3.8551 8.183   312 330  (K)HVPGGGSVQIVYKPVDLSK(V)                                   54.8431    255090.0 Pass One Match  23
2.0  1578.8234 2.2025 6.5409  367 382  (K)IGSLDNITHVPGGGNK(K)                                      49.1046     28447.0 Pass One Match   3
2.51  978.5367 3.1284 6.6822  389 396  (K)LTFRENAK(A)                                              36.5317     11732.0 Pass One Match   2
3.98 4326.1406 3.6128 8.3519  409 451  (K)SPVVSGDTSPRHLSNVSSTGSIDMVDSPQLATLADEVSASLAK(Q)           78.2705    523278.0 Pass One Match  77
2.23 1954.9604 1.5157 8.5957   81 100  (K)STPTAEDVTAPLVDEGAPGK(Q)                                  53.6107    101581.0 Pass One Match  31
3.0  1718.9548 -9.231 6.0695  177 193  (K)GQANATRIPAKTPPAPK(T) DAM N(4), N-Gl(4)                   83.1139       959.0 PTM             4
3.0  3277.7175 8.9407 6.4825  367 396  (K)IGSLDNITHVPGGGNKKIETHKLTFRENAK(A)DAM N(6),DAM N(15),DAM N(28),N-Gl(6) 81.9366   1034.0 PTM   4
3.0  1126.6327 8.7894 6.3092  177 187  (K)GQANATRIPAK(T)         N-Glycosylation (4)               79.964      1050.0 PTM             6
3.0  1561.7969 3.0383 0.0     367 382  (K)IGSLDNITHVPGGGNK(K)                                      51.339       1105.0 Neut.Loss (NH3) 0
4.11 4384.0635 3.6413 0.0     101 143  (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)            57.293      15016.0 Neut.Loss (NH3) 13
3.0  1534.841  2.2333 0.0     254 267  (K)SRLQTAPVPMPDLK(N)                                        53.9068      3234.0 Neut.Loss (H2O) 2
2.0  1113.6262 3.1038 0.0     294 303  (K)KLDLSNVQSK(C)                                            45.6391      4130.0 Neut.Loss (H2O) 1
4.0  1962.0807 3.6814 0.0     312 330  (K)HVPGGGSVQIVYKPVDLSK(V)                                   54.0401     17421.0 Neut.Loss (H2O) 40
3.0  1560.8129 3.9564 0.0     367 382  (K)IGSLDNITHVPGGGNK(K)                                      47.0769     10820.0 Neut.Loss (H2O) 19
3.0  1245.7062 2.01   0.0     164 176  (K)IATPRGAAPPGQK(G)                                         36.649       1296.0 Neut.Loss (H2O) 0
5.8  3581.8054 7.6835 6.9426  204 238  (K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKK(V)                    45.2921     28599.0 Missed Clvg.    8
2.41 1131.6368 2.5575 7.0518  294 303  (K)KLDLSNVQSK(C)                                            41.2296     32499.0 Missed Clvg.    8
3.0  3139.6494 2.8421 6.476   354 382  (K)SEKLDFKDRVQSKIGSLDNITHVPGGGNK(K)                         8.2237      3931.0 Missed Clvg.    4
3.27 1706.9185 5.102  7.8956  367 383  (K)IGSLDNITHVPGGGNKK(I)                                     46.4164    153805.0 Missed Clvg.    11
3.0  4326.974  8.0964 6.5382   58 100  (K)ESPLQTPTEDGSEEPGSETSDAKSTPTAEDVTAPLVDEGAPGK(Q)           10.5798      4033.0 Missed Clvg.    3
```

Lys-C/rTau Digest – Control Non-Incubated

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z    MH+(Da)   Error(ppm)/Score/Start/End      Sequence                                            RT(min) Intensity  Peptide Type   Products
2.31 1263.7168 1.0854 6.879   164 176  (K)IATPRGAAPPGQK(G)                                         5.082     10110.0 Pass Two Match   3
5.24 3453.7104 5.0778 7.4589  204 237  (K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPK(K)                    47.9864    34997.0 Pass Two Match  12
1.76 1132.5634 4.1754 6.8786  399 408  (K)TDHGAEIVYK(S)                                            5.118      4550.0 Pass Two Match   4
3.99 4326.1406 4.4386 8.8263  409 451  (K)SPVVSGDTSPRHLSNVSSTGSIDMVDSPQLATLADEVSASLAK(Q)           80.3235 3501308.0 Pass Two Match 109
2.74 2391.0317 4.3757 7.4615   58  80  (K)ESPLQTPTEDGSEEPGSETSDAK(S)                               44.869     14195.0 Pass One Match   6
2.34 1954.9604 1.7578 8.8962   81 100  (K)STPTAEDVTAPLVDEGAPGK(Q)                                  54.1792   135586.0 Pass One Match  31
4.43 4401.09  -1.6737 7.2069  101 143  (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)           56.5268    11179.0 Pass One Match   3
2.36 1126.6327 3.3629 6.736   177 187  (K)GQANATRIPAK(T)                                           5.0801      8711.0 Pass One Match   4
1.21  610.3559 2.1542 7.6427  188 193  (K)TPPAPK(T)                                                5.0687      8128.0 Pass One Match   6
2.17  966.6095 3.2297 6.907   239 247  (K)VAVVRTPPK(S)                                             5.086      16093.0 Pass One Match   6
2.46 1552.8516 3.2854 7.4884  254 267  (K)SRLQTAPVPMPDLK(N)                                        5.3768     19664.0 Pass One Match   8
1.32  714.4509 1.3552 7.9938  288 293  (K)VQIINK(K)                                                5.1287     10223.0 Pass One Match   8
3.56 1980.0913 3.8738 7.5841  312 330  (K)HVPGGGSVQIVYKPVDLSK(V)                                   56.0201    36004.0 Pass One Match   9
2.88 1578.8234 3.6405 7.9734  367 382  (K)IGSLDNITHVPGGGNK(K)                                      51.6862    80258.0 Pass One Match  15
2.23  978.5367 2.2557 6.9989  389 396  (K)LTFRENAK(A)                                              5.1134      5634.0 Pass One Match   3
2.97 2165.904  3.799  7.0908   38  57  (K)DQGGYTMHQDQEGDTDAGLK(E)                                  42.4383    19379.0 Pass One Match   9
4.09 4384.0635 3.7282 0.0     101 143  (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)           58.0421    84408.0 Neut. Loss (NH3)22
5.8  3581.8054 3.2781 6.8304  204 238  (K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKK(V)                   46.3018    13053.0 Missed Cleavage  3
1.97 1131.6368 6.8671 6.4745  294 303  (K)KLDLSNVQSK(C)                                            5.1295      7792.0 Missed Cleavage  5
1.0   755.441 -0.9216 6.636   383 388  (K)KIETHK(L)                                                5.0829      1303.0 Missed Cleavage  4
3.0  5519.6104 2.5869 6.4635  101 154  (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSKSKDGTGSDDKK(A) 5.1099    1189.0 Missed Cleavage  4
```

Lys-C/rTau Digest – Control Incubated at 60°C

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z    MH+(Da)   Error(ppm)/Score/Start/End Sequence                                            RT (min)Intensity Peptide Type    Products
5.05 3453.7104 5.4338 6.6437  204 237  (K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPK(K)                    10.9627   74903.0 Pass Two Match  17
2.0  1954.9604 2.1807 5.3686   81 100  (K)STPTAEDVTAPLVDEGAPGK(Q)                                  4.9306     1087.0 Pass One Match   3
4.91 4401.09   3.765  5.8173  101 143  (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)           57.8039    9757.0 Pass One Match   4
2.09  966.6095 2.2486 6.1583  239 247  (K)VAVVRTPPK(S)                                             4.8993     4963.0 Pass One Match   7
2.28 1552.8516 1.1678 5.8334  254 267  (K)SRLQTAPVPMPDLK(N)                                        4.9106     2828.0 Pass One Match   6
2.28 1578.8234 1.098  6.1885  367 382  (K)IGSLDNITHVPGGGNK(K)                                      4.9219     2205.0 Pass One Match   5
4.0  4326.1406 3.3416 6.2482  409 451  (K)SPVVSGDTSPRHLSNVSSTGSIDMVDSPQLATLADEVSASLAK(Q)           81.1491   42396.0 Pass One Match  15
2.37 2391.0317 1.379  5.4553   58  80  (K)ESPLQTPTEDGSEEPGSETSDAK(S)                               10.5684    3318.0 Pass One Match   3
4.06 4384.0635 -0.7438 0.0    101 143  (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)           58.1089    4328.0 Neut. Loss(NH3)  1
5.52 3581.8054 7.6662 6.2145  204 238  (K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKK(V)                   11.1402   63784.0 Missed Cleavage 10
```

9.6 Approach B/Proof of concept experiment

9.6.1 Theoretical ionization of peptides

List of ionized species of relevant peptides was generated with MasLynx software.

A7731 (CBTau 28.1)

Frag#	Res#	Sequence	Theor (Bo)	[M+H]	[M+2H]	[M+3H]	[M+4H]	[M+5H]	[M+6H]	[M+7H]	[M+8H]	[M+9H]
▪M1	1-20	(-) TEDGSEEPGSETSDAKS TPT (-)	2363.00	2364.00	1182.51	788.67	591.76	473.61	394.84	338.58	296.38	263.56

V1089-023 (CBTau 24.1)

Frag#	Res#	Sequence	Theor (Bo)	[M+H]	[M+2H]	[M+3H]	[M+4H]	[M+5H]	[M+6H]	[M+7H]	[M+8H]	[M+9H]
▪M1	1-18	(-) VVRTPPKSPSJAKSRLQ T (-)	2357.23	2358.24	1179.62	786.75	590.32	472.45	393.88	337.75	295.66	262.92

A6907 (CBTau 24.1)

Frag#	Res#	Sequence	Theor (Bo)	[M+H]	[M+2H]	[M+3H]	[M+4H]	[M+5H]	[M+6H]	[M+7H]	[M+8H]	[M+9H]
▪M1	1-68	(-) GEPPKSGDRSGYSSPGS PGTPGSRSRTPSLPTPPTRE PKKVAVVRTPPKSPSSAKSR LQTAPVPMPDL (-)	7338.84	7339.84	3670.43	2447.29	1835.72	1468.77	1224.15	1049.41	918.36	816.43

B1014 (CBTau 28.1)

Frag#	Res#	Sequence	Theor (Bo)	[M+H]	[M+2H]	[M+3H]	[M+4H]	[M+5H]	[M+6H]	[M+7H]	[M+8H]	[M+9H]
▪M1	1-30	(-) LQTPTEDGSEEPGSETS DAKSTPTAEDVTA (-)	3388.50	3389.51	1695.26	1130.51	848.13	678.71	565.76	485.08	424.57	377.51

A6984 (NC)

Frag#	Res#	Sequence	Theor (Bo)	[M+H]	[M+2H]	[M+3H]	[M+4H]	[M+5H]	[M+6H]	[M+7H]	[M+8H]	[M+9H]
▪M1	1-57	(-) GSGMAEPRQEFEVMEDH AGTYGLGDRRKDQGGYTMHQD QEGDTDAGLKESPLQTPTED (-)	6549.86	6550.87	3275.94	2184.30	1638.47	1310.98	1092.65	936.70	819.74	728.77

A8119 (CBTau 27.1)

Frag#	Res#	Sequence	Theor (Bo)	[M+H]	[M+2H]	[M+3H]	[M+4H]	[M+5H]	[M+6H]	[M+7H]	[M+8H]	[M+9H]
▪M1	1-20	(-) HVPGGGSVQIVYKPVDL SKV (-)	2417.31	2418.32	1209.66	806.78	605.34	484.47	403.89	346.34	303.17	269.60

B1002 (CBTau 27.1)

Frag#	Res#	Sequence	Theor (Bo)	[M+H]	[M+2H]	[M+3H]	[M+4H]	[M+5H]	[M+6H]	[M+7H]	[M+8H]	[M+9H]
▪M1	1-30	(-) HVPGGGSVQIVYKPVDL SKVTSKCGSLGNI (-)	3377.78	3378.79	1689.90	1126.94	845.45	676.56	563.97	483.55	423.23	376.32

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9.6.2 Peptide species identified in fractions B, C and D.

Sample 1: CB tau 28.1

Table 4: Summary of the peptide species detected within fraction 1B.

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	10.80	A7731	788.67	3	788.67	2363.0	2.59E+03
	-	A7731	-	2	1182.51	2363.0	-
CB tau 24.1	11.25	V1089-023	590.32	4	590.32	2357.22	1.59E+05
	11.25	V1089-023	786.76	3	786.75	2357.22	3.83E+04
CB tau 24.1	11.44	A6907	918.36	8	918.36	7338.83	2.09E+04
	11.44	A6907	816.43	9	816.43	7338.83	2.09E+04
CB tau 28.1	12.40	B1014	848.13	4	848.13	3388.50	2.90E+03
	-	B1014	-	3	1130.51	3388.50	-
None - NC	12.83	A6984	1092.65	6	1092.65	6549.86	4.23E+04
	12.83	A6984	1310.99	5	1310.98	6549.86	4.68E+03
CB tau 27.1	13.40	A8119	605.34	4	605.34	2417.31	7.37E+04
	13.40	A8119	806.78	3	806.78	2417.31	3.77E+04
CB tau 27.1	13.75	B1002	845.46	4	845.45	3377.78	2.04E+03
	-	B1002	-	5	676.56	3377.78	-

Table 5: Summary of the peptide species detected within fraction 1B.

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	10.87	A7731	788.68	3	788.67	2363.0	1.17E+03
	-	A7731	-	2	1182.51	2363.0	-
CB tau 24.1	11.34	V1089-023	590.32	4	590.32	2357.22	6.18E+03
	11.32	V1089-023	786.76	3	786.75	2357.22	1.25E+03
CB tau	-	A6907	-	8	918.36	7338.83	

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
24.1	-	A6907	-	9	816.43	7338.83	-
CB tau 28.1	12.45	B1014	848.14	4	848.13	3388.50	1.16E+03
	-	B1014	-	3	1130.51	3388.50	-
None - NC	12.87	A6984	1092.66	6	1092.65	6549.86	1.73E+03
	-	A6984	-	5	1310.98	6549.86	-
CB tau 27.1	-	A8119	-	4	605.34	2417.31	-
	-	A8119	-	3	806.78	2417.31	-
CB tau 27.1	-	B1002	-	4	845.45	3377.78	-
	-	B1002	-	5	676.56	3377.78	-

Table 6: Summary of the peptide species detected within fraction 1E.

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	10.83	A7731	788.67	3	788.67	2363.0	4.86E+03
	-	A7731	-	2	1182.51	2363.0	-
CB tau 24.1	11.27	V1089-023	590.32	4	590.32	2357.22	5.78E+03
	11.27	V1089-023	786.76	3	786.75	2357.22	1.07E+03
CB tau 24.1	-	A6907	-	8	918.36	7338.83	-
	11.45	A6907	816.44	9	816.43	7338.83	1.28E+03
CB tau 28.1	12.41	B1014	848.14	4	848.13	3388.50	3.32E+03
	12.41	B1014	1130.51	3	1130.51	3388.50	1.27E+03
None - NC	-	A6984	-	6	1092.65	6549.86	-
	-	A6984	-	5	1310.98	6549.86	-
CB tau 27.1	13.40	A8119	605.34	4	605.34	2417.31	1.36E+03
	-	A8119	-	3	806.78	2417.31	-
CB tau 27.1	-	B1002	-	4	845.45	3377.78	-
	-	B1002	-	5	676.56	3377.78	-

Sample 2: CB tau 27.1

Table 7: Summary of the peptide species detected within fraction 2B.

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	10.83	A7731	788.67	3	788.67	2363.0	9.03E+03
CB tau 28.1	-	A7731	-	2	1182.51	2363.0	-
CB tau 24.1	11.27	V1089-023	590.32	4	590.32	2357.22	1.46E+05
CB tau 24.1	11.27	V1089-023	786.76	3	786.75	2357.22	3.82E+04
CB tau 24.1	11.47	A6907	918.37	8	918.36	7338.83	5.67E+03
CB tau 24.1	11.47	A6907	816.44	9	816.43	7338.83	6.08E+03
CB tau 28.1	12.43	B1014	848.14	4	848.13	3388.50	7.75E+03
CB tau 28.1	12.43	B1014	1130.52	3	1130.51	3388.50	3.90E+03
None - NC	12.82	A6984	1092.66	6	1092.65	6549.86	3.05E+04
None - NC	12.82	A6984	1310.99	5	1310.98	6549.86	4.23E+03
CB tau 27.1	13.37	A8119	605.34	4	605.34	2417.31	2.69E+04
CB tau 27.1	13.37	A8119	806.78	3	806.78	2417.31	1.36E+04
CB tau 27.1	-	B1002	-	4	845.45	3377.78	-
CB tau 27.1	-	B1002	-	5	676.56	3377.78	-

Table 8: Summary of the peptide species detected within fraction 2C.

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	-	A7731	-	3	788.67	2363.0	-
CB tau 28.1	-	A7731	-	2	1182.51	2363.0	-
CB tau 24.1	11.28	V1089-023	590.32	4	590.32	2357.22	1.61E+04
CB tau 24.1	11.28	V1089-023	786.76	3	786.75	2357.22	3.21E+03
CB tau 24.1	-	A6907	-	8	918.36	7338.83	-
CB tau 24.1	-	A6907	-	9	816.43	7338.83	-

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	-	B1014	-	4	848.13	3388.50	-
	-	B1014	-	3	1130.51	3388.50	-
None - NC	12.84	A6984	1092.65	6	1092.65	6549.86	2.52E+03
	-	A6984	-	5	1310.98	6549.86	-
CB tau 27.1	13.41	A8119	605.33	4	605.34	2417.31	6.16E+03
	13.41	A8119	806.78	3	806.78	2417.31	2.83E+03
CB tau 27.1	-	B1002	-	4	845.45	3377.78	-
	-	B1002	-	5	676.56	3377.78	-

Table 9: Summary of the peptide species detected within fraction 2E.

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	-	A7731	-	3	788.67	2363.0	-
	-	A7731	-	2	1182.51	2363.0	-
CB tau 24.1	11.26	V1089-023	590.32	4	590.32	2357.22	2.68E+03
	-	V1089-023	-	3	786.75	2357.22	-
CB tau 24.1	11.45	A6907	918.37	8	918.36	7338.83	1.75E+03
	11.45	A6907	816.44	9	816.43	7338.83	1.55E+03
CB tau 28.1	-	B1014	-	4	848.13	3388.50	-
	-	B1014	-	3	1130.51	3388.50	-
None - NC	-	A6984	-	6	1092.65	6549.86	-
	-	A6984	-	5	1310.98	6549.86	-
CB tau 27.1	13.37	A8119	605.34	4	605.34	2417.31	6.68E+04
	13.37	A8119	806.78	3	806.78	2417.31	3.34E+04
CB tau 27.1	13.74	B1002	845.45	4	845.45	3377.78	3.17E+04
	13.74	B1002	676.56	5	676.56	3377.78	1.14E+04

Sample 3: mCB tau 24.1

Table 10: Summary of the peptide species detected within fraction 3B.

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	10.80	A7731	788.67	3	788.67	2363.0	1.44E+04
	-	A7731	-	2	1182.51	2363.0	-
CB tau 24.1	11.24	V1089-023	590.32	4	590.32	2357.22	3.20E+04
	11.24	V1089-023	786.76	3	786.75	2357.22	7.15E+03
CB tau 24.1	-	A6907	-	8	918.36	7338.83	-
	-	A6907	-	9	816.43	7338.83	-
CB tau 28.1	12.39	B1014	848.13	4	848.13	3388.50	1.10E+04
	12.39	B1014	1130.51	3	1130.51	3388.50	5.40E+03
None - NC	12.80	A6984	1092.65	6	1092.65	6549.86	5.12E+04
	12.80	A6984	1310.99	5	1310.98	6549.86	7.56E+03
CB tau 27.1	13.37	A8119	605.33	4	605.34	2417.31	6.42E+04
	13.37	A8119	806.78	3	806.78	2417.31	3.29E+04
CB tau 27.1	-	B1002	-	4	845.45	3377.78	-
	-	B1002	-	5	676.56	3377.78	-

Table 11: Summary of the peptide species detected within fraction 3C.

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	-	A7731	-	3	788.67	2363.0	-
	-	A7731	-	2	1182.51	2363.0	-
CB tau 24.1	11.26	V1089-023	590.32	4	590.32	2357.22	1.55E+04
	11.26	V1089-023	786.76	3	786.75	2357.22	3.63E+03
CB tau 24.1	-	A6907	-	8	918.36	7338.83	-
	-	A6907	-	9	816.43	7338.83	-

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	-	B1014	-	4	848.13	3388.50	-
CB tau 28.1	-	B1014	-	3	1130.51	3388.50	-
None - NC	12.82	A6984	1092.66	6	1092.65	6549.86	3.27E+03
None - NC	-	A6984	-	5	1310.98	6549.86	-
CB tau 27.1	-	A8119	-	4	605.34	2417.31	-
CB tau 27.1	-	A8119	-	3	806.78	2417.31	-
CB tau 27.1	-	B1002	-	4	845.45	3377.78	-
CB tau 27.1	-	B1002	-	5	676.56	3377.78	-

Table 12: Summary of the peptide species detected within fraction 3E.

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	-	A7731	-	3	788.67	2363.0	-
CB tau 28.1	-	A7731	-	2	1182.51	2363.0	-
CB tau 24.1	11.26	V1089-023	590.32	4	590.32	2357.22	2.55E+05
CB tau 24.1	11.26	V1089-023	786.76	3	786.75	2357.22	7.28E+04
CB tau 24.1	11.45	A6907	918.37	8	918.36	7338.83	3.51E+04
CB tau 24.1	11.45	A6907	816.44	9	816.43	7338.83	4.47E+04
CB tau 28.1	-	B1014	-	4	848.13	3388.50	-
CB tau 28.1	-	B1014	-	3	1130.51	3388.50	-
None - NC	-	A6984	-	6	1092.65	6549.86	-
None - NC	-	A6984	-	5	1310.98	6549.86	-
CB tau 27.1	13.39	A8119	605.33	4	605.34	2417.31	1.07E+03
CB tau 27.1	-	A8119	-	3	806.78	2417.31	-
CB tau 27.1	-	B1002	-	4	845.45	3377.78	-
CB tau 27.1	-	B1002	-	5	676.56	3377.78	-

Sample 4: Negative control – CR8043

Table 13: Summary of the peptide species detected within fraction 4B.

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	10.82	A7731	788.67	3	788.67	2363.0	1.48E+04
	10.82	A7731	1182.51	2	1182.51	2363.0	1.06E+03
CB tau 24.1	11.26	V1089-023	590.32	4	590.32	2357.22	2.14E+05
	11.26	V1089-023	786.76	3	786.75	2357.22	5.90E+04
CB tau 24.1	11.45	A6907	918.37	8	918.36	7338.83	2.21E+04
	11.45	A6907	816.43	9	816.43	7338.83	2.41E+04
CB tau 28.1	12.41	B1014	848.13	4	848.13	3388.50	7.48E+03
	12.41	B1014	1130.51	3	1130.51	3388.50	5.70E+03
None - NC	12.80	A6984	1092.66	6	1092.65	6549.86	4.27E+04
	12.80	A6984	1310.99	5	1310.98	6549.86	8.56E+03
CB tau 27.1	13.39	A8119	605.34	4	605.34	2417.31	5.78E+04
	13.39	A8119	806.78	3	806.78	2417.31	2.88E+04
CB tau 27.1	-	B1002	-	4	845.45	3377.78	-
	-	B1002	-	5	676.56	3377.78	-

Table 14: Summary of the peptide species detected within fraction 4C.

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	10.82	A7731	788.68	3	788.67	2363.0	1.52E+03
	-	A7731	-	2	1182.51	2363.0	-
CB tau 24.1	-	V1089-023	-	4	590.32	2357.22	-
	-	V1089-023	-	3	786.75	2357.22	-
CB tau 24.1	-	A6907	-	8	918.36	7338.83	-
	-	A6907	-	9	816.43	7338.83	-

Antibody		Peptide		Charge +	Theor. m/z	Mass [Da]	
CB tau 28.1	-	B1014	-	4	848.13	3388.50	-
	-	B1014	-	3	1130.51	3388.50	-
None - NC	-	A6984	-	6	1092.65	6549.86	-
	-	A6984	-	5	1310.98	6549.86	-
CB tau 27.1	-	A8119	-	4	605.34	2417.31	-
	-	A8119	-	3	806.78	2417.31	-
CB tau 27.1	-	B1002	-	4	845.45	3377.78	-
	-	B1002	-	5	676.56	3377.78	-

Table 15: Summary of the peptide species detected within fraction 4E.

Antibody	RT [min]	Peptide	Experim. m/z	Charge +	Theor. m/z	Mass [Da]	Intensity
CB tau 28.1	-	A7731	-	3	788.67	2363.0	-
	-	A7731	-	2	1182.51	2363.0	-
CB tau 24.1	11.26	V1089-023	590.32	4	590.32	2357.22	1.37E+04
	11.26	V1089-023	786.76	3	786.75	2357.22	3.05E+03
CB tau 24.1	-	A6907	-	8	918.36	7338.83	-
	-	A6907	-	9	816.43	7338.83	-
CB tau 28.1	-	B1014	-	4	848.13	3388.50	-
	-	B1014	-	3	1130.51	3388.50	-
None - NC	-	A6984	-	6	1092.65	6549.86	-
	-	A6984	-	5	1310.98	6549.86	-
CB tau 27.1	-	A8119	-	4	605.34	2417.31	-
	-	A8119	-	3	806.78	2417.31	-
CB tau 27.1	-	B1002	-	4	845.45	3377.78	-
	-	B1002	-	5	676.56	3377.78	-

9.7 Approach B/epitope mapping experiment

9.7.1 Theoretical ionization

List of ionized species of relevant peptides was generated with MasLynx software.

CB tau 28.1 + rTau/Lys-C:

Res#	Sequence	Theor (Bo)	[M+H]	[M+2H]	[M+3H]	[M+4H]	[M+5H]	[M+6H]	[M+7H]	[M+8H]	[M+9H]	[M+10H]
58-80	(K)ESPLQTPTEDGSEEPGSETSDAK(S)	2390.02	2391.03	1196.02	797.68	598.51	479.01	399.35	342.44	299.76	266.57	240.01
81-100	(K)STPTAEDVTAPLVDEGAPGK(Q)	1953.95	1954.96	977.98	652.33	489.50	391.80	326.67	280.14	245.25	218.11	196.40
101-143	(K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)	4400.08	4401.09	2201.05	1467.70	1101.03	881.02	734.35	629.59	551.02	489.91	441.02

CB tau 28.1 + rTau/Asp-N:

Res#	Sequence	Theor (Bo)	[M+H]	[M+2H]	[M+3H]	[M+4H]	[M+5H]	[M+6H]	[M+7H]	[M+8H]	[M+9H]	[M+10H]
53-66	(T)DAGLKESPLQTPTE(D)	1484.74	1485.74	743.38	495.92	372.19	297.95	248.46	213.11	186.60	165.98	149.48
67-77	(E)DGSEEPGSETS(D)	1093.40	1094.41	547.71	365.48	274.36	219.69	183.24	157.21	137.68	122.50	110.35
78-86	(S)DAKSTPTAE(D)	918.43	919.44	460.22	307.15	230.62	184.69	154.08	132.21	115.81	103.06	92.85
87-93	(E)DVTAPLV(D)	713.40	714.40	357.71	238.81	179.36	143.69	119.91	102.92	90.18	80.27	72.35
94-122	(V)DEGAPGKQAAAQPHTEIPEGTTAEEAGIG(D)	2831.32	2832.33	1416.67	944.78	708.84	567.27	472.89	405.48	354.92	315.60	284.14

CB tau 27.1 + rTau/Lys-C:

Res#	Sequence	Theor (Bo)	[M+H]	[M+2H]	[M+3H]	[M+4H]	[M+5H]	[M+6H]	[M+7H]	[M+8H]	[M+9H]	[M+10H]
312-330	(K)HVPGGGSVQIVYKPVDLSK(V)	1979.08	1980.09	990.55	660.70	495.78	396.82	330.86	283.73	248.39	220.91	198.92
331-334	(K)VTSK(C)	433.25	434.26	217.63	145.43	109.32	87.66	73.22	62.90	55.16	49.15	44.33

CB tau 27.1 + rTau/Asp-N:

Res#	Sequence	Theor (Bo)	[M+H]	[M+2H]	[M+3H]	[M+4H]	[M+5H]	[M+6H]	[M+7H]	[M+8H]	[M+9H]	[M+10H]
308-326	(K)DNIKHVPGGGSVQIVYKPV(D)	2006.09	2007.10	1004.06	669.71	502.53	402.23	335.36	287.59	251.77	223.91	201.62
327-357	(V)DLSKVTSKCGSLGNIHHKPGGGQVEVKSEKL(D)	3231.70	3232.71	1616.86	1078.24	808.93	647.35	539.63	462.68	404.97	360.09	324.18

CB tau 24.1 + rTau/Lys-C:

Res#	Sequence	Theor (Bo)	[M+H]	[M+2H]	[M+3H]	[M+4H]	[M+5H]	[M+6H]	[M+7H]	[M+8H]	[M+9H]	[M+10H]
248-253	(K)SPSSAK(S)	575.29	576.30	288.65	192.77	144.83	116.07	96.89	83.19	72.92	64.93	58.54
254-267	(K)SRLQTAPVPMPDLK(N)	1551.84	1552.85	776.93	518.29	388.97	311.38	259.65	222.70	194.99	173.43	156.19

CB tau 24.1 + rTau/Asp-N:

Res#	Sequence	Theor(Bo)	[M+H]	[M+2H]	[M+3H]	[M+4H]	[M+5H]	[M+6H]	[M+7H]	[M+8H]	[M+9H]	[M+10H]
206-264	(G)DRSGYSSPGSPGTPGSR SRTPSLPTPPTREPKKVAVV RTPPKSPSSAKSRLQTAPVP MP(D)	6119.25	6120.25	3060.63	2040.76	1530.82	1224.86	1020.88	875.19	765.91	680.92	612.93

9.7.2 PLGS output

The lists below show all the peptides detected during the MS runs. PLGS software was used for the analysis of the spectra. Fractions which are not listed did not contain any peptide species.

Control Samples

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                                     rTau/Lys-C Control Sample ~ 130 pmoles

MH+ (Da)    z    Error(ppm  Score  Start  End   Sequence                                          RT(min)  Intensity  Peptide Type    Products
1578.8217   2.84 -1.1467    8.7729  367   382   (K)IGSLDNITHVPGGGNK(K)                             10.6522  337700.0   Pass One Match   25
4326.1577   4.11  4.0125    8.6971  409   451   (K)SPVVSGDTSPRHLSNVSSTGSIDMVDSPQLATLADEVSASLAK(Q)  14.371   479028.0   Pass One Match   73
1980.087    3.39 -2.1892    8.6702  312   330   (K)HVPGGGSVQIVYKPVDLSK(V)                          11.2939  262397.0   Pass One Match   27
4401.097    4.93  1.6391    8.6535  101   143   (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)  11.789   465506.0   Pass One Match   64
3453.7112   5.2   0.2023    8.6524  204   237   (K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPK(K)           10.2786  279071.0   Pass One Match   31
1552.8478   2.6  -2.4184    8.6077  254   267   (K)SRLQTAPVPMPDLK(N)                               11.5084  182898.0   Pass One Match   17
1954.9684   2.32  4.0787    8.4875   81   100   (K)STPTAEDVTAPLVDEGAPGK(Q)                         11.4462  249186.0   Pass One Match   28
2766.2544   4.4  -2.3847    8.4165   14    37   (K)MAEPRQEFEVMEDHAGTYGLGDRK(D)                     11.5086  302306.0   Pass One Match   28
2391.0366   2.6   1.967     8.3152   58    80   (K)ESPLQTPTEDGSEEPGSETSDAK(S)                      10.1775  131494.0   Pass One Match   19
3581.798    5.72 -2.0596    8.2172  204   238   (K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKK(V)          9.9879   216624.0   Missed Cleavage  23
2165.905    2.88  0.4707    8.1091   38    57   (K)DQGGYTMHQDQEGDTDAGLK(E)                         9.7204   145535.0   Pass One Match   21
1263.7101   2.86 -5.2684    7.4918  164   176   (K)IATPRGAAPPGQK(G)                                8.7514   125372.0   Pass Two Match    9
861.4639    1.97 -4.3112    7.265   273   280   (K)IGSTENLK(H)                                     2.1318   53216.0    Pass One Match    3
2373.027    3.0   2.4597    0.0      58    80   (K)ESPLQTPTEDGSEEPGSETSDAK(S)                      10.1869  1308.0     Neut.Loss(H2O)    1
1936.9517   3.0   0.8961    0.0      81   100   (K)STPTAEDVTAPLVDEGAPGK(Q)                         11.446   1247.0     Neut.Loss(H2O)    0
4383.074    6.84 -1.151     0.0     101   143   (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)  11.789   3962.0     Neut.Loss(H2O)    1
4384.083    4.34  4.504     0.0     101   143   (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)  12.0982  138686.0   Neut.Loss(NH3)   27
3435.6946   6.31 -1.5306    0.0     204   237   (K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPK(K)           10.2777  3846.0     Neut.Loss(H2O)    0
843.4539    2.0  -3.6859    0.0     273   280   (K)IGSTENLK(H)                                     2.1274   1148.0     Neut.Loss(H2O)    0
1962.0739   4.0  -3.4945    0.0     312   330   (K)HVPGGGSVQIVYKPVDLSK(V)                          11.2932  31422.0    Neut.Loss(H2O)    1
1560.8109   3.0  -1.2927    0.0     367   382   (K)IGSLDNITHVPGGGNK(K)                             10.6526  1844.0     Neut.Loss(H2O)    0
2147.89     4.0  -1.6744    0.0      38    57   (K)DQGGYTMHQDQEGDTDAGLK(E)                         9.7204   1474.0     Neut.Loss(H2O)    0
```

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                                     Asp-N Control Sample ~ 130 pmoles

MH+ (Da)    z    Error(ppm) Score  Start  End   Sequence                                               RT(min)  Intensity  Peptide Type    Products
2007.1049   3.27  1.325     8.3312  308   326   (K)DNIKHVPGGGSVQIVYKPV(D)                               11.1485  414402.0   Pass One Match   32
1217.637    1.92 -0.1653    8.1318  443   454   (A)DEVSASLAKQGL(-)                                      10.816   169739.0   Pass One Match   16
4613.4873   7.03 -0.7663    7.8387  159   205   (A)DGKTKIATPRGAAPPGQKGQANATRIPAKTPPAPKTPPSSGEPPKSG(D)  9.8079   187527.0   Pass One Match   29
1485.7485   2.02  3.5846    7.8324   53    66   (T)DAGLKESPLQTPTE(D)                                    10.5149  105644.0   Pass One Match   18
714.4068    1.0   4.9275    7.8098   87    93   (E)DVTAPLV(D)                                           10.5447  124005.0   Pass Two Match   15
2832.3337   3.17  1.927     7.8023   94   122   (V)DEGAPGKQAAAQPHTEIPEGTTAEEAGIG(D)                    10.9053  97340.0    Pass One Match   23
1557.7947   2.34  2.5247    7.7626  400   414   (T)DHGAEIVYKSPVVSG(D)                                   10.5691  67687.0    Pass One Match   17
1102.6191   2.02 -2.077     7.6986  361   370   (K)DRVQSKIGSL(D)                                        9.0792   99890.0    Pass One Match   16
1657.8127   2.6  -0.7471    7.6825  415   430   (G)DTSPRHLSNVSSTGSI(D)                                  9.6912   150670.0   Pass One Match   27
661.3049    1.0   1.5248    7.4024  123   128   (G)DTPSLE(D)                                            3.1941   20015.0    Pass One Match    6
1814.9102   3.79 -4.1994    7.2968  129   145   (E)DEAAGHVTQARMVSKSK(D)                                7.9952   207456.0   Pass One Match    9
915.4831    1.02  5.3399    7.1603  434   442   (V)DSPQLATLA(D)                                         10.5978  55323.0    Pass Two Match   12
919.4359    1.83 -0.878     6.9946   78    86   (S)DAKSTPTAE(D)                                         0.9847   11586.0    Pass One Match    3
6120.2456   7.76 -1.0883    6.7031  206   264   (G)DRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKKVAVVRTPPKSPSSAKSRLQTAPVPMP(D) 10.8084  299520.0  Pass One Match   19
1467.7312   3.0  -0.915     0.0      53    66   (T)DAGLKESPLQTPTE(D)                                    10.5111  1745.0     Neut.Loss(H2O)    1
901.4248    2.0  -1.4601    0.0      78    86   (S)DAKSTPTAE(D)                                         0.9849   808.0      Neut.Loss(H2O)    0
2814.3174   4.0  -0.1074    0.0      94   122   (V)DEGAPGKQAAAQPHTEIPEGTTAEEAGIG(D)                    10.9062  1014.0     Neut.Loss(H2O)    0
1796.9003   4.13 -3.8456    0.0     129   145   (E)DEAAGHVTQARMVSKSK(D)                                7.9901   4682.0     Neut.Loss(H2O)    0
1990.0732   4.0  -1.2422    0.0     308   326   (K)DNIKHVPGGGSVQIVYKPV(D)                               11.1487  4003.0     Neut.Loss(NH3)    2
1539.7802   3.0  -0.0334    0.0     400   414   (T)DHGAEIVYKSPVVSG(D)                                   10.5689  1168.0     Neut.Loss(H2O)    0
1639.8025   3.0  -0.5708    0.0     415   430   (G)DTSPRHLSNVSSTGSI(D)                                  9.691    11688.0    Neut.Loss(H2O)    1
897.4642    2.0  -3.8289    0.0     434   442   (V)DSPQLATLA(D)                                         10.6082  897.0      Neut.Loss(H2O)    0
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Sample 1: CB tau 28.1

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                                   1Ai - CBTau 28.1 + rTau/Lys-C   -   Unbound Peptides

MH+ (Da)   z  Error(ppm) Score  Start   End    Sequence                                                     RT(min)   Intensity   Peptide Type Products
4326.153  4.12  2.8249   8.6339 409     451    (K)SPVVSGDTSPRHLSNVSSTGSIDMVDSPQLATLADEVSASLAK(Q) 14.3891   332894.0    Pass One Match  65
4401.096  4.93  1.406    8.6139 101     143    (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S) 11.7999   365653.0    Pass One Match  60
1552.8492 2.64 -1.5471   8.6111 254     267    (K)SRLQTAPVPMPDLK(N)                              11.5301   145460.0    Pass One Match  18
1578.821  2.85 -1.5051   8.5746 367     382    (K)IGSLDNITHVPGGGNK(K)                            10.6739   269354.0    Pass One Match  20
3453.7153 5.22  1.3824   8.4654 204     237    (K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPK(K)          10.2936   221426.0    Pass One Match  30
1954.9735 2.35  6.7305   8.3378 81      100    (K)STPTAEDVTAPLVDEGAPGK(Q)                        11.4607   205336.0    Pass Two Match  27
2766.2576 4.4  -1.2291   8.303  14      37     (K)MAEPRQEFEVMEDHAGTYGLGDRK(D)                    11.5216   246214.0    Pass One Match  24
1980.0874 3.43 -1.9697   8.2512 312     330    (K)HVPGGGSVQIVYKPVDLSK(V)                         11.3158   137060.0    Pass One Match  19
3581.7966 5.73 -2.4997   8.0844 204     238    (K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKK(V)         10.0019   179432.0    Missed Cleavage 24
2165.906  2.89  0.9083   7.8752 38      57     (K)DQGGYTMHQDQEGDTDAGLK(E)                        9.7397    113483.0    Pass One Match  18
1263.7134 2.87 -2.7029   7.601  164     176    (K)IATPRGAAPPGQK(G)                               8.7798    102315.0    Pass One Match  9
861.4648  1.97 -3.2981   7.0559 273     280    (K)IGSTENLK(H)                                    2.1553    43386.0     Pass One Match  2
1936.9575 3.0   3.9821   0.0    81      100    (K)STPTAEDVTAPLVDEGAPGK(Q)                        11.4589   1085.0      Neut. Loss(H2O) 0
4383.0728 -1.5185         0.0   101     143    (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S) 11.797    3455.0      Neut. Loss(H2O) 0
1245.7014 -3.8581         0.0   164     176    (K)IATPRGAAPPGQK(G)                               8.7803    14504.0     Neut. Loss(H2O) 0
3435.6865 -3.8973         0.0   204     237    (K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPK(K)          10.2944   2926.0      Neut. Loss(H2O) 0
3563.7844 -2.9354         0.0   204     238    (K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKK(V)         10.0031   11913.0     Neut. Loss(H2O) 1
1534.8385 -1.6097         0.0   254     267    (K)SRLQTAPVPMPDLK(N)                              11.5316   811.0       Neut. Loss(H2O) 0
1962.0753 -2.7678         0.0   312     330    (K)HVPGGGSVQIVYKPVDLSK(V)                         11.3164   17596.0     Neut. Loss(H2O) 0
1560.8121 -0.4732         0.0   367     382    (K)IGSLDNITHVPGGGNK(K)                            10.6749   1476.0      Neut. Loss(H2O) 0
2147.8855 -3.746          0.0   38      57     (K)DQGGYTMHQDQEGDTDAGLK(E)                        9.7418    1246.0      Neut. Loss(H2O) 0
```

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                            1Aii - CBTau 28.1 + rTau/Lys-C   -   Unbound Peptides - 1PBS:1H2O wash #1

MH+ (Da)   z    Error(ppm) Score  Start End   Sequence                                           RT (min) Intensity      Peptide Type Products
1954.9576 2.67 -1.3807    6.3573  81    100   (K)STPTAEDVTAPLVDEGAPGK(Q)                         11.4586  13914.0        Pass One Match  4
4401.0957 4.99  1.3309    6.1862  101   143   (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)  11.7961  30200.0        Pass One Match  8
1552.8497 2.85 -1.1776    6.0834  254   267   (K)SRLQTAPVPMPDLK(N)                               11.5433  11412.0        Pass One Match  5
1578.8207 2.94 -1.7268    6.0834  367   382   (K)IGSLDNITHVPGGGNK(K)                             10.6746  19807.0        Pass One Match  3
3453.7068 5.36 -1.0954    5.729   204   237   (K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPK(K)           10.2914  14088.0        Pass One Match  3
2766.2568 4.55 -1.4744    5.5919  14    37    (K)MAEPRQEFEVMEDHAGTYGLGDRK(D)                     11.5227  26465.0        Pass One Match  3
4384.0786 4.39  3.5155    0.0     101   143   (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)  12.0988  6902.0         Neut. Loss(NH3) 1
```

```
1Aiv - CBTau 28.1 + rTau/Lys-C   -   Bound Peptides

MH+ (Da)    z    Error(ppm) Score  Start End   Sequence                     RT (min)      Intensity Peptide Type  Products
22391.0327 2.69  0.3479    6.8915  58    80    (K)ESPLQTPTEDGSEEPGSETSDAK(S) 10.1685 PLGS 84000.0   Pass One Match  14
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                               1Bi - CBTau 28.1 + rTau/Asp-N   -   Unbound Peptides

MH+ (Da)   z    Error(ppm) Score Start  End   Sequence                                                      RT(min)  Intensity Peptide Type    Products
2007.1049 3.31  1.3363    8.2024 308    326   (K)DNIKHVPGGGSVQIVYKPV(D)                                      11.1857  293343.0  Pass One Match  30
4613.4907 7.03 -0.0585    7.8212 159    205   (A)DGKTKIATPRGAAPPGQKGQANATRIPAKTPPAPKTPPSSGEPPKSG(D)          9.8252   158952.0  Pass One Match  33
1217.6404 1.93  2.598     7.772  443    454   (A)DEVSASLAKQGL(-)                                             10.8455  135270.0  Pass One Match  15
714.4069  1.0   5.1417    7.7698 87     93    (E)DVTAPLV(D)                                                  10.5758  96277.0   Pass Two Match  14
1485.7463 2.02  2.1617    7.6807 53     66    (T)DAGLKESPLQTPTE(D)                                           10.5437  87742.0   Pass One Match  16
1102.6215 2.03  0.0284    7.62   361    370   (K)DRVQSKIGSL(D)                                               9.1162   70568.0   Pass One Match  13
1657.8148 2.62  0.4658    7.604  415    430   (G)DTSPRHLSNVSSTGSI(D)                                         9.7143   124311.0  Pass One Match  26
2832.342  3.19  4.8862    7.4916 94     122   (V)DEGAPGKQAAAQPHTEIPEGTTAEEAGIG(D)                            10.9323  79559.0   Pass Two Match  23
1557.7963 2.37  3.5673    7.4388 400    414   (T)DHGAEIVYKSPVVSG(D)                                          10.6015  54408.0   Pass One Match  14
661.3051  1.0   1.7416    7.2502 123    128   (G)DTPSLE(D)                                                   3.2041   12569.0   Pass One Match  4
915.4836  1.02  5.887     6.9886 434    442   (V)DSPQLATLA(D)                                                10.6263  39034.0   Pass Two Match  12
6120.2607 7.77  1.3498    6.5235 206    264   (G)DRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKKVAVVRTPPKSPSSAKSRLQTAPVPMP(D) 10.8408 233295.0 Pass One Match  17
2814.3257 4.0   2.9029    0.0    94     122   (V)DEGAPGKQAAAQPHTEIPEGTTAEEAGIG(D)                            10.9342  943.0     Neut.Loss(H2O)  0
1990.0746 4.0  -0.5353    0.0    308    326   (K)DNIKHVPGGGSVQIVYKPV(D)                                      11.187   2986.0    Neut.Loss(NH3)  2
1539.7789 3.0  -0.8328    0.0    400    414   (T)DHGAEIVYKSPVVSG(D)                                          10.6025  1012.0    Neut.Loss(H2O)  0
1639.8042 3.0   0.4746    0.0    415    430   (G)DTSPRHLSNVSSTGSI(D)                                         9.7143   10166.0   Neut.Loss(H2O)  2
897.4655  2.0  -2.3598    0.0    434    442   (V)DSPQLATLA(D)                                                10.6349  791.0     Neut.Loss(H2O)  0
1467.7281 3.0  -3.0316    0.0    53     66    (T)DAGLKESPLQTPTE(D)                                           10.5407  1937.0    Neut.Loss(H2O)  0
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                            1Bii - CBTau 28.1 + rTau/Asp-N   -   Unbound Peptides - 1PBS:1H2O wash #1

MH+ (Da)   z    Error(ppm) Score Start  End   Sequence                                             RT(min)  Intensity Peptide Type    Products
4613.4814 7.17 -2.0963    5.0733 159    205   (A)DGKTKIATPRGAAPPGQKGQANATRIPAKTPPAPKTPPSSGEPPKSG(D)  9.8211   18148.0   Pass One Match  4
714.406   1.0   3.8644    4.786  87     93    (E)DVTAPLV(D)                                          10.5969  2179.0    Pass One Match  2
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Sample 2: CB tau 27.1

2Ai – CBTau 27.1 + rTau/Lys-C   –   Unbound Peptides

MH+ (Da)	z	Error(ppm)	Score	Start	End	Sequence	RT(min)	Intensity	Peptide Type	Products
1578.8195	2.82	-2.5168	8.7794	367	382	(K)IGSLDNITHVPGGGNK(K)	10.5803	487020.0	Pass One Match	28
4401.099	4.92	2.1091	8.7219	101	143	(K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)	11.7387	579021.0	Pass One Match	71
1552.8488	2.55	-1.7588	8.7211	254	267	(K)SRLQTAPVPMPDLK(N)	11.4438	242461.0	Pass One Match	19
1954.9648	2.28	2.2302	8.7104	81	100	(K)STPTAEDVTAPLVDEGAPGK(Q)	11.3835	359739.0	Pass One Match	29
4326.167	4.1	6.0636	8.6209	409	451	(K)SPVVSGDTSPRHLSNVSSTGSIDMVDSPQLATLADEVSASLAK(Q)	14.3427	552288.0	Pass Two Match	75
2766.2563	4.39	-1.6631	8.5573	14	37	(K)MAEPRQEFEVMEDHAGTYGLGDRK(D)	11.4523	380695.0	Pass One Match	29
3453.7117	5.22	0.3353	8.5501	204	237	(K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPK(K)	10.2097	363976.0	Pass One Match	32
3581.8025	5.72	-0.8303	8.4933	204	238	(K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKK(V)	9.9135	280132.0	Missed Cleavage	30
2391.0376	2.53	2.4595	8.3851	58	80	(K)ESPLQTPTEDGSEEPGSETSDAK(S)	10.106	192884.0	Pass One Match	24
1980.0823	3.41	-4.5261	8.3272	312	330	(K)HVPGGGSVQIVYKPVDLSK(V)	11.2409	194626.0	Pass One Match	20
2165.909	2.85	2.295	8.0197	38	57	(K)DQGGYTMHQDQEGDTDAGLK(E)	9.6429	211486.0	Pass One Match	23
861.4663	1.95	-1.5447	7.8944	273	280	(K)IGSTENLK(H)	2.0571	81863.0	Pass One Match	5
1263.7126	2.85	-3.3167	7.8755	164	176	(K)IATPRGAAPPGQK(G)	8.636	183830.0	Pass One Match	11
714.4463	1.89	-6.347	7.6115	288	293	(K)VQIINK(K)	2.6967	30347.0	Pass Two Match	6
610.3567	1.0	1.3055	7.5337	188	193	(K)TPPAPK(T)	0.8905	3823.0	Pass One Match	4
996.4979	2.0	-1.7607	7.1805	194	203	(K)TPPSSGEPPK(S)	1.6729	34981.0	Pass One Match	2
1131.636	2.08	-0.6975	7.0426	294	303	(K)KLDLSNVQSK(C)	8.018	64503.0	Missed Cleavage	5
2147.8892	4.0	-2.0232	0.0	38	57	(K)DQGGYTMHQDQEGDTDAGLK(E)	9.6444	1963.0	Neut. Loss(H2O)	0
2373.0283	3.0	2.9532	0.0	58	80	(K)ESPLQTPTEDGSEEPGSETSDAK(S)	10.1268	1821.0	Neut. Loss(H2O)	0
1936.945	3.0	-2.4999	0.0	81	100	(K)STPTAEDVTAPLVDEGAPGK(Q)	11.3823	1596.0	Neut. Loss(H2O)	0
4383.0674	6.53	-2.7217	0.0	101	143	(K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)	11.738	7136.0	Neut. Loss(H2O)	2
4384.078	4.32	3.3054	0.0	101	143	(K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)	12.0496	218573.0	Neut. Loss(NH3)	35
1245.7006	3.0	-4.5544	0.0	164	176	(K)IATPRGAAPPGQK(G)	8.6362	25546.0	Neut. Loss(H2O)	0
3563.7878	6.49	-1.9665	0.0	204	238	(K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKK(V)	9.9145	20185.0	Neut. Loss(H2O)	0
843.4539	2.0	-3.7509	0.0	273	280	(K)IGSTENLK(H)	2.0562	1662.0	Neut. Loss(H2O)	0
1962.0717	4.0	-4.5974	0.0	312	330	(K)HVPGGGSVQIVYKPVDLSK(V)	11.2418	23592.0	Neut. Loss(H2O)	0
1560.8086	3.0	-2.7383	0.0	367	382	(K)IGSLDNITHVPGGGNK(K)	10.579	2482.0	Neut. Loss(H2O)	2

2Aii – CBTau 27.1 + rTau/Lys-C   –   Unbound Peptides - 1PBS:1H2O Wash #1

MH+ (Da)	z	Error(ppm)	Score	Start	End	Sequence	Retention Time (min)	Query Tool	Intensity	Peptide Type	Products
1954.9607	2.61	0.1633	7.1619	81	100	(K)STPTAEDVTAPLVDEGAPGK(Q)	11.36		36905.0	Pass One Match	7
4401.095	4.99	1.1657	7.1383	101	143	(K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)	11.6902		67437.0	Pass One Match	21
1552.8488	2.8	-1.7577	7.138	254	267	(K)SRLQTAPVPMPDLK(N)	11.4403		25673.0	Pass One Match	8
4326.148	4.21	1.7026	7.034	409	451	(K)SPVVSGDTSPRHLSNVSSTGSIDMVDSPQLATLADEVSASLAK(Q)	14.2893		48417.0	Pass One Match	22
2391.0337	2.85	0.84	6.9087	58	80	(K)ESPLQTPTEDGSEEPGSETSDAK(S)	10.1115		20057.0	Pass One Match	4
1980.0858	3.55	-2.7586	6.7664	312	330	(K)HVPGGGSVQIVYKPVDLSK(V)	11.2226		27502.0	Pass One Match	4
2766.2551	4.52	-2.1513	6.737	14	37	(K)MAEPRQEFEVMEDHAGTYGLGDRK(D)	11.4176		61045.0	Pass One Match	6
1578.8188	2.93	-2.8914	6.7064	367	382	(K)IGSLDNITHVPGGGNK(K)	10.5882		52327.0	Pass One Match	4
3453.7034	5.26	-2.0628	6.5681	204	237	(K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPK(K)	10.2131		33716.0	Pass One Match	5
1263.7075	2.91	-7.3531	6.1411	164	176	(K)IATPRGAAPPGQK(G)	8.6997		16242.0	Pass Two Match	3
3581.79	5.82	-4.3249	6.0687	204	238	(K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKK(V)	9.9266		24501.0	Missed Cleavage	3
1962.075	4.0	-2.8953	0.0	312	330	(K)HVPGGGSVQIVYKPVDLSK(V)	11.2227		4216.0	Neut. Loss(H2O)	0
4384.071	4.42	1.688	0.0	101	143	(K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)	11.9922		17407.0	Neut. Loss(NH3)	5

2Aiv – CBTau 27.1 + rTau/Lys-C   –   Bound Peptides - 0.1% FA Elution #1

MH+ (Da)	z	Error(ppm)	Score	Start	End	Sequence	RT(min)	Intensity	Peptide Type	Products
1980.0869	3.56	-2.2028	5.7099	312	330	(K)HVPGGGSVQIVYKPVDLSK(V)	11.343	13113.0	Pass One Match	4
1962.0756	4.0	-2.5871	0.0	312	330	(K)HVPGGGSVQIVYKPVDLSK(V)	11.3434	2068.0	Neut. Loss(H2O)	0

2Bi – CBTau 27.1 + rTau/Asp-N   –   Unbound Peptides

MH+ (Da)	z	Error(ppm)	Score	Start	End	Sequence	RT(min)	Intensity	Peptide Type	Products
2007.1057	3.31	1.7349	8.0577	308	326	(K)DNIKHVPGGGSVQIVYKPV(D)	11.2058	275371.0	Pass One Match	29
1217.6398	1.93	2.1561	7.7733	443	454	(A)DEVSASLAKQGL(-)	10.8635	120970.0	Pass One Match	14
714.406	1.0	3.8503	7.7345	87	93	(E)DVTAPLV(D)	10.5914	98106.0	Pass One Match	15
1485.7476	2.02	2.9311	7.5181	53	66	(T)DAGLKESPLQTPTE(D)	10.565	77890.0	Pass One Match	13
1657.8126	2.61	-0.8526	7.5169	415	430	(G)DTSPRHLSNVSSTGSI(D)	9.7316	129198.0	Pass One Match	25
1557.7947	2.37	2.5475	7.4892	400	414	(T)DHGAEIVYKSPVVSG(D)	10.6236	49419.0	Pass One Match	14
1102.6202	2.03	-1.0981	7.4817	361	370	(K)DRVQSKIGSL(D)	9.1179	74548.0	Pass One Match	13
2832.3384	3.18	3.558	7.4208	94	122	(V)DEGAPGKQAAAQPHTEIPEGTTAEEAGIG(D)	10.9574	77555.0	Pass One Match	24
661.3065	1.0	3.9678	7.0854	123	128	(G)DTPSLE(D)	3.1596	12883.0	Pass One Match	4
915.4835	1.02	5.8439	6.9504	434	442	(V)DSPQLATLA(D)	10.6429	38644.0	Pass Two Match	12
1814.91	3.78	-4.2603	6.9226	129	145	(E)DEAAGHVTQARMVSKSK(D)	8.0248	159407.0	Pass One Match	6
6120.2544	7.77	0.3354	6.7812	206	264	(G)DRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKKVAVVRTPPKSPSSAKSRLQTAPVPMP(D)	10.8707	221077.0	Pass One Match	19
2814.3264	4.0	3.0718	0.0	94	122	(V)DEGAPGKQAAAQPHTEIPEGTTAEEAGIG(D)	10.9591	821.0	Neut.Loss(H2O)	0
1796.9042	4.17	-1.6759	0.0	129	145	(E)DEAAGHVTQARMVSKSK(D)	8.0228	3785.0	Neut.Loss(H2O)	0
1990.0746	4.0	-0.5283	0.0	308	326	(K)DNIKHVPGGGSVQIVYKPV(D)	11.2064	2859.0	Neut.Loss(NH3)	1
1539.7756	3.0	-2.9658	0.0	400	414	(T)DHGAEIVYKSPVVSG(D)	10.6238	955.0	Neut.Loss(H2O)	0
1639.8018	3.0	-1.0477	0.0	415	430	(G)DTSPRHLSNVSSTGSI(D)	9.732	10286.0	Neut.Loss(H2O)	2
897.4646	2.0	-3.4223	0.0	434	442	(V)DSPQLATLA(D)	10.6496	767.0	Neut.Loss(H2O)	0
1467.7303	3.0	-1.481	0.0	53	66	(T)DAGLKESPLQTPTE(D)	10.5603	1126.0	Neut.Loss(H2O)	0

2Bii – CBTau 27.1 + rTau/Asp-N   –   Unbound Peptides - 1PBS:1H2O wash #1

MH+ (Da)	z	Error(ppm)	Score	Start	End	Sequence	RT(min)	Intensity	Peptide Type	Products
1485.7458	2.15	1.8038	6.0678	53	66	(T)DAGLKESPLQTPTE(D)	10.5383	9389.0	Pass One Match	5
1217.6364	2.04	-0.7686	5.9377	443	454	(A)DEVSASLAKQGL(-)	10.8354	22303.0	Pass One Match	6
4613.479	7.14	-2.6214	5.7599	159	205	(A)DGKTKIATPRGAAPPGQKGQANATRIPAKTPPAPKTPPSSGEPPKSG(D)	9.8059	29361.0	Pass One Match	7
714.4045	1.0	1.8034	5.7491	87	93	(E)DVTAPLV(D)	10.5788	4492.0	Pass One Match	4

Sample 3: CB tau 24.1

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                          3Ai - CBTau 24.1 + rTau/Lys-C   -   Unbound Peptides

MH+ (Da)   z   Error(ppm)Score  Start  End    Sequence                                              RT(min)  Intensity  Peptide Type  Products
4401.09    4.91 -0.0308  8.9602  101    143   (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)      11.7674  551424.0   Pass One Match  71
1980.0906  3.38 -0.3389  8.793   312    330   (K)HVPGGGSVQIVYKPVDLSK(V)                             11.2758  242677.0   Pass One Match  24
1578.82    2.83 -2.2486  8.7728  367    382   (K)IGSLDNITHVPGGGNK(K)                                10.6267  469899.0   Pass One Match  24
1552.8479  2.56 -2.3457  8.7399  254    267   (K)SRLQTAPVPMPDLK(N)                                  11.4842  233150.0   Pass One Match  19
4326.1567  4.1   3.6972  8.735   409    451   (K)SPVVSGDTSPRHLSNVSSTGSIDMVDSPQLATLADEVSASLAK(Q)     14.3566  525062.0   Pass One Match  76
1954.9675  2.29  3.6584  8.6114  81     100   (K)STPTAEDVTAPLVDEGAPGK(Q)                            11.421   339614.0   Pass One Match  29
3453.7134  5.21  0.8696  8.5756  204    237   (K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPK(K)              10.2566  346773.0   Pass One Match  29
2766.2544  4.39 -2.3446  8.5673  14     37    (K)MAEPRQEFEVMEDHAGTYGLGDRK(D)                        11.4877  372155.0   Pass One Match  28
2391.0366  2.53  2.0404  8.5176  58     80    (K)ESPLQTPTEDGSEEPGSETSDAK(S)                         10.1576  177753.0   Pass One Match  25
3581.7979  5.71 -2.1284  8.3725  204    238   (K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKK(V)             9.9645   268209.0   Missed Cleavage 29
2165.9043  2.85  0.1004  8.3056  38     57    (K)DQGGYTMHQDQEGDTDAGLK(E)                            9.7015   194040.0   Pass One Match  26
714.4482   1.89 -3.7356  7.9226  288    293   (K)VQIINK(K)                                          2.7365   27721.0    Pass One Match  7
861.4644   1.95 -3.7182  7.8212  273    280   (K)IGSTENLK(H)                                        2.0817   69805.0    Pass One Match  5
1263.7096  2.84 -5.6878  7.7446  164    176   (K)IATPRGAAPPGQK(G)                                   8.7143   162666.0   Pass Two Match  11
610.3579   1.0   3.2429  7.4868  188    193   (K)TPPAPK(T)                                          0.8936   3580.0     Pass One Match  4
1131.637   2.06  0.1267  7.2997  294    303   (K)KLDLSNVQSK(C)                                      8.1307   60237.0    Missed Cleavage 4
2147.8862  4.0  -3.3736  0.0     38     57    (K)DQGGYTMHQDQEGDTDAGLK(E)                            9.7033   1838.0     Neut.Loss(H2O)  0
2373.0283  3.0   2.951   0.0     58     80    (K)ESPLQTPTEDGSEEPGSETSDAK(S)                         10.1714  1729.0     Neut.Loss(H2O)  0
1936.9491  3.0  -0.3588  0.0     81     100   (K)STPTAEDVTAPLVDEGAPGK(Q)                            11.4201  1641.0     Neut.Loss(H2O)  0
4383.065   6.52 -3.3266  0.0     101    143   (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)     11.7657  6695.0     Neut.Loss(H2O)  3
4384.0786  4.31  3.4249  0.0     101    143   (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)     12.0767  201844.0   Neut.Loss(NH3)  35
3435.6958  6.31 -1.2048  0.0     204    237   (K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPK(K)              10.2561  5041.0     Neut.Loss(H2O)  0
1534.8374  3.0  -2.3582  0.0     254    267   (K)SRLQTAPVPMPDLK(N)                                  11.4834  1202.0     Neut.Loss(H2O)  0
843.4533   2.0  -4.4246  0.0     273    280   (K)IGSTENLK(H)                                        2.08     1791.0     Neut.Loss(H2O)  0
1962.0732  4.0  -3.7922  0.0     312    330   (K)HVPGGGSVQIVYKPVDLSK(V)                             11.2757  28750.0    Neut.Loss(H2O)  1
1560.8087  3.0  -2.6552  0.0     367    382   (K)IGSLDNITHVPGGGNK(K)                                10.6261  2468.0     Neut.Loss(H2O)  2
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                          3Aii - CBTau 24.1 + rTau/Lys-C   -   Unbound Peptides - 1PBS:1H2O Wash #1

MH+ (Da)   z    Error(ppm) Score  Start End   Sequence                                              RT(min) Intensity  Peptide Type Products
4401.0947  4.98  1.0364   7.245   101   143   (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)     11.7864 69396.0    Pass One Match  21
1552.8496  2.78 -1.2476   7.1697  254   267   (K)SRLQTAPVPMPDLK(N)                                  11.5279 25684.0    Pass One Match  9
1954.9587  2.61 -0.8547   7.0629  81    100   (K)STPTAEDVTAPLVDEGAPGK(Q)                            11.4422 38108.0    Pass One Match  8
4326.1484  4.2   1.8132   7.056   409   451   (K)SPVVSGDTSPRHLSNVSSTGSIDMVDSPQLATLADEVSASLAK(Q)     14.3829 50928.0    Pass One Match  23
2766.2568  4.52 -1.542    6.8035  14    37    (K)MAEPRQEFEVMEDHAGTYGLGDRK(D)                        11.5084 61567.0    Pass One Match  6
2391.0354  2.84  1.1467   6.7984  58    80    (K)ESPLQTPTEDGSEEPGSETSDAK(S)                         10.1769 22143.0    Pass One Match  4
1980.0835  3.53 -3.9416   6.7028  312   330   (K)HVPGGGSVQIVYKPVDLSK(V)                             11.3046 26105.0    Pass One Match  4
1578.8193  2.93 -2.6352   6.568   367   382   (K)IGSLDNITHVPGGGNK(K)                                10.659  53472.0    Pass One Match  3
3453.7107  5.26  0.0668   6.4204  204   237   (K)SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPK(K)              10.2822 36261.0    Pass One Match  4
1962.0756  4.0  -2.6196   0.0     312   330   (K)HVPGGGSVQIVYKPVDLSK(V)                             11.3055 4078.0     Neut.Loss(H2O)  0
4384.082   4.41  4.2179   0.0     101   143   (K)QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSK(S)     12.0877 18616.0    Neut.Loss(NH3)  5
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                          3Bi - CBTau 24.1 + rTau/Asp-N   -   Unbound Peptides

MH+ (Da)   z     Error(ppm)  Score Start End   Sequence                                              RT(min) Intensity Peptide Type Product
2007.1017  3.28  -0.2638   8.2998  308   326   (K)DNIKHVPGGGSVQIVYKPV(D)                             11.1471 303652.0 Pass One Match 30
1217.6375  1.93   0.2618   8.091   443   454   (A)DEVSASLAKQGL(-)                                    10.8058 202845.0 Pass One Match 16
714.4054   1.0    3.0389   7.8558  87    93    (E)DVTAPLV(D)                                         10.5368 102634.0 Pass One Match 15
2832.334   3.17   2.0381   7.7463  94    122   (V)DEGAPGKQAAAQPHTEIPEGTTAEEAGIG(D)                  10.8972 95984.0  Pass One Match 24
1485.7463  2.01   2.1666   7.7402  53    66    (T)DAGLKESPLQTPTE(D)                                  10.5105 92022.0  Pass One Match 15
4613.4746  7.02  -3.5791   7.5111  159   205   (A)DGKTKIATPRGAAPPGQKGQANATRIPAKTPPAPKTPPSSGEPPKSG(D) 9.8019  177880.0 Pass One Match 35
1557.7957  2.35   3.1288   7.4835  400   414   (T)DHGAEIVYKSPVVSG(D)                                 10.5683 58417.0  Pass One Match 14
1102.6184  2.03  -2.8206   7.4335  361   370   (K)DRVQSKIGSL(D)                                      9.0632  77274.0  Pass One Match 15
1657.8094  2.6   -2.7506   7.3785  415   430   (G)DTSPRHLSNVSSTGSI(D)                                9.6846  138164.0 Pass One Match 27
661.3055   1.0    2.3846   7.2845  123   128   (G)DTPSLE(D)                                          3.1741  15634.0  Pass One Match 4
915.483    1.02   5.2389   7.0221  434   442   (V)DSPQLATLA(D)                                       10.588  45998.0  Pass Two Match 12
1814.91    3.77  -4.283    6.8328  129   145   (E)DEAAGHVTQARMVSKSK(D)                               7.9688  171146.0 Pass One Match 7
6120.2393  7.81  -2.1669   6.0233  206   264   (G)DRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKKVAVVRTPPKSPSSAKSRLQTAPVPMP(D) 10.8239 14917.0 Pass One Match 3
1467.7284  3.0   -2.867    0.0     53    66    (T)DAGLKESPLQTPTE(D)                                  10.5073 1130.0   Neut. Loss(H2O)0
2814.3108  4.0   -2.4513   0.0     94    122   (V)DEGAPGKQAAAQPHTEIPEGTTAEEAGIG(D)                  10.8979 1016.0   Neut. Loss(H2O)0
1796.8989  4.0   -4.6025   0.0     129   145   (E)DEAAGHVTQARMVSKSK(D)                               7.9674  3320.0   Neut. Loss(H2O)0
1990.0708  4.0   -2.4246   0.0     308   326   (K)DNIKHVPGGGSVQIVYKPV(D)                             11.1476 3141.0   Neut. Loss(NH3)3
1539.7756  3.0   -2.9716   0.0     400   414   (T)DHGAEIVYKSPVVSG(D)                                 10.5691 998.0    Neut. Loss(H2O)0
1639.7981  3.0   -3.2384   0.0     415   430   (G)DTSPRHLSNVSSTGSI(D)                                9.6845  11147.0  Neut. Loss(H2O)1
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                          3Bii - CBTau 24.1 + rTau/Asp-N   -   Unbound Peptides - 1PBS:1H2O Wash #1

MH+ (Da)   z    Error(ppm)Score  Start  End   Sequence                                              RT(min) Intensity  Peptide Type Products
2832.3381  3.36  3.4915   6.334   94    122   (V)DEGAPGKQAAAQPHTEIPEGTTAEEAGIG(D)                  10.8817 7719.0     Pass One Match  4
1485.7465  2.15  2.1941   6.2679  53    66    (T)DAGLKESPLQTPTE(D)                                  10.5102 8721.0     Pass One Match  4
4613.486   7.12 -1.1137   6.132   159   205   (A)DGKTKIATPRGAAPPGQKGQANATRIPAKTPPAPKTPPSSGEPPKSG(D) 9.779   28435.0    Pass One Match  8
714.4061   1.0   4.0251   5.9069  87    93    (E)DVTAPLV(D)                                         10.5488 4355.0     Pass One Match  4
1557.7874  2.66 -2.1392   5.7503  400   414   (T)DHGAEIVYKSPVVSG(D)                                 10.5786 5926.0     Pass One Match  4
2007.0948  3.51 -3.6499   5.6988  308   326   (K)DNIKHVPGGGSVQIVYKPV(D)                             11.1609 22455.0    Pass One Match  5
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                          3Biv - CBTau 24.1 + rTau/Asp-N   -   Bound Peptides - 0.1%FA Elution #1

MH+ (Da)   z    Error(ppm) Score Start End    Sequence                                              RT(min)  Intensity Peptide Type   Products
6120.252   7.91 -0.084    6.2622 206   264    (G)DRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKKVAVVRTPPKSPSSAKSRLQTAPVPMP(D) 10.7069 240707.0  Pass One Match  22
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