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Myelin basic protein, an autoantigen in multiple sclerosis, is selectively processed by human trypsin 4

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Abstract **Demyelination, the proteolytic degradation of the major membrane protein in central nervous system, myelin, is involved in many neurodegenerative diseases. In the present in vitro study the proteolytic actions of calpain, human trypsin 1 and human trypsin 4 were compared on lipid bound and free human myelin basic proteins as substrates. The fragments formed were identified by using N-terminal amino acid sequencing and mass spectrometry. The analysis of the degradation products showed that of these three proteases human trypsin 4 cleaved myelin basic protein most specifically. It selectively cleaves the Arg79-Thr80 and Arg97-Thr98 peptide bonds in the lipid bound form of human myelin basic protein. Based on this information we synthesized peptide IVTPRTPPPSQ that corresponds to sequence region 93–103 of myelin basic protein and contains one of its two trypsin 4 cleavage sites, Arg97-Thr98. Studies on the hydrolysis of this synthetic peptide by trypsin 4 have confirmed that the Arg97-Thr98 peptide bond is highly susceptible to trypsin 4. What may lend biological interest to this finding is that the major autoantibodies found in patients with multiple sclerosis recognize sequence 85–96 of the protein. Our results suggest that human trypsin 4 may be one of the candidate proteases involved in the pathomechanism of multiple sclerosis.**
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Keywords: Serine protease; Trypsin; Proteolysis; Central nervous system; Myelin degradation; Autoimmune peptide

1. Introduction

Multiple sclerosis (MS), an inflammatory disease of the human central nervous system, is associated with demyelination (for reviews see Refs. [1–6]). Although autoreactive CD4$^+$ T cells are considered central to initiate inflammation, analysis of tissue lesions indicates the contribution of myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein specific CD8$^+$ T cells and antibodies as well [6]. Because myelin proteins are targets of proteolysis in MS, several brain proteases that degrade these proteins in vitro or in vivo, like matrix metalloproteinase-9 [7], calpain [8], myelencephalon-specific protease (MSP) [9] and plasminogen activators [10], have been implicated in myelinolysis. To this list now we add a new protease, human trypsin 4 that due to its immunohistochemical localization in the brain and to its substrate specificity [11] appears to be a further candidate to process the immunodominant MBP fragments in MS.

Human trypsinogen 1 and 2 genes (PRSS1 and PRSS2, respectively) are located on chromosome 7, whereas the gene (PRSS3) encoding mesotrypsinogen and human trypsinogen 4 is located on chromosome 9 [12]. Mesotrypsin or human brain trypsin (human trypsin 4) is a unique isoform of trypsin in which an arginine replaces the conserved glycine at position 193. This explains both the enhanced substrate restriction and enhanced inhibitor resistance of the protease [13,14]. Human trypsinogen 4 mRNA was the only typical trypsin-related mRNA found in the brain [15].

In the present study we have compared the proteolytic action of human trypsin 4 on human MBP in both lipid free (LF) and lipid bound (LB) forms with those of human trypsin 1 and calpain. The generated fragments and cleavage sites were identified by using N-terminal amino acid sequencing and electrospray ionization mass spectrometry (ESI-MS) analysis. The results have demonstrated that human MBP is a good substrate for human trypsin 4 and that human trypsin 4 selectively cleaves peptide bonds Arg79-Thr80 and Arg97-Thr98 of LB MBP. Thus it processes a fragment that, in line with some previous and recent proposals [1,4,5], may contain immunodominant epitopes for CD4$^+$ and CD8$^+$ myelin reactive T cell clones from MS patients.

2. Materials and methods

2.1. Isolation of MBP

Myelin basic protein was purified from a 10.4 g sample of human occipital cortex white matter. Human brain samples were obtained from Prof. Miklós Palkovits (Laboratory of Neuromorphology,

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Abbreviations: B, bound; Cbz, carbobenzyloxy; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; ESI-MS, electrospray ionization mass spectrometry; F, free; HPLC, high-performance liquid chromatography; LB, lipid bound; LF, lipid free; MBP, myelin basic protein; MS, multiple sclerosis; pNA, *para*-nitroanilide; SDS–PAGE, sodium dodecyl sulfate polyacrilamide gel electrophoresis; Tris–HCl, α,α,α-Tris-(hydroxymethyl)-methylamine hydrochloride

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Department of Anatomy, Semmelweis University, Budapest) and stored at −80 °C until use. LF MBP extraction procedure was performed by the chloroform method of Maatta et al. [16]. The total yield of LF MBP was 11.6 mg as determinated by the bicinchoninic acid method (Sigma) according to the manufacturer's instruction. The LF MBP isoforms were separated on a high-performance liquid chromatography (HPLC) column (Aquapore OD-300 220×4.6 mm, Perkin–Elmer) by elution of a gradient of acetonitrile from 30% to 60% in 0.1% trifluoroacetic acid. The purity of the separated LF MBP isoforms was tested by sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS–PAGE). The identification was performed by ESI-MS. MBP was also purified in lipid bound form by using a procedure based on 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps) solubilization according to Riccio et al. [17].

2.2. Digestion of MBP with human trypsin 4, human trypsin 1 and m-calpain

The enzyme:substrate molar ratios of 1:70 000, 1:10 000 and 1:50 for human trypsin 1, human trypsin 4 and m-calpain, respectively, were chosen to result in similar extents of digestion of human MBP. Recombinant human trypsins 1 and 4 were prepared as described previously [18], and m-calpain was obtained from Dr. Péter Tompa, Institute of Enzymology, Budapest. Buffers contained 20 mM, pH 7.5, α,α,α-Tris-(hydroxymethyl)-methylamine hydrochloride (Tris–HCl) and 10 mM $CaCl_2$ for all digestions and additional 5 mM 2-mercaptoethanol for the calpain digestion. The digestions were carried out at room temperature and aliquots were taken at 5, 15, 30 and 60 min. The resulting proteolytic fragments were separated by SDS–PAGE (17%), electroblotted onto ProBlot polyvinylidene difluoride membranes (PE Biosystems) and then subjected to N-terminal amino acid sequencing by a Procise sequencer (ABI 494, Applied Biosystems). The 1 hour aliquots of the human trypsin 4 and m-calpain digests were also analyzed by a Hewlett Packard HPLC-MS/ESI system.

2.3. Solid-phase peptide synthesis

Oligopeptides (MBP-fragments IVTPRTPPPSQ and IVTPR-Phospho-T-PPPSQ and internal standard HAAPPSADIQIDI) were synthesized by an ABI 431A Automated Peptide Synthesizer with Fmoc chemistry. All chemicals used were of analytical or sequencing grade. HPLC-grade acetonitrile was obtained from Romil (Great Britain). *N*,*N*-diisopropylethylamine, trifluoroacetic acid and all chemicals for the peptide synthesis and sequencing were obtained from ABI-Perkin–Elmer (USA).

2.4. Enzymatic digestion of synthesized oligopeptides

56 μM of both synthetic MBP fragments was digested with 0.009 μM human trypsin 4 and human trypsin 1, while the concentration of m-calpain was 1.9 μM. Composition of the reaction buffers were 0.05 M Tris–HCl of pH 8.0 and 0.02 M $CaCl_2$ for human trypsins and 0.02 M Tris–HCl, pH 7.5, 0.01 M $CaCl_2$ and 0.005 M β-mercaptoethanol for the human m-calpain digestion. Activites of human trypsins 4 and 1 were the same on the Cbz-Ala-Ala-Pro-Arg-pNA substrate (Sigma). Three independent digestions were carried out with 5 μl of enzymes in 150 μl reaction volume at 25 °C. The digestions were terminated at 0, 10, 30 and 60 min by transferring 30 μl aliquots to the injector tubes containing 10 μl of 5 M acetic acid. The aliquots were analyzed by an automated HPLC system.

2.5. RP-HPLC analysis

A PE-ABI automated HPLC instrument with a 140 C Microgradient System, a Series 200 Autosampler, a 785 A Programmable Absorbance Detector, and a 900 Series Interface equipped with an Aquapore OD 300 reversed phase column (2.1×220 mm, Perkin–Elmer) was used for the separations. 15 μl aliquots of the terminated digestion mixtures were loaded onto the column. Chromatographic runs were carried out with the general conditions as follows: 300 μl/min flow rate; eluent A was water containing 0.1% (v/v) trifluoroacetic acid; eluent B was 80% acetonitrile and 20% water containing 0.1% (v/v) trifluoroacetic acid. A 10 min equilibration followed by a 0–35% B linear gradient was applied at 220 nm detection wavelength. Chromatographic data

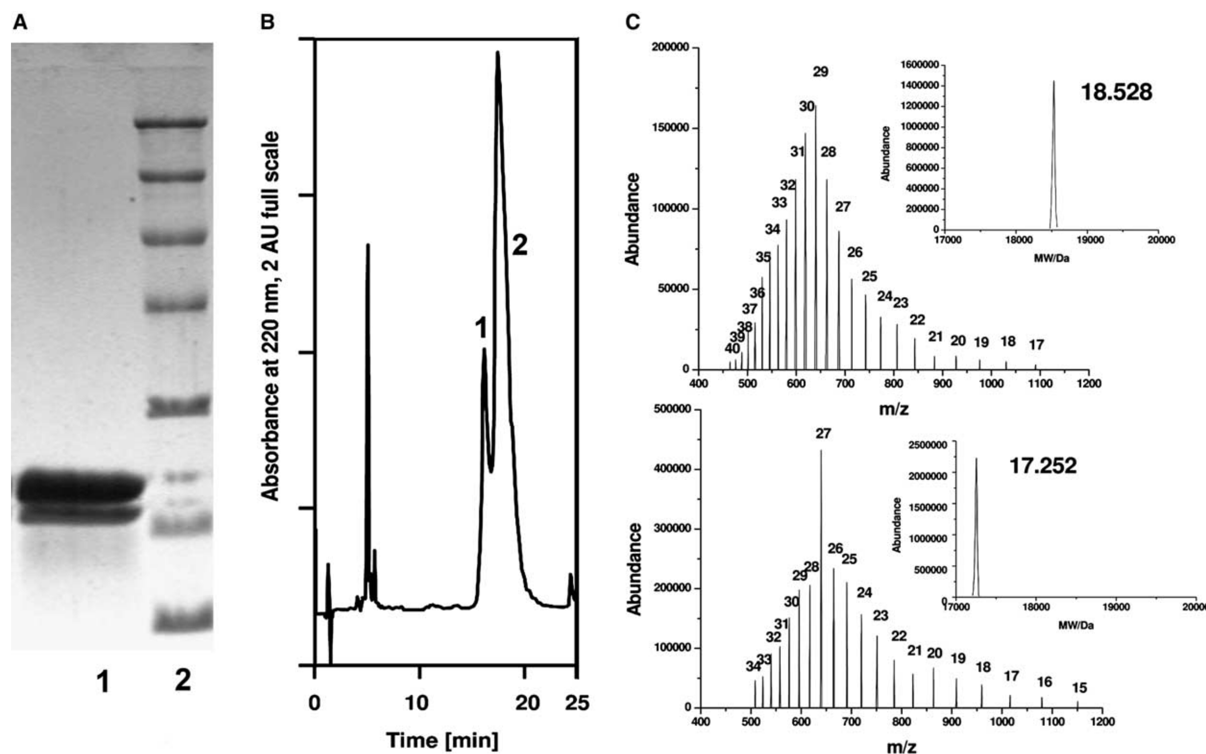

Fig. 1. SDS–PAGE of LF human myelin basic protein isoforms (lane 1: myelin basic proteins; lane 2: protein molecular weight markers (Fermentase), β-galactosidase 116 kDa, bovine serum albumin 66.2 kDa, ovalbumin 45 kDa, lactate dehydrogenase 35 kDa, restriction endonuclease Bsp98I 25 kDa, β-lactoglobulin 18.4 kDa, lysozyme 14.4 kDa.) (A). HPLC chromatography of human myelin basic protein isoforms. Peak 1 contains the 17.2 kDa LF MBP isoform and peak 2 the 18.5 kDa LF MBP isoform (B). Mass spectrogram of myelin basic protein isoforms, with molecular masses of 18.52 and 17.25 kDa, respectively (C).

obtained by RP-HPLC were collected and quantitated by the Perkin–Elmer Turbochrom 4 chromatography software.

3. Results

3.1. Isolation and identification of MBP

LF and LB human myelin basic proteins were purified from occipital cortex white matter with the chloroform [16] and Chaps extraction methods [17], respectively. Two bands were identified in the LF MBP by SDS–PAGE (Fig. 1A). After HPLC separation of the two proteins (Fig. 1B) their molecular masses were determined by ESI-MS analysis (Fig. 1C) and found to be 17.25 (Peak 1) and 18.52 kDa (Peak 2), respectively. These correspond to the two major human myelin basic protein isoforms, MBP4 (isoform 6) and MBP3 (isoform 5) (Fig. 1C).

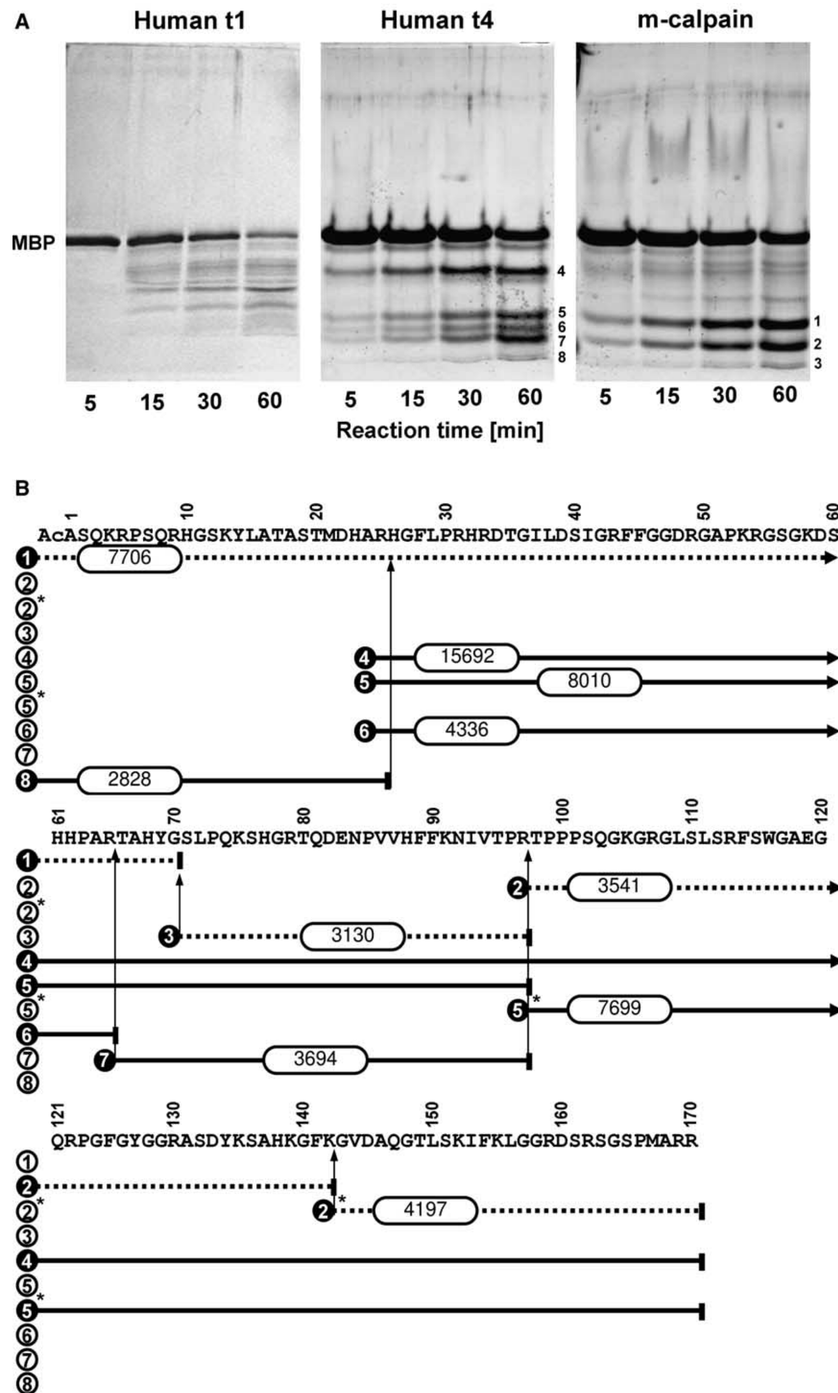

Fig. 2. As followed by SDS–PAGE, the time-course of the digestion of LF MBP3 by human trypsin 1, human trypsin 4 and m-calpain. The enzyme:substrate ratios (mol:mol) were 1:70 000, 1:10 000 and 1:50, respectively. The bands corresponding to the newly formed fragments are marked with arabic numbers on the gels (A). The fragments isolated from the m-calpain and human trypsin 4 digests with their molecular masses shown and the identified cleavage sites in the structure of LF MBP3 (see Table 1) are demonstrated in (B).

3.2. Digestion of MBP with human trypsin 1, human trypsin 4 and m-calpain

Fig. 2A shows the time-course of degradation of human LF MBP3 with three different proteases. Human trypsin 1 was used as a reference protease in order to compare its specificity to that of human trypsin 4. During the incubation period distinct patterns of fragments emerged. Keeping the different protease to substrate ratios in mind (see Section 2), it appears that human trypsin 1 hydrolyzes MBP3 much less selectively than human trypsin 4 and m-calpain. Cleavage patterns by the latter two proteases, however, significantly differ from each other. It is interesting to note that a similar extent of digestion of human MBP3 by human trypsin 4 requires about 200 times less human trypsin 4 than m-calpain (Fig. 2A). Identification of the LF MBP fragments generated by m-calpain (fragments 1–3 in Table 1 and Fig. 2B) and human trypsin 4 (fragments 4–8 in Table 1 and Fig. 2B) was performed by N-terminal amino acid sequencing and mass spectrometry of the electroblotted fragments. According to this analysis calpain cleaves peptide bonds Gly70-Ser71, Arg97-Thr98, Ala131-Ser132 generating fragments 1–70 (fragment 1), 71–97 (fragment 3), 98–131 (fragment 2) and 132–170 (fragment 2*) with molecular masses of 7706, 3130, 3541 and 4200 Da (the last value was estimated by SDS–PAGE only). By using the same strategy the following peptide bonds were located as human trypsin 4 cleavage sites for LF MBP: Arg25-His26, Arg65-Thr66 and Arg97-Thr98 (Table 1 and Fig. 2B). Human trypsin 4 and m-calpain have one common cleavage site, peptide bond Arg97-Thr98 that is, in fact, the major cleavage site for both human trypsin 4 and m-calpain. The digestions by human

Table 1
LF MBP fragments obtained by human trypsin 4 and m-calpain digestions

Bands in the m-calpain digest	Fragment	Molecular mass (Da)	Amino acid positions	Bands in the human t4 digest
	HGFLP	15692	26–	4
1	N-terminus[b]	7706	1–70	
	(HGFLP) ND[c]	8010	26–97	5
	TPPPSQ	7699	98–	5[a]
	HGFLPR	4366	26–65	6
2[a]	SDYKS	(4197) ND[d]	132–	
2	TPPPSQ	3541	98–131	
	TAHYGS	3694	66–97	7
3	SLPQK	3130	71–97	
	N-terminus[b]	2828	1–25	8

[a]Second fragment in the same band.
[b]Blocked (acetyled) N-terminus, not detectable by amino acid sequencing.
[c]Not detected by sequencing.
[d]Not detected by MS.

Table 2
Comparison of fragments obtained by digestion with human trypsin 4 of LF and LB MBP

Bands from the LB MBP digest with human t4	Fragment	Molecular mass (Da)	Amino acid positions	Bands from LF MBP digest with human t4
	HGFLP	15692	26–	4
A	N-terminus[c]	10821[e]	1–97	
A[a]	TQDEN	9823[e]	80–	
A[b]	N-terminus[c]	8697[e]	1–79	
	(HGFLP) ND[d]	8010	26–97	5
	TPPPSQ	7699	98–	5[a]
	HGFLPR	4366	26–65	6
B	GLSLSR	6693[e]	108–	
	TAHYGS	3694	66–97	7
C	TQDEN	2141[e]	80–97	
C[a]	TPPPSQ	1024[e]	98–107	
	N-terminus[c]	2828	1–25	8

[a]Second fragment in the same band.
[b]Third fragment in the same band.
[c]Blocked (acetyled) N-terminus, not detectable by sequencing.
[d]Not identified by sequencing.
[e]Not determined by MS.

trypsin 4 of LB MBP and LF MBP were also compared (Table 2, Fig. 3A and B). Fragments originating from the Arg25-His26 and Arg65-Thr66 peptide bond cleavages (see LF MBP processing) could not be detected in the LB MBP digest. Instead, the Arg79-Thr80 and Arg107-Gly108 cleavage sites are unique to LB MBP. These are close in the amino acid sequence to peptide bond Arg97-Thr98 that in the case of LB MBP also appears to be the most susceptible cleavage site for trypsin 4.

3.3. Digestion of synthetic MBP fragments by human trypsin 4, human trypsin 1 and calpain

As Fig. 4 shows, hydrolysis of human MBP fragment **IVTPRTPPPSQ** by the same amounts of human trypsins 4 and 1 result in comparable extents of cleavage: 65% and 77% of the peptide remained intact, respectively. With this experiment for the first time we demonstrated a higher hydrolytic activity of human trypsin 4 than trypsin 1 on a peptide substrate. In contrast, m-calpain did not attack this fragment at all (Fig. 4). As data in Table 3 show, the Thr98-phosphorylated fragment of MBP, **IVTPR-Phospho-T-PPPSQ** resists all proteases tested.

4. Discussion

Several lines of evidence support that T cell responses to certain proteins of the protective myelin sheath around neuronal axons play an important role in the pathomechanism of MS. Though debated by some authors [2,3,6], increased levels and characteristic specificitiy profiles of anti-MBP response in the cerebrospinal fluid and brain of patients with MS have also been reported [1,4,5]. The myelin sheath may by demaged by several mechanisms such as digestion of surface myelin by proteases in macrophages and glial cells, by cytokine-mediated injuries or even via direct attacks by $CD8^+$ T cells, by antibodies and complement [6]. Different proteolytic enzymes have been implicated in myelinolysis [7–10]. In an in vitro study, here we have shown that the proteolytic processing of LB MBP by recombinant human trypsin 4 generates an MBP fragment, residues 80–97 (Table 2, Fig. 3), that comprises the structure of a putative immunodominant T cell epitope of MBP, residues 85–96 [1,4–6]. We have also shown that human trypsin 4 digests MBP more efficiently than calpain (Fig. 2), another protease implicated in the pathomechanism of MS [8]. Our preliminary studies on the localization of trypsinogen 4- and trypsin 4-like immunoreactivities in glial cells of human spinal cord and brain [11] lend further support to the possibility that trypsin 4 may function as a myelin processing protease of microglia and oligodendroglia.

Recently, a significantly increased $CD8^+$ T cell response has been described to MBP peptides containing residues 87–95 and 111–119 [4]. Based on this finding the possibility should also be considered that trypsin 4 may be involved the generation of a cytotoxic T cell response attacking oligodendrocytes that express both MHC class I molecules and MBP.

From chemical point of view human MBP is a 170-amino acid protein, containing 19 arginyl and 12 lysyl residues which account for its basic character. MBP strongly interacts with the lipids in the multilamellar myelin sheath that surrounds the axons. Though the exact secondary and tertiary structures of MBP within the myelin sheath have not been elucidated yet, a common feature of the proposed structural models is that the immunodominant epitope center of the protein, or at least a part of it, is located at the cytoplasmic surface of oligodendrocytes [1,19,20]. This suggestion is in line with our results

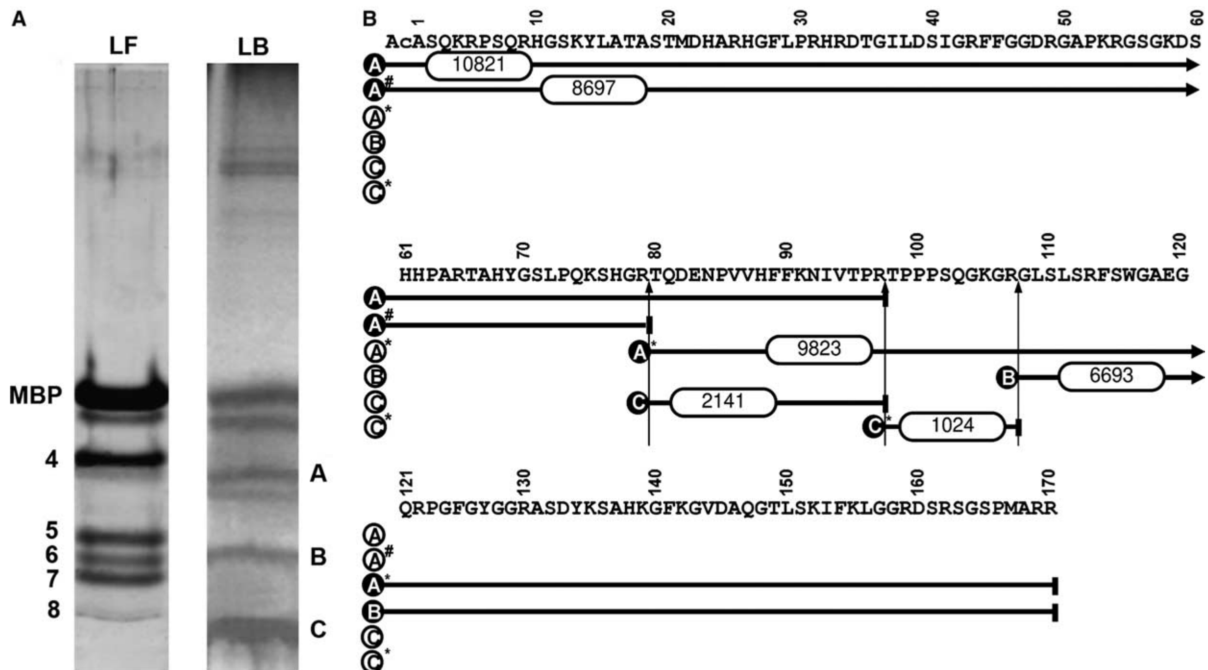

Fig. 3. Comparison by SDS–PAGE of the LF and LB MBP digestion by human trypsin 4. Arabic numbers and capital letters refer the proteolytic fragments formed from LF and LB MBP, respectively (A). (B) The proteolytic fragments from LB MBP with their molecular masses and the trypsin 4 cleavage sites in the structure of MBP.

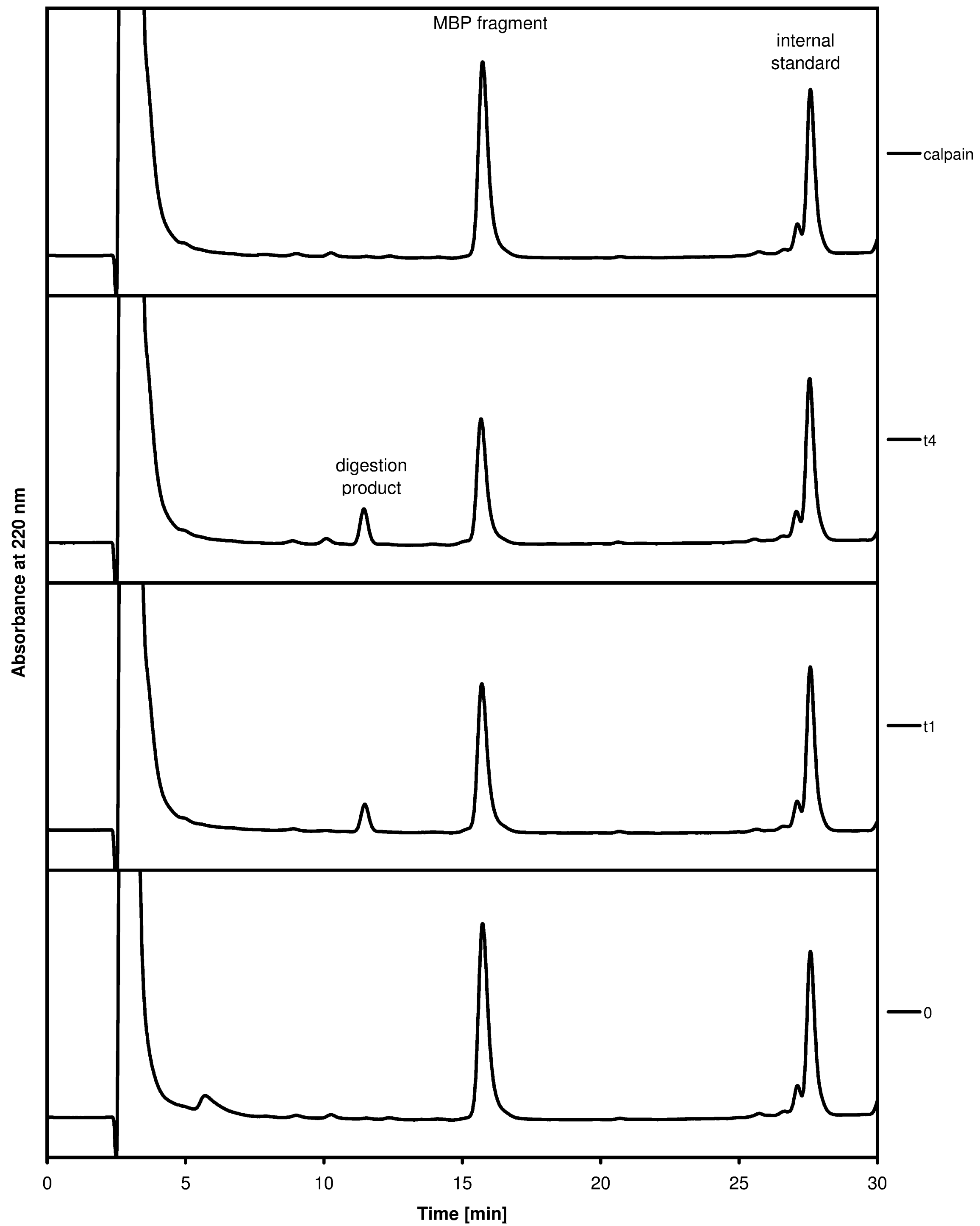

Fig. 4. Hydrolysis of the non-phosphorylated synthetic MBP fragment IVTPRTPPPSQ by calpain, human trypsin 4 (t4) and human trypsin 1 (t1) as followed by RP-HPLC. '0' is the untreated MBP peptide.

Table 3
The percentage of residual peak area of the undigested synthetic MBP fragments in the different digests

	Trypsin 1	Trypsin 4	Calpain
IVTPRTPPPSQ	77.25	65.08	100
IVTPR-Phospho-T-PPPSQ	100	100	100

With the same amount of proteases IVTPRTPPPSQ digestion was carried out for 10 min and IVTPR-Phospho-T-PPPSQ digestion for 60 min.

according to which peptide bond Arg97-Thr98 in both LF and LB MBP are preferentially cleaved by the proteases examined (Figs. 2 and 3). The protective effect of lipids against proteolysis is clearly illustrated by the difference of cleavage patterns of LF and LB MBPs (Fig. 3). It may follow from these considerations that the antibody response in MS is directed either to this exposed region of intact LB MBP, or to the processed epitope. Another consequence of the unique structural feature of this region, residues 93–103, is that some of its residues are subject to post-translational modifications, like phosphorylation of Thr98 by a mitogen-activated protein kinase [21]. Post-translational modifications of MBP are thought to play a key role in the demyelinating process [22]. In this context it is interesting to note that in our model experiments with synthetic peptides trypsin 4 cleaved the Arg97-Thr98 bond in MBP fragment IVTPRTPPPSQ (residues 93–103) more efficiently than human trypsin 1 (Fig. 4 and Table 3). The phosphorylation of Thr98 to yield peptide IVTPR-phospho-T-PPPSQ, however, rendered peptide bond Arg-phospho-Thr, residues 97–98, in the peptide resistant toward all three proteases tested (Table 3).

Concerning the biological function of trypsin 4 in human brain only two possibilities have been reported so far. In astrocytes of transgenic mice expressing human trypsinogen 4 enhanced GFAP expression and an increase in the amount of amyloid fragments were found [23]. A recent study on human cell lines suggests that human trypsin 4 might function as an agonist of protease-activated receptors, PAR 2 and 4 [24]. As a further possibility, our present study suggests that human trypsin 4 may be one of the proteases involved in the pathomechanism of multiple sclerosis. This proposal, together with our preliminary data on the detection and immunohistochemical localization of the active enzyme in some glial cells of human brain [11], is based on the present in vitro experiments that demonstrate efficient and specific actions of trypsin 4 on LF and LB MBP and small synthetic peptides containing a physiologically (or pathologically) crucial processing site in MBP [21,22].

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