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QUALITY EVALUATION OF MONOCLONAL ANTIBODIES SUITABLE FOR IMMUNOMAGNETIC PURIFICATION OF NATIVE TAU PROTEIN

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Tau protein plays a crucial role in the neuronal cytoskeleton stabilization. Under the pathological conditions, it can be abnormally phosphorylated which leads to the aggregation and formation of neurofibrillary tangles representing pathological hallmark of Alzheimer's disease (AD). For its association with neurodegenerative diseases, tau protein is intensively studied in various diagnostic and therapeutic applications. Since there is no standard of tau protein involving essential post-translational modifications, it is often necessary to purify it directly from cerebrospinal fluid (CSF) or blood of healthy or AD clinical signs exhibiting organism. The immunomagnetic purification based on the isolation of the target protein using a specific antibody bound to a magnetic carrier is the most effective

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tool for this purpose. High quality antibodies are the main prerequisite of successful purification, but many commercial antibodies do not comply with the challenging requirements for the immunosorbent preparation. In this work, we compared four different anti-tau monoclonal antibodies currently available on the market (clones HT7, BT2, 8F10, 7E5). The evaluation criteria were set along the intended use for the preparation of specific magnetic immunosorbent subsequently applicable for the native tau protein purification. We evaluated the characteristics declared by producers as specificity, purity and homogeneity. We also tested the binding affinity and IgG stability during the covalent immobilization to the surface of magnetic microparticles and during the immunoprecipitation of intact tau protein or tryptic tau fragments. The results are summarized and discussed here.

Introduction

Tau proteins belong to the microtubule-associated proteins (MAPs) family operating *in vivo* as a significant regulatory element for microtubule assembly in cells by inducing the tubule formation [1]. In adult human brain, there are six major isoforms of tau generated by alternative mRNA splicing, which are phosphorylated. Since site-specific phosphorylation clearly modulates the function and intracellular localization of tau, inappropriate hyperphosphorylation caused by an imbalance in the activity of specific protein kinases or phosphatases is probably one of the key events in the neurodegenerative process. Hyperphosphorylated tau protein has a lower affinity to the microtubules and drastically increases the ability to self-associate and create the cytotoxic aggregates [2]. The formation of hyperphosphorylated, insoluble and filamentous tau protein is a common feature of many human neurodegenerative diseases, collectively referred to as tauopathies [3], of which the most common is Alzheimer's disease (AD) [4]. Additionally, increased phosphorylated tau and total tau levels, combined with reduced concentrations of amyloid-β 1-42 (Aβ42) in cerebrospinal fluid (CSF), but not in plasma or serum, have been generally accepted as sensitive AD diagnostic markers [5].

Since phosphorylation may change some physico-chemical properties and functions of protein [6,7], it is necessary to operate under *in vitro* studies also with the natural structures of target molecules, including post-translational modifications (PTM). On the market there is no standard of phosphorylated tau protein, and *in vitro* phosphorylated recombinant molecules can differ in some way from tau protein naturally occurring in the organism. Therefore, tau protein isolated directly from biological materials is useful. The isolation procedure should be specific providing high yields and purity without the risk of the structural or functional modification of the isolated protein.

Purifications based on affinity chromatography represent the most powerful

tool available in terms of the selectivity and recovery. A batch separation with specific antibodies as an affinity ligand immobilized on magnetic carriers (i.e., immunomagnetic separation, IMS) has several advantages in the comparison with standard column separation procedures [8]. Separation techniques using the magnetically active microparticles are fast, gentle, easily automated and applicable in a wide range of disciplines [9]. Magnetic particles coated with antibody molecules are incubated with a protein source, after protein binding the beads are washed to remove contaminants, and molecules of the target protein are subsequently eluted in small volumes. The selection of high-quality antibodies represents fundamental prerequisite for an effective immunopurification of the target antigen. Antibodies with the desired specificity are usually provided by several different manufacturers and companies, but the quality of the antibodies and their suitability for the designate application can significantly differ.

In this paper, we evaluated four various commercial monoclonal antibodies with the specificity against all six isoforms ensuring the total tau protein isolation. The specificity of monoclonal antibody clone 8F10 is directed against the C-terminus of the microtubule binding-domain of the tau protein molecule (epitope: aa 428-437), epitopes of antibody clones HT7 and 7E5 are included in the N-terminal projection domain and epitope of antibody clone BT2 is located between these two domains (see Table I). The specific immunoreactivity with tau protein and affinity of the antibodies was verified by a dot-blot analysis [10]. Consequently, immobilization of the antibodies onto magnetic microparticles with carboxyl function groups was performed and these immunosorbents (IS) were applied for the immunomagnetic separation of recombinant tau protein or tryptic peptide fragments of tau. The efficiency of antibodies immobilization was evaluated by a simple BCA test. Elution fractions collected during the IMS experiments were analyzed by dot-blot and mass spectrometry (MS). The main goal of this work was to select IgG molecules suitable for intended tau protein purification from liquid biological materials such as CSF or serum. The scheme of the whole experiment is given in Fig. 1.

Experimental

Materials and Equipment

Four types of monoclonal anti-tau antibodies were compared (see Table I): clones HT7, BT2 were obtained from Pierce (Rockford, IL, USA), clones 8F10, 7E5 from AJ Roboscreen GmbH (Leipzig, Germany). Recombinant tau protein (human 2N4R variant, isoform 441) was supplied by rPeptide (Bogart, GA, USA). SeraMag Speed Beads Magnetic Carboxylate-Modified Particles (0.816 μm) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Tau monoclonal antibody (Tau 5) applied as detection antibody in the quantitative dot-blot analysis

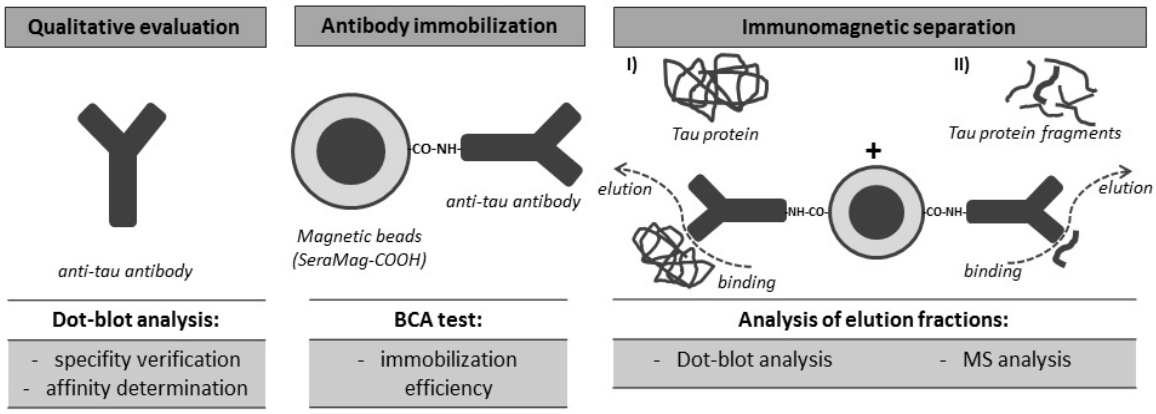

Fig. 1 Schematic representation of comparative experiment

Table I Characteristics of tested monoclonal anti-human tau antibodies

Clone	Isotype	Antigen	Declared purity	Epitope*	Supplier
HT7	Mouse IgG1κ	Human tau	> 95 % (SDS-PAGE), PBS	PPGQK (aa 159-163)	Pierce
BT2	Mouse IgG1κ	Bovine tau	> 95 % (SDS-PAGE), PBS	RSGYS (aa 194-198)	Pierce
8F10	Mouse IgG1κ	Recombinant human tau 441	> 95 % (SDS-PAGE), PBS pH 7.4	LADEVSASLA (aa 428-437)	AJ Roboscreen
7E5	Mouse IgG3κ	Human tau 441	> 95 % (SDS-PAGE), carbonate buffer pH 9.6	RGAAPPGQKGQA (aa 156-165)	AJ Roboscreen

* numbering according to human Tau isoform 441; aa – amino acid residues

was a gift from Francisco Garcia-Sierra (Center of Research and Advanced Studies of the National Polytechnic Institute, Mexico). Polyclonal rabbit anti-mouse IgG/HRP antibodies were obtained from Dako (Glostrup, Denmark). Bovine serum albumin (BSA), L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (EC 3.4.22.2, 12,700 IU mg^{-1} solid), DL-dithiothreitol (DTT), iodoacetic acid (IAA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC), *N*α-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BApNA), 2-(*N*-morpholino)ethanesulfonic acid sodium salt (MES), acetonitrile (ACN), benzamidine and diammonium hydrogen citrate (DAHC) were produced by Sigma–Aldrich (St. Louis, MO, USA). *N*-hydroxysulfosuccinimide sodium salt (Sulfo-NHS) and trifluoroacetic acid (TFA) were obtained from Fluka (Buchs, Switzerland). The Micro BCA protein assay reagent kit was purchased from Pierce (Rockford, IL, USA). The PVDF membrane (Immuno-Blot PVDF Membrane, 0.2 μm), the Clarity Western ECL substrate and Immun-Blot Opti-4CN Colorimetric kit were acquired from Bio-Rad (Hercules, CA, USA). Poros Oligo R3 reversed-phase material was purchased from Life Technologies (Carlsbad, CA, USA).

Matrices for MS analysis, α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) were from LaserBio Labs (Sophia-Antipolis, France). All other chemicals were of reagent grade.

The magnetic separator was acquired from Dynal (Carlsbad, CA, USA). Amicon® Ultra 0.5 ml filters were purchased from Merck Millipore (Billerica, MA, USA). GELoader tips were from Eppendorf (Hamburg, Germany). An MS instrument MALDI LTQ Orbitrap XL was obtained from Thermo Scientific (Waltham, MA, USA), A SpeedVac RVC 2-18 from Christ (Osterode am Harz, Germany) was connected with a vacuum pump KNF Neuberger (Freiburg, Germany). The dot-blot DHM-96 unit manifold was purchased from Scie-Plast (Cambridge, UK).

SDS-PAGE Analysis

1 μg of each tested antibody (Table I)/tau protein sample was mixed with Laemmli sample buffer/Tricine sample buffer with or without β-mercaptoethanol (1:1), boiled at 100 °C for 3 min and then loaded onto a 0.75 mm Tris-glycine gel (10 % [w/v] separating gel) according to Laemmli [11]/Tris-tricine gel (16.5 % T, 3 % C [w/v] separating gel) according to Schägger [12]. Electrophoresis was performed using a Mini-Protean system (Bio-Rad, Hercules, CA, USA) at 180 V in Tris-glycine-SDS/30 and 100 V in Tris-tricine-SDS running buffer. Proteins in the gels were visualized by a silver staining method [13] and pictures were taken with a digital camera Nikon Coolpix 5000 (Nikon, Tokyo, Japan).

Immobilization of Antibodies to Magnetic Microparticles

20 μg monoclonal anti-tau antibodies (Table I) were covalently bound to 0.5 mg SeraMag magnetic microparticles (MPs) by two-step carbodiimide method using EDAC (120 mM)/Sulfo-NHS (20 mM) [14,15] in 50 mM MES buffer pH 6.0 overnight at 4 °C and under mild stirring. Prepared immunosorbents were stored in 0.1 M phosphate buffer pH 7.0 containing 0.05 % sodium azide at 4 °C. The immobilization efficiency was evaluated from the difference of antibody concentration in the solution before and after immobilization determined by a BCA test. The BCA assay was performed in 96-well plate arrangement according to manufacturer's recommendations.

Proteolytic Digestion of Tau Protein

Trypsin specific magnetic carrier was prepared using the TPCK-treated trypsin (9 mg) pre-incubated for 10 min at room temperature (RT) with benzamidine (2.5

mM) and consequently mixed with SeraMag MPs (3 mg). The formation of the covalent bonds was performed by one-step carbodiimide method using EDAC (120 mM)/Sulfo-NHS (20 mM) [14,15] in 0.1 M phosphate buffer pH 7.3 overnight at RT under mild stirring. The activity of immobilized TCPK-trypsin was determined in a 96-well plate by hydrolysis of chromogenic substrate BApNA [16]. Biofunctionalised beads were stored in 0.1 M phosphate buffer pH 7.0 containing reversible enzyme inhibitor benzamidine (2.5 mM) at 4 °C.

The protein to be digested (recombinant tau protein, isoform 441) was first unfolded by reductive alkylation using DTT and IAA [17] in 50 mM ammonium bicarbonate solution and subsequently digested by TPCK-trypsin immobilized on magnetic particles in molar ratio E : S of 1 : 20 for 2 h at 37 °C under mild stirring. The efficiency of digestion was verified by a Tricin/SDS-PAGE electrophoresis [12] and MALDI-MS analysis. The mixture of tryptic tau fragments was also used for the immunomagnetic separation.

Immunomagnetic Separation

5μg tau protein (recombinant, isoform 441) or mixture of tryptic tau fragments was added to the immunosorbents (0.5 mg) prepared by immobilization of four various monoclonal anti-tau antibodies and pre-washed with 0.1 M phosphate buffer pH 7.0. Incubation for 4 h, at RT under stirring followed. Intensive washing was carried out by 0.1 M phosphate buffer pH 7.0 (containing 0.2 M /1 M NaCl), 0.01 M phosphate buffer pH 7.0 and ultra-pure water. Finally, the elution of immunocaptured tau molecules or tryptic tau fragments was performed three times for 20 min by 0.2 ml of 0.05 % TFA at RT and under stirring. Pooled eluted fractions were dried in a speed-vac and analyzed by the dot-blot analysis (proteins) or by MS-analysis (peptides).

The experiment with tryptic tau fragments was repeated also with incubation (10 min, RT, under stirring) of immunosorbents after the immunocomplex formation (4 h incubation) with 8M urea to minimize unspecific protein-protein interactions.

Dot-blot Analysis

The dot-blot experiment was performed according to already published protocol [10] with slight modifications. The analyzed samples dissolved in 100 μl 0.1 M phosphate buffer pH 7.0 (PB buffer) were spotted on the equilibrated PVDF membrane and washed with 100 μl PBS-T buffer. Then the membrane was blocked using 5 % BSA in PBS-T for 60 min, and incubation with primary antibody diluted in the ratio 1:30000/1:10000 for 60 min was performed. Washing

with PBS-T and incubation for 60 min with secondary antibody (anti-mouse IgG/HRP) diluted in the ratio 1:10000/1:1000 followed. Finally, washing with PBS-T and the chemiluminescence detection by a Clarity western ECL substrate/colorimetric detection using the Opti-4CN kit according to the manufacturer's instructions was carried out.

For affinity evaluation of monoclonal anti-tau antibodies (Table I), recombinant tau-441 protein (0.03 μg per spot) was applied, and chaotropic step using 0-2 M NH_4SCN was included in addition [10]. ChemiDoc™ XRS+ Imaging System with Image Lab™ Software (Bio-Rad, Hercules, CA, USA) was applied for the documentation and dot-blot analysis.

Mass Spectrometry Analysis

Chromatographic reversed-phase (Poros Oligo R3) microcolumns used for desalting and concentration of peptides were prepared using GELoader tips as previously described [18]. Dried tau peptide fragments were dissolved in 0.1% TFA and applied onto Poros Oligo R3 microcolumns using gentle air pressure. The columns were washed with 15 μl 0.1% TFA, and retained peptides were eluted directly onto MALDI-targets by 1 μl CHCA solution (5 mg ml^{-1} of 70 % ACN/0.1 % TFA + 2 mM DAHC). A MALDI-Orbitrap MS instrument was used for the measurement in the positive mode with pulsed nitrogen laser operating at 337 nm.

Results and Discussion

Two conditions have to be met for the successful immunomagnetic purification: i) reactivity with biospecific paired molecules retained even after the covalent binding of ligand (IgG) to the solid phase, and ii) the interaction between the ligand and the target molecule must be reversible to allow the target molecules to be released in an active form. IgG molecules providing the desired specificity and appropriate affinity are the most suitable ligand for the immunosorbent preparation. Monoclonal antibodies compared with polyclonal are more desirable due to their lack of variability which allows preparing a robust and more uniform carrier [19]. In this work, we evaluated and compared four mouse monoclonal antibodies from two suppliers with specificity for tau protein differing in their isotype, epitope reactivity or used immunogen (see Table I).

The rating quality of antibodies, it means purity, affinity, specificity and no cross-reactivity, should be considered prior to covalent immobilization to the solid phase. We used the SDS-PAGE and dot-blot analysis for the qualitative evaluation of all the tested antibodies. As can be seen in Fig. 2, all antibodies are pure, homo-

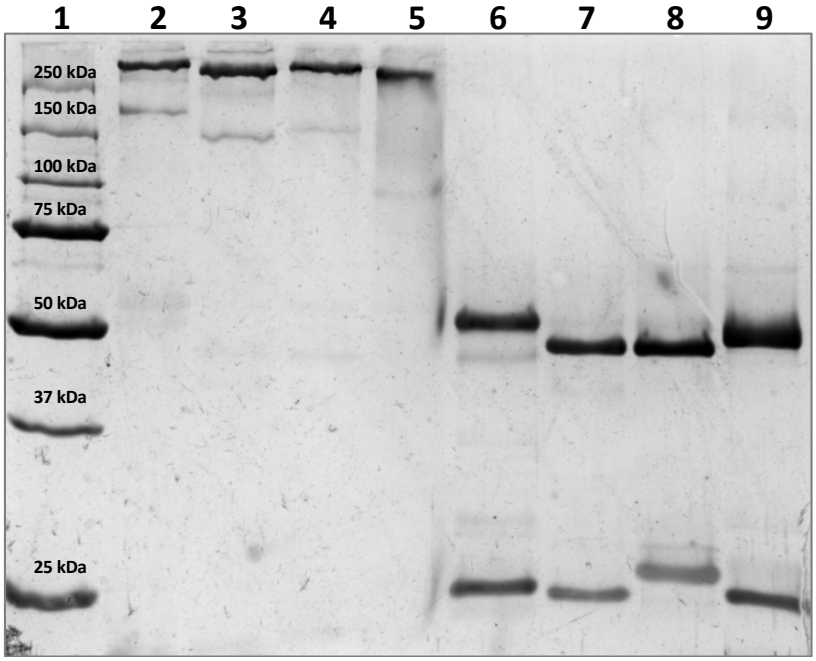

Fig. 2 SDS-PAGE analysis of mouse monoclonal anti-tau protein antibodies followed by silver staining; lanes: 1 – marker of molecular weights (10-250 kDa), 2 and 6 – clone HT7, 3 and 7 – clone BT2, 4 and 8 – clone 8F10, 5 and 9 – clone 7E5; positions 2 – 5 are without β-mercaptoethanol, positions 6 – 9 are with β-mercaptoethanol

geneous and non-fragmented. After the reduction and alkylation of disulfide bonds by β-mercaptoethanol (positions 6-9), the differences in molecular weights corresponding to the light and heavy chains are observed. These discrepancies can be due to the different carbohydrate content or isotype affiliation.

The dot-blot results (Fig. 3A) confirmed the specific reactivity of all antibodies with recombinant tau protein isoform 441, but BT2 antibodies in the comparison with others show lower level of signal, using 0.025 μg antigen the signal was almost zero (spot No. 2). Moreover, no antibodies showed cross-reactivity with BSA, which was spotted on the membrane in position 3 as a negative control. The dot-blot experiment supplemented by chaotropic step with different concentrations of ammonium thiocyanate (NH_4SCN) in the range of 0-2 M was applied for the determination of the affinity index of tested antibodies (Fig. 3B) [10]. The affinity index is expressed as the molarity of chaotropic reagent causing 50% reduction of the initial signal [20]. Clones BT2, 8F10 and HT7 showed similar value of the affinity index around 0.75 mol l^{-1}, the signal corresponding to the amount of immunocomplex decreased significantly with an increasing concentration of ammonium thiocyanate. The higest affinity index (1.3 mol l^{-1}) was observed for 7E5 clone, which indicates its strongest interaction with recombinant tau protein.

The type of covalent bonds and the orientation of affinity ligand are very important when designing an affinity chromatography in a preparative mode.

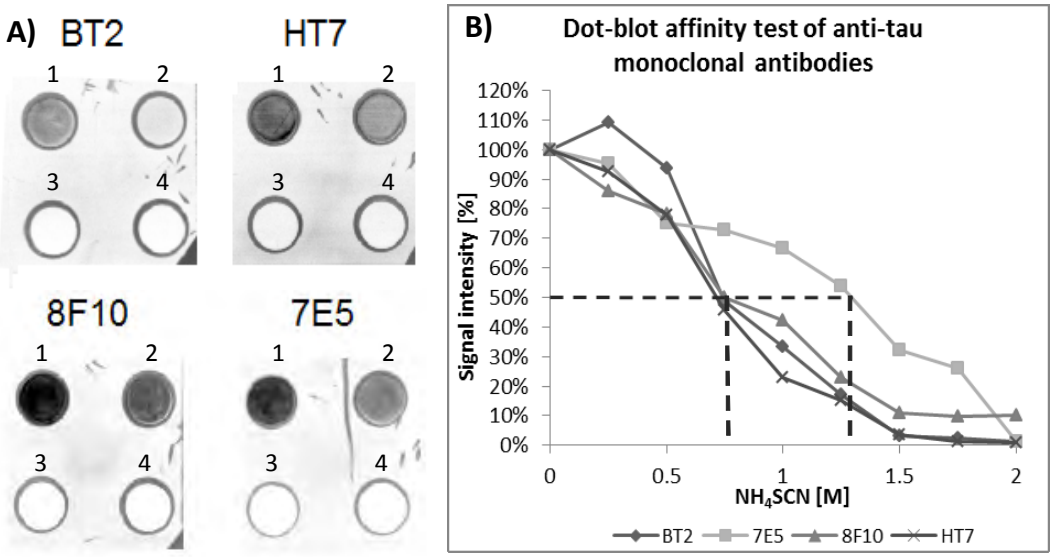

Fig. 3 Dot-blot analysis: A – immunoreactivity of monoclonal anti-tau protein antibodies (clones HT7, BT2, 8F10 and 7E5) with recombinant tau protein isoform 441, list of samples and controls spotted on the PVDF membrane: 1 – recombinant tau protein isoform 441 (0.05 μg); 2 – recombinant tau protein isoform 441 (0.025 μg), 3 – bovine serum albumin (1 μg), 4 – phosphate buffer; B – results of dot-blot affinity test determining affinity index of monoclonal anti-tau protein antibodies (clones HT7, BT2, 8F10 and 7E5), 0.03 μg recombinant tau protein isoform 441 was applied in triplicate for each concentration of NH_4SCN (average values are in graph); primary antibody: HT7, BT2, 8F10, 7E5, 0.1 μg ml^{-1}; secondary antibody: anti-mouse IgG/HRP 1:1000; detection: Opti-4CN kit

Antibody immobilization onto the solid surface has been studied extensively for a number of applications [21]. There are a number of immoblization strategies, including covalent coupling, adsorption or affinity binding, each of which has its benefits and drawbacks. Covalent coupling is one of the most common ways of attaching an affinity ligand to a solid support material and we use it when a very active and stable microsphere reagent is required [22]. There is a wide range of coupling chemistries available when considering covalent immobilization methods. Random covalent immobilization methods generally link antibodies to the solid support *via* their free amine groups [23]. Anti-tau IgG molecules can be attached to the surface of carrier through the widely occurred COOH or NH_2 groups or by specific functional groups, e.g., generated by oxidation of terminal galactoses of glycosidic carbohydrate chains localized in Fc part of the antibody molecule. When immobilizing an affinity ligand, care must be taken to the steric accessibility of sites intended for the biospecific interaction. Also the binding affinity of ligand to the target antigen has to be maintained [19].

In our work, we used a common carbodiimide method (EDAC) for crosslinking of carboxyl groups placed on the magnetic beads with amino groups

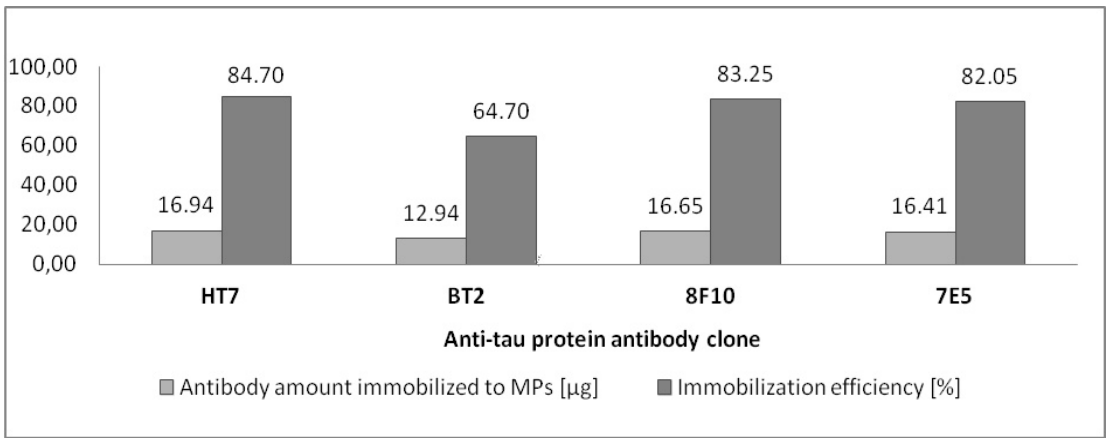

Fig. 4 Binding efficiency of monoclonal anti-tau antibodies immobilized to the SeraMag MPs, protein content was determined in solution before and after IgG immobilization by BCA kit, absorbance at 570 nm was measured

of antibody molecules. Sulfo-NHS was added for improving the binding efficiency [14]. Optimization of the coating protocol (amounts and ratios of all reagents, 1-step/2-step procedure, and incubation time) should lead to the high-quality immunosorbent. In our experiment, 20 µg of each monoclonal anti-tau protein antibody was bound on 0.5 mg SeraMag beads. The binding efficiency was determined by an SDS-PAGE (data not shown) and a simple BCA method estimating the protein content in the solution before and after the immobilization. The results in Fig. 4 show that all antibodies were bound to SeraMag MPs in quite a large amount, the lowest efficiency (65 %) was obtained with BT2 antibodies, the rest of the antibodies was bound in a quantity greater than 80 %.

The prepared magnetic immunosorbents comprising MPs with specific antibodies were applied to the immunomagnetic purification of recombinant tau protein isoform 441 (5 µg). The binding capacity of the immunosorbents was determined by the dot-blot analysis of captured and consequently eluted tau protein. Dried binding fractions, first washing fractions and pooled eluted fractions from the IMS experiments performed with the whole tau protein were dissolved in 100 µl PB buffer and spotted on the equilibrated PVDF membrane, as well as samples for the calibration curve construction (0.0625-1 µg of recombinant Tau 441/100 µl PB buffer) and negative control (1 µg of BSA/100 µl of PB buffer). The high-sensitive chemiluminescence detection was applied in this case. The results of dot-blot quantification (Fig. 5) show that all immunosorbents are able to effectively bind tau protein and could be used for the immunomagnetic purification. The highest binding capacity was observed using the immunosorbents with 7E5 and 8F10 antibodies.

We tried to repeat the immunomagnetic separation experiment also with the mixture of tau protein fragments generated by tryptic digestion. All antibodies have defined specific epitope corresponding to the amino acid sequence in the tau protein structure to which antibodies are specifically bound. The goal was to verify

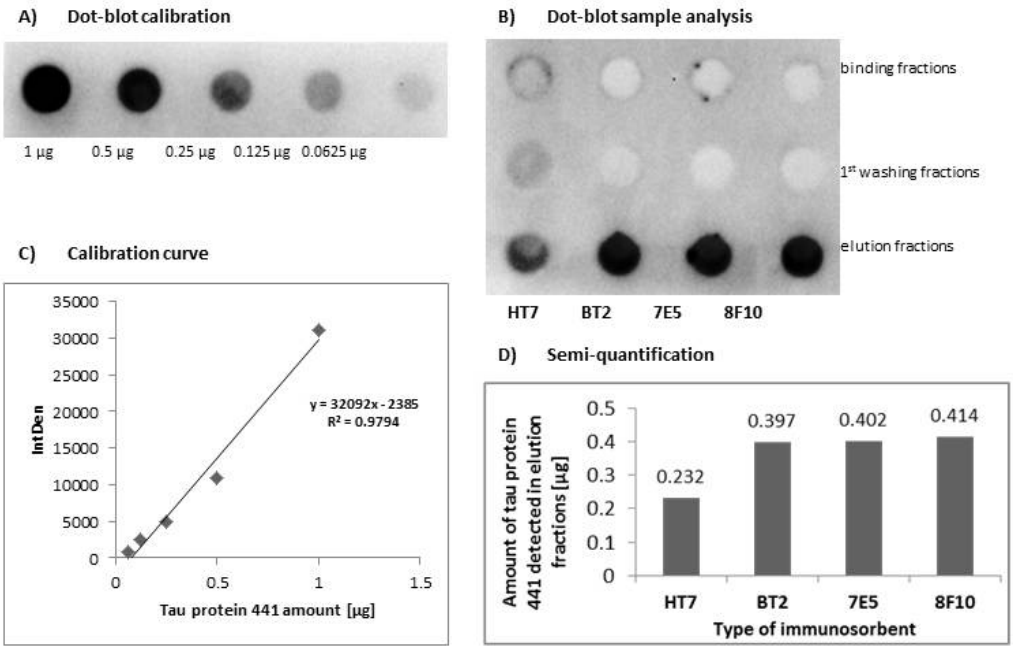

Fig. 5 Determination of binding capacity of anti-tau protein immunosorbents: A – dot-blot analysis of different amounts of recombinant tau protein 441; B – dot-blot analysis of fractions collected during immunomagnetic separation of recombinant tau protein 441; C – calibration curve constructed for semi-quantification of tau protein amount in samples; D – amount of tau protein calculated in elution fractions; primary antibody: Tau 5, 1:30000; secondary antibody: anti-mouse IgG/HRP 1:10000; detection: Clarity western ECL substrate

the immunoreactivity of antibodies also at the peptide level, because *in vivo* tau protein can also be found in truncated forms. In recent years, besides the well documented role of abnormal phosphorylation of tau, other modifications such as proteolytic cleavage at the C-terminus of the molecule have been linked also to the pathogenesis of AD [24]. Mass spectrometry (MS) was used for the detection of peptide samples. Theoretical peptide fragments of tau protein 441 obtained by digestion with trypsin, which include within the sequence epitopes of individual antibodies and can be expected in the MS spectra, are listed in Table II.

For tau the protein fragmentation, we applied highly specific TPCK-trypsin immobilized on MPs. When trypsin is immobilized, it can be easily removed from the final peptide mixture using a magnetic separator, so the sample is free of trypsin contaminants; moreover, we can use it repeatedly and minimum of autolytic products are generated. The final activity of immobilized trypsin determined by synthetic substrate BApNA was 996 IU mg^{-1} MPs. Proteolytic digestion of tau protein was carried out for 2 hours at 37 °C and with a molar enzyme-to-substrate ratio of 1:20. The reduction and alkylation of disulfide bonds using DTT and IAA, respectively, were performed prior the digestion which helps to denature proteins, making their proteolytic sites more accessible for proteolysis. The efficiency of tryptic digestion was confirmed by Tris-tricine-SDS-PAGE and

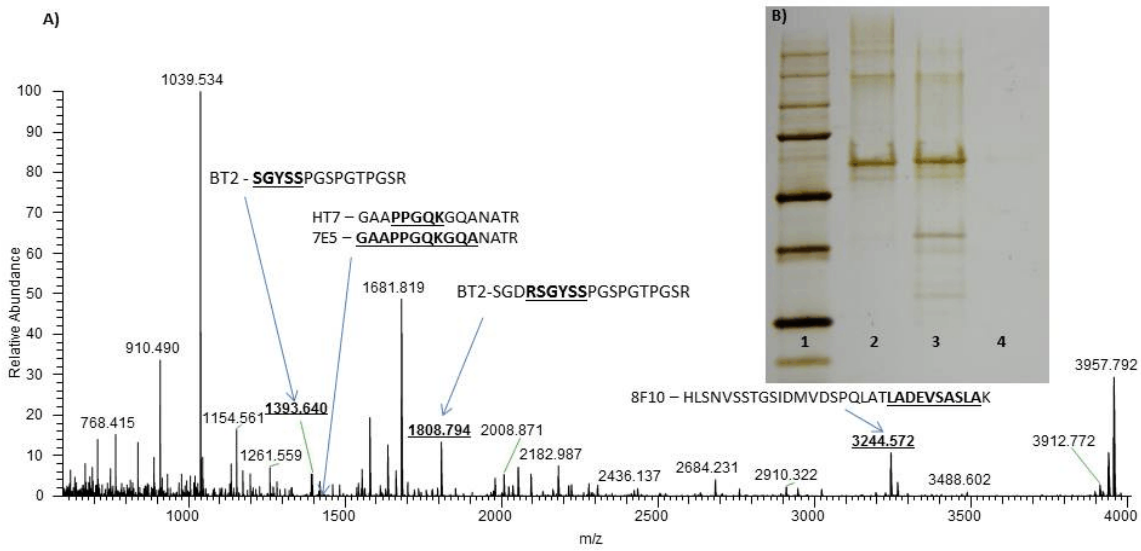

Fig. 6 Analysis of recombinant tau protein 441 digestion using immobilized TPCK-trypsin: A – MS analysis; B - Tris–tricine–SDS-PAGE followed by silver staining, lanes: 1 – marker of molecular weights (10-250 kDa), 2 – recombinant tau protein 441, 3 – unfolded recombinant tau protein 441 using DTT and IAA, 4 – unfolded tau protein after digestion with immobilized trypsin

Table II List of tryptic fragments of tau protein 441 including highlighted epitopes of tested antibodies, generated by PeptideMass tool, set parameters — maximum number of missed cleavages (MC): 2, all cysteines have been treated with iodoacetic acid to form carboxymethyl-cysteine (Cys_CM), methionines were oxidized to form methionine sulfoxide (MSO), displaying peptides with mass higher than 500 Dalton, using monoisotopic masses of occurring amino acid residues and giving peptide masses as $[M+H]^+$

Antibody clone	$[M+H]^+$	Position	MC	Sequence
HT7	1423.7400	156-170	1	GAA**PPGQK**GQANATR
	725.3940	156-163	0	GAA**PPGQK**
BT2	1808.8158	191-209	1	SGD**RSGYS**SPGSPGTPGSR
	434.1994	191-194	0	SGD**R**
	1393.6342	195-209	0	**SGYS**SPGSPGTPGSR
8F10	3541.769	407-441	1	HLSNVSSTGSIDMVDSPQLAT**LADEVSASLA**KQGL
	3243.6049	407-438	0	HLSNVSSTGSIDMVDSPQLAT**LADEVSASLA**K
7E5	1962.0627	151-170	2	IATP**RGAAPPGQKGQA**NATR
	1263.7167	151-163	1	IATP**RGAAPPGQK**
	1423.7400	156-170	1	**GAAPPGQKGQA**NATR
	557.3405	151-155	0	IATP**R**
	725.3940	156-163	0	**GAAPPGQK**
	717.3638	164-170	0	**GQA**NATR

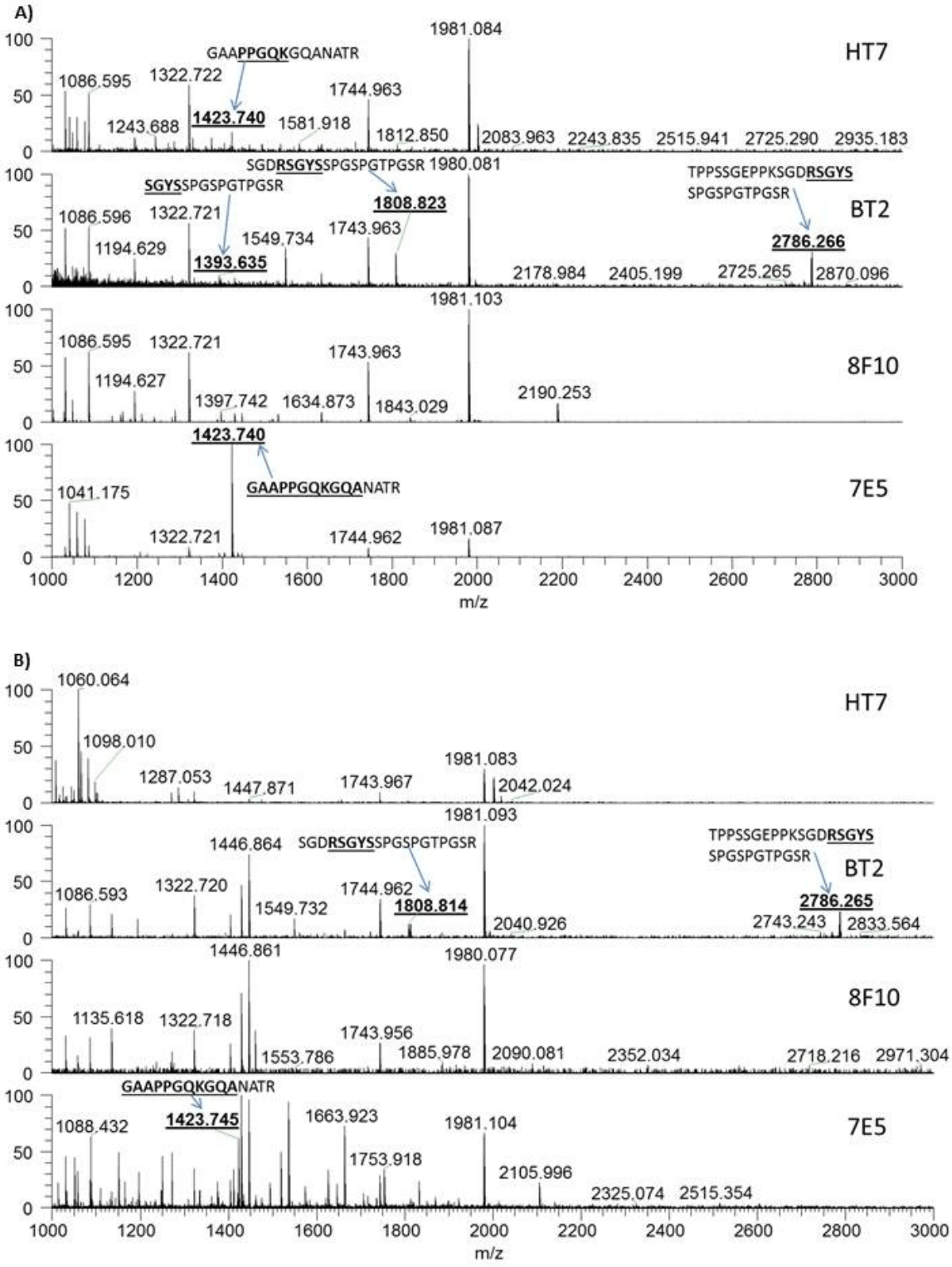

Fig. 7 MS analysis of elution fractions collected within IMS using four different anti-tau immunosorbents: A – without 8M urea incubation step, B – with 8M urea incubation step; peptides containing specific epitope sequence are highlighted in bold

MALDI-MS analysis. The results of Tris-tricine-SDS-PAGE (Fig. 6B) demon strated complete tau protein digestion. In the MS spectrum (Fig. 6A), we can see

Table III Summary of achieved results

Anti-tau mAb	HT7	BT2	8F10	7E5
Purity	pure, homogeneous and non-fragmented	pure, homogeneous and non-fragmented	pure, homogeneous and non-fragmented	pure, homogeneous and non-fragmented
Affinity index	0.75 mol l^{-1} (+)	0.75 mol l^{-1} (+)	0.75 mol l^{-1} (+)	1.3 mol l^{-1} (++)
Specific reactivity	strong	weaker	strong	strong
Cross-reactivity	-	-	-	-
Immobilization efficiency	84.7 %	64.7 %	83.25 %	82.05 %
Binding capacity (μg tau/0.5 mg IS)	0.274 μg	0.614 μg	0.497 μg	0.49 μg
Peptide reactivity (without urea)	+	+	-	+ +
Peptide reactivity (without urea)	-	+	-	+

specific tau fragments including epitopes, but also some non-specific fragments are present.

The mixture of peptide fragments obtained by tryptic digestion of tau protein (5 μg) was applied to the individual anti-tau immunosorbents. Only peptides including specific epitope should be captured by antibody, other peptides are washed away. The captured pepides are subsquently eluted with acid pH and analysed by MS. Two experiments with or without 8M urea incubation step were performed and compared.

The results of MS analysis (Fig. 7) show the most effective and specific immunocapturing peptide reaction using the immunosorbent with 7E5 antibodies, specific peak m/z = 1423.74 dominates in the spectrum of the elution fraction collected in the IMS experiment without urea incubation (Fig. 7A). Residues of this peptide are visible also in the elution fraction after treatment with 8M urea (Fig. 7B) which confirms a higher affinity of these antibodies to the specific peptide fragment. In other elution fractions, specific peptide fragments are also detected but with lower intensity and together with non-specific peptides. In the case of 8F10, peptide including specific epitope was not observed. 8M urea was applied to remove peptide fragments non-specifically adsorbed to the carrier for increasing the purity of the elution fractions, which should contain only the specific epitope-containing peptide fragments. As seen in Fig. 7B, some non-specific fragments resist the urea effect and they are still observed in the spectra, on the contrary, weaker specific interactions were suppressed which was demonstrated by the reduction (BT2, 7E5) or disappearance of specific fragments (HT7). All the obtained results are summarized in Table III.

Conclusion

Our results show that mouse monoclonal antibody, specific clone 7E5, directed against the N-terminal domain of tau protein (aa 156-165), is the most suitable from among tested anti-tau IgG molecules for the immunosorbent preparation and subsequent immunomagnetic purification of tau protein. It shows the strongest immunoreactivity with both whole tau protein as well as the specific peptide fragments. As the producer declared, these IgG molecules are phospho-insensitive. Therefore, clone 7E5 is a good candidate for the immunomagnetic purification of all forms, including hyperphosphorylated, of native tau protein from complex biological material such as cerebrospinal fluid or serum.

Abbreviations:
amino acid residues (aa); amyloid-β 1-42 (Aβ42); acetonitrile (ACN); Alzheimer's disease (AD); Nα-benzoyl-DL-arginine p-nitroanilide hydrochloride (BApNA); bicinchoninic acid (BCA); bovine serum albumin (BSA); α-cyano-4-hydroxycinnamic acid (CHCA); carboxyl group (COOH); cerebrospinal fluid (CSF); carboxymethyl-cysteine (Cys_CM); diammonium hydrogen citrate (DAHC); 2,5-dihydroxybenzoic acid (DHB); dot-blot hybridization manifold (DHM); DL-dithiothreitol (DTT); electrochemiluminescence (ECL); 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC); crystallizable fragment (Fc); horseradish peroxidase (HRP); iodoacetic acid (IAA); immunoglobulin G (IgG); immunomagnetic separation (IMS); immunosorbent (IS); linear trap quadrupole (LTQ); matrix-assisted laser desorption/ionization (MALDI); microtubule-associated proteins (MAPs); missed cleavages (MC); 2-(*N*-morpholino)ethanesulfonic acid sodium salt (MES); magnetic microparticles (MPs); messenger RNA (mRNA); mass spectrometry (MS); methionine sulfoxide (MSO); mass-to-charge ratio (*m*/*z*); amine group (NH_2); ammonium thiocyanate (NH_4SCN); phosphate buffer (PB); phosphate-buffered saline with Tween 20 (PBS-T); post-translational modifications (PTMs); polyvinylidene difluoride (PVDF); room temperature (RT); sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE); *N*-hydroxysulfosuccinimide sodium salt (Sulfo-NHS); trifluoroacetic acid (TFA); L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK); 4-chloro-1-naphthol (4CN)

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