Journal of Peptide Science
J. Pept. Sci. 2007; **13**: 803–810
Published online 8 October 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.902

JOURNAL OF PEPTIDE SCIENCE

Identification, affinity characterisation and biological interactions of lectin-like peptide–carbohydrate complexes derived from human TNF-α using high-resolution mass spectrometry

ANDREAS MARQUARDT, BOGDAN BERNEVIC and MICHAEL PRZYBYLSKI*

Department of Chemistry, Laboratory of Analytical Chemistry and Biopolymer Structure Analysis, University of Konstanz, 78457 Konstanz, Germany

Received 20 January 2007; *Revised 21 February 2007*; *Accepted 27 February 2007*

Abstract: A cyclic disulfide heptadecapeptide (TIP17ox; **2**) derived from the lectin-like 17-amino acid domain of human tumor necrosis factor-α [TNF-α (100–116)] was synthesised and demonstrated to bind specifically to *N*,*N*-diacetylchitobiose, a disaccharide present in many glycan structures of glycoproteins. Although the TIP domain forms a loop structure in the native TNF-α protein, we show in this study by high-resolution ESI-FTICR mass spectrometry that a homologous linear heptadecapeptide (TIP17rd; **1**) binds with comparable affinity to chitobiose, suggesting that cyclisation is not essential for carbohydrate binding. ESI-FTICR-MS was used as an efficient tool for the direct molecular characterisation of TIP peptide–carbohydrate complexes. The specific binding of the TNF-TIP domain to chitobiose and other carbohydrate motifs in glycoproteins may explain the high proteolytic stability of these peptides in biological fluids. A considerably higher proteolytic stability in human plasma was found by mass spectrometric analysis for the cyclic TIP peptide **2**, compared to the linear peptide **1**. Furthermore, affinity-proteomics studies using immobilised cyclic TIP peptide **2** provided the identification of specific interacting glycoproteins in plasma. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: TNF-α; lectin-like peptide domain; cyclic TIP peptide–carbohydrate complexes; ESI-FTICR mass spectrometry; proteolytic stability; glycoprotein interactions

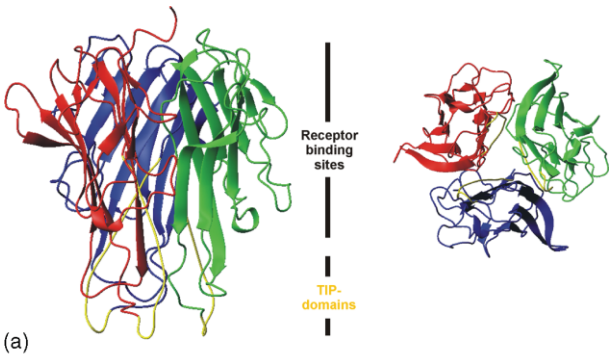
INTRODUCTION

The human tumour necrosis factor (hTNF) TNF-α is a member of the cytokine family which is involved in the regulatory control of cellular and organismal inflammatory processes [1]. TNF-α is secreted by many different cell types, especially by activated macrophages and lymphocytes [2], which among others induce hemorrhagic necrosis of certain tumours *in vivo* by binding to the high-affinity receptors (TNFR1 and TNFR2) present on most cell membranes [3]. Overproduction of TNF-α as a result of age or genetic defects has been implicated in several inflammatory diseases, including multiple sclerosis, Crohn's disease and rheumatoid arthritis, the latter caused by TNF-induced apoptosis of cartilage cells [4,5].

In addition to its receptor-binding activities, TNF-α also exerts lectin-like affinity of hitherto undefined molecular specificity; e.g. TNF-α has been shown to interact with uromodulin, an immunosuppressive glycoprotein in human urine [6]. The binding to uromodulin can be blocked by carbohydrates such as *N*,*N*-diacetylchitobiose and branched mannoses, while other oligosaccharides have no inhibitory effect [6]. TNF-α has also been found to inhibit the development of certain parasites, e.g. trypanosomes [7]. Both the trypanolytic and the lectin-like activities are mediated by a 15-amino acid peptide domain ('TIP domain' TNF-α(101–115); CQRETPEGAEAKPWY), which is functionally and spatially distinct from the receptor binding sites [8], as shown by the X-ray crystal structure of human TNF-α (PDB code 1TNF; Figure 1(a)) [9].

Recent studies have shown that peptides derived from the TIP domain of TNF-α can lead to resorption of lung fluids by activating sodium channels in a mouse lung model [10]. Interestingly, these peptides are active only if injected directly into the lung, whereas the activity is lost after intravenous administration. These findings and recent studies of lectin-derived peptide–carbohydrate interactions in our laboratory prompted our interest to characterise the carbohydrate affinities of linear and cyclic TNF-TIP peptides, and investigate their proteolytic stabilities and specific biological interactions. In the present study we report the application of high-resolution FTICR mass spectrometry as a powerful tool for the direct characterisation of TIP peptide–carbohydrate complexes, their proteolytic degradation in plasma and identification of specific glycoprotein interactions.

*Correspondence to: Michael Przybylski, Department of Chemistry, Laboratory of Analytical Chemistry, University of Konstanz, Universitaetsstrasse 10, 78457 Konstanz, Germany;
e-mail: Michael.Przybylski@uni-konstanz.de



(a)

1 VRSSSRTPSDKPVAHVVANPQAEGQLQWLN
31 RRANALLANGVELRDNQLVVPSEGLYLIYS
61 QVLFKGQGCPSTHVLLTHTISRIAVSYQTK
91 VNLLSAIKSPCQRETPEGAEAKPWYEPIYL
121 GGVFQLEKGDRLSAEINRPDYLDFAESGQV
(b) 151 YFGIIAL

Figure 1 (a) Ribbon representation of the X-ray crystal structure of the trimeric complex of human TNF-α (PDB 1TNF [9]. The TIP-domains are coloured in yellow. (b) Sequence of the human TNF-α. The TIP sequence is highlighted in yellow.

MATERIALS AND METHODS

Peptide Synthesis

All peptides were synthesised as carboxamides on a semi-automatic peptide synthesiser (EPS 221; Intavis, Langenfeld, Germany) according to the Fmoc-synthesis strategy (Table 1) on a NovaSyn TGR resin. Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium-hexafluorophosphate (PyBOP) and *N*-methyl-morpholine were used as coupling reagents. All coupling steps were carried out in dimethylformamide (DMF). *N*-α-Fmoc-protected amino acids were used with the following side-chain protections: Arg(Pmc), Lys(Boc), Thr(tBu), Glu(OtBu), Gln(Trt), Cys(Trt), Cys(Acm), Tyr(tBu). Deprotection of Fmoc was carried out with 2% piperidine and 2% 1,8-diazabicyclo(5.4.0)undec-7-ene (DBU) in DMF. *N*-Terminal acetylation of peptide **3** (Table 1) was performed following SPPS with acetic anhydride (1 ml) and diisopropylethylamine (1 ml) in DMF for 1 h to yield acetylated 17-peptide **5**. Cleavage of the peptides from the resin and simultaneous side-chain deprotection (except for Cys-acetamidomethyl) was performed with a mixture of 90% trifluoracetic acid (TFA), 5% triethylsilane and 5% water. Molecular masses of all peptides were confirmed by MALDI-MS.

HPLC Purification

HPLC was performed with a Waters–Millipore M590/510 instrument equipped with an M–490 UV detector. Peptides were purified on a 25 × 0.4 cm Vydac RP-C_{18} nucleosil column (Macherey-Nagel, Duisburg, Germany) using a linear binary gradient of 0.1% aqueous TFA (A) and 0.1% TFA in 80% acetonitrile (B), 10–40% B within 30 min at a flow rate of 1 ml/min. The detection wavelength was 220 nm.

Oxidation of Terminal Cysteine–Thiol Groups

The oxidation of peptide **1** (TIP17rd) was carried out in a mixture of 50% 50 mM NH_4OAc (pH 6) and 50% DMSO with a peptide concentration of 1 mg/ml; the reaction mixture was shaken overnight at 25 °C and then lyophilised. The remaining solid was dissolved in 0.1% aqueous TFA and purified by RP-HPLC. The formation of cyclic disulfide **2** (TIP17ox) was ascertained by high-resolution MALDI-FTICR-MS and by proteolytic digestion.

Proteolytic Digestion in Human Plasma

To examine the proteolytic stability, cyclic peptide **2** was dissolved in human plasma with a concentration of 10 µM. The digestion was carried out at 37 °C. After different reaction times, ranging from 30 min to 4 h, aliquots of the reaction mixture were taken out, mixed with an equal volume of 0.1% TFA to stop the digestion and concentrated using ultra-centrifugation units with a cut-off mass of 10 000 Da. Prior to mass spectrometric analysis, a further desalting step was applied using Millipore ZipTip-C_{18} tips.

In-gel Digestion of TIP Peptide Affinity-isolated Plasma Glycoproteins

For affinity experiments, a peptide micro-column was employed by immobilising the cyclic TIP peptide **2** on NHS-activated Sepharose according to the instructions of the supplier. Affinity experiments were performed by loading 100 µl of

Table 1 Sequences and monoisotopic masses of the synthesised peptide amides used

No.	Name	Sequence	Monoisotopic molecular mass		
			calc.	exp.	Δm (ppm)
1	TIP17rd	H-CGQRETPEGAEAKPWYC-NH_2	1922.8403	1922.8447	2.3
2	TIP17ox	H-CGQRETPEGAEAKPWYC-NH_2	1920.8247	1920.8265	0.9
3	TIP17lr	H-CGQRETPEGAEAKPWYC(Acm)-NH_2	1993.8775	1993.8721	2.7
4	TIP15wt	H-CQRETPEGAEAKPWY-NH_2	1762.8097	1762.8166	3.9
5	TIP17mut	Ac-CGQREAPAGAAAKPWYC-NH_2	1816.8137	1816.8197	3.3

DOI: 10.1002/psc

human plasma onto the affinity column. The column was gently shaken for 2 h at 20 °C and then washed repeatedly with 20 ml PBS buffer to remove unbound proteins. The affinity-bound proteins were then eluted with 10 ml of 0.1% aqueous TFA. The collected fractions were lyophilised and characterised by 1D gel electrophoresis.

For manual in-gel digestion and subsequent MALDI-MS analysis, the gels were washed with water for 15 min, and the gel spots were cut out and destained by addition of 60% acetonitrile in MilliQ water for 20 min at 25 °C. After removal of supernatant and lyophilisation of the gel spot, a solution of 50 mM NH_4HCO_3 was added for rehydration and incubated for 20 min at 25 °C. This procedure was repeated two times and the final rehydration was performed with the protease solution (12.5 ng/μl trypsin in 50 mM NH_4HCO_3) at 4 °C for 45 min. The gel spots were then incubated for 16 h at 37 °C in 50 mM NH_4HCO_3, and protein fragments were eluted three times with 60% acetonitrile in water. The eluates were lyophilised to dryness and dissolved immediately before MALDI-MS analysis in 5 μl acetonitrile/0.1% TFA in water (2 : 1).

Database Search Engines and Procedures for Identification of Plasma Proteins

The Mascot peptide mass fingerprint (PMF) search engine was employed for the identification of affinity-isolated proteins from human plasma, as previously described [11]. The search was carried out using the NCBI database [12].

FTICR Mass Spectrometry

FTICR mass spectra were recorded on a Bruker Daltonics APEX II instrument (Bremen, Germany) equipped with a 7 T, actively shielded, superconducting magnet (Magnex, Oxford, UK), and either a Bruker Apollo ESI or a Scout 100 MALDI source with an API100 controller and a UNIX-based Silicon Graphics OS2 workstation data system. Acquisition of spectra was performed with the Bruker Daltonics software XMass and corresponding programs for mass calculation, data calibration and processing. Calibration was carried out with a standard peptide mixture within an m/z range from approximately 1000 to 3500.

For electrospray measurements of non-covalent complexes, the peptides and carbohydrates were dissolved in a solution consisting of 80% 10 mM ammonium acetate (pH 6) with the addition of 20% methanol; the samples were mixed together, and after 2 h of reaction they were analysed at a flow rate of 2 μl/min. Sixty four single scans were accumulated to get a final spectrum. The capillary voltage was set to 4200 V with an end plate voltage of 3800 V and a cap exit voltage of 75 V. The accumulation time in the hexapole was 1.5 s. A nebulizing gas with a flow rate of 10 l/h was used for quicker evaporation of the samples.

For MALDI-FTICR measurement, 2,4-dihydroxy-benzoic acid (DHB) was used as the matrix with a concentration of 50 mg/ml in acetonitrile/H_2O (2 : 1) and 0.1% TFA. Before mass spectrometric analysis, 0.3 μl of matrix solution was mixed with 0.3 μl sample solution directly on the MALDI target and allowed to dry.

RESULTS AND DISCUSSION

Synthesis and Structural Characterisation of TNF-TIP Peptides

Several linear and cyclic peptide amides derived from the TIP domain (C^{101}QRETPEGAEAKPWY^{115}) of human TNF-α were synthesised by semi-automated SPPS using the Fmoc protection strategy. Table 1 summarises the sequences and the mass spectrometric characterisation of the HPLC-purified peptides. Cyclic peptides (**2, 5**) were generally designed with a disulfide linkage between the *N*-terminal Cys and an additional *C*-terminal Cys residue introduced. The cyclisation of linear TIP peptides appeared to be useful to mimic and optimise the original loop conformation in human TNF-α. In order to facilitate the cyclisation, the Pro-100 residue of the original hTNF sequence (100–116) was replaced by a cysteine residue, the original Cys-101 by a glycine residue and Glu-116 by a cysteine residue, resulting in the initial linear peptide sequence **1**. The cyclic disulfide **2** was obtained by the oxidation of the terminal Cys residues. Several methods have been previously reported for the oxidation of cysteine residues, which showed disadvantages such as slow reaction rates and the formation of by-products [13–15]. In the present study we used DMSO as the oxidising reagent under mild conditions favouring intra-molecular disulfide formation, while application of slightly acidic reaction media and dilution greatly reduced the possible concomitant inter-molecular by-products [16]. The correct formation of intra-molecular disulfide bridges was ascertained by high-resolution MALDI-FTICR mass spectrometric analysis, which in all cases provided ions due to 2 amu lower molecular masses than those for the linear sequences (Table 1).

The finding that the linear peptide **1** was slowly oxidised in solution prompted our interest to synthesise and evaluate linear TIP peptide analogues with potentially higher oxidative stabilities, but identical amino acid sequence as in peptide **1**. To this purpose, the *C*-terminal Cys residue was modified by an acetamidomethyl group (TIP17lr, **3**; Table 1), which is cleavable only under reductive conditions but not at the acidic conditions used for cleavage of the peptide from the resin with simultaneous side-chain deprotection [17]. The acetamidomethyl-protecting group thus inhibits oxidation of peptide **3** while maintaining a linear structure.

ESI-Mass Spectrometric Characterisation of TIP–Carbohydrate Complexes

TIP peptides derived from TNF-α have been shown to exert specific binding to plasma proteins, which pointed our interest toward a detailed study of the interactions of TIP peptides with carbohydrates and the direct characterisation of peptide–carbohydrate complexes.

DOI: 10.1002/psc

In a previous work, TIP peptides have been reported to bind to specific carbohydrates including chitobiose and branched mannose, and these observations have been supported by inhibition studies, showing that the lectin-like affinity can be blocked by addition of certain carbohydrates [18]. The interaction of TIP peptides with chitobiose may be of considerable biological interest since this disaccharide motif is present in many glycan structures of glycoproteins [19]. However, no structural characterisation of interactions between TIP peptides and carbohydrates has been hitherto reported.

High-resolution ESI-FTICR-MS was employed in order to characterise the specific peptide–carbohydrate interactions by direct identification of the non-covalent complexes between three different TIP peptides (**2,4,5**) and chitobiose (GlcNAc(β1–4)GlcNAc). Peptide **2** was used as the cyclic 17-peptide and 15-peptide **4** as a linear variant, corresponding to the original TIP sequence present in the hTNF protein. In addition, the 17-peptide variant **5** was used, in which the polar amino acids of the potential carbohydrate-binding motif (TPEGAE) were replaced by alanine residues (APAGAA). An exchange of the polar amino acids by alanine would be expected to diminish polar inter-molecular interactions [20,] thereby resulting in a potentially decreased carbohydrate binding affinity for this peptide. Although native TNF-α *in vivo* is present as a trimer, there was no indication for the formation of trimers of the TIP peptides from the mass spectrometric data in this study. In none of the mass spectrometric experiments were TIP oligomers observed, suggesting that the monomeric peptides are responsible for the lectin-like affinity.

The mild electrospray ionisation technique has been successfully applied in recent studies to the direct characterisation of non-covalent biopolymer complexes [21–23]. It has been shown that the mass spectrometric conditions used for detection of non-covalent complexes (e.g. flow rate, spray duration) may have a strong effect on the relative abundances of ions of complexes [24,25], and therefore the conditions for obtaining quantitative yields of non-covalent complexes have not been clarified. To keep unspecific binding to the lowest possible extent, aqueous solutions of the peptides were prepared with a 5 molar excess of carbohydrate at low micro-molar concentrations; the use of excess carbohydrate concentrations was found essential to obtain significant yields of peptide–carbohydrate complex formation. Competitive ESI-mass spectrometric analyses with mixtures of chitobose and cellobiose consistently showed specific binding of all three TIP peptides **2**, **4** and **5** to chitobiose (albeit with different affinities), while cellobiose showed substantially lower (>20-fold) binding to the peptides. To assess the effect of amino acids and the structure of TIP peptides on the affinity to chitobiose, an equimolar mixture of the three

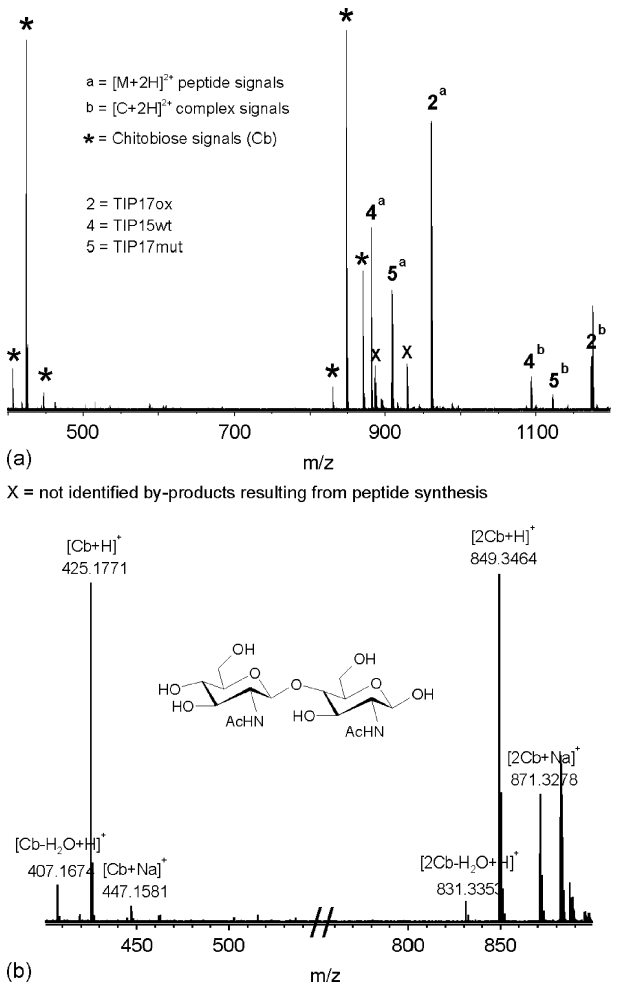


Figure 2 (a) ESI-FTICR-MS of non-covalent complexes of a mixture of chitobiose with the TIP peptides **2**, **4** and **5**. The masses of peptide ions and ions of the corresponding peptide–carbohydrate complexes are summarised in Table 2. (b) ESI-FTICR-MS analysis of chitobiose (Cb), showing the protonated and sodiated molecular ions of Cb dimers.

peptides (3.3 μM) was subjected to carbohydrate binding (50 μM) (Figure 2(a); see the monoisotopic masses of the $[C + 2H]^{2+}$ ions of the complexes and $[M + 2H]^{2+}$ ions of the free peptides in Table 2). In addition to the peptide–carbohydrate complex ions, ions derived from chitobiose were observed (Figure 2(b)), showing formation of a non-covalent dimer of the disaccharide. However, no complex formation between the chitobiose dimer and TIP peptides could be detected.

To obtain an estimation of the relative yields of the complexes, the integrals of ion signals for the free peptide and the complex were determined using the XMASS software by integrating individual isotopes of ion signals and subsequent accumulation of isotopic integrals to a final integral for the peptide–carbohydrate complexes. These results (Table 3) suggested approximately equal affinities for the cyclic and linear TIP peptides **2** and **4**, while the mutant peptide **5** showed a lower (*ca* 30–40%) affinity, in agreement with competitive binding studies

DOI: 10.1002/psc

Table 2 Masses of peptides and peptide–carbohydrate complexes of the signals present in the ESI-FTICR spectrum shown in Figure 2(a)

No.	Peptide	Monoisotopic mass of peptide			Monoisotopic mass of complex			% Yield of complex (absolute/**relative**)
		$[M+2H]^{2+}_{calc}$	$[M+2H]^{2+}_{exp}$	Δm (ppm)	$[C+2H]^{2+}_{calc}$	$[C+2H]^{2+}_{exp}$	Δm (ppm)	
2	TIP17ox	961.4196	961.4202	0.6	1173.5043	1173.5018	2.1	15,8/**92**
4	TIP15wt	882.4121	882.4111	1.2	1094.4968	1094.4946	2.0	17,1/**100**
5	TIP17mut	909.9181	909,9217[a)]	3.9	1122.0027	1122,0084[a)]	5.1	11,2/**66**

[a)] Most abundant isotope.

Table 3 Monoisotopic molecular masses and amino acid sequences of proteolytic fragments of TIP peptide **3** in human plasma identified by MALDI-FTICR-MS (Figure 4(b))

Fragment	$[M+H]^+$ measured	$[M+H]^+$ calculated	$[M+Na]^+$ measured	$[M+Na]^+$ calculated	Sequence
8–17	1152.5162	1152.5150	—	—	EGAEAKPWYC
8–17	—	—	1174.4916	1174.4962	EGAEAKPWYC
8–17	1223.5536	1223.5491	—	—	EGAEAKPWYC(Acm)
8–17	—	—	1245.5345	1245.5333	EGAEAKPWYC(Acm)
6–17	1350.6131	1350.6147	—	—	TPEGAEAKPWYC
6–17	1421.6542	1421.6476	—	—	TPEGAEAKPWYC(Acm)
5–17	1479.6622	1479.6569	—	—	ETPEGAEAKPWYC
5–17	1550.6985	1550.6960	—	—	ETPEGAEAKPWYC(Acm)
5–17	—	—	1572.6763	1572.6646	ETPEGAEAKPWYC(Acm)
1–17	1921.8386	1921.8421	—	—	CGQRETPEGAEAKPWYC (cyclic)
1–17	1994.8820	1994.8868	—	—	CGQRETPEGAEAKPWYC(Acm)

by affinity mass spectrometry and with biological experiments, indicating significantly reduced binding of the mutant peptide [21]. Control experiments of the complex formation in a strongly acidic solution (2% acetic acid: methanol, 1 : 1) at identical peptide and carbohydrate concentrations showed essentially diminished complex formation for all peptides, suggesting the specificity of the peptide–carbohydrate interaction. Affinity MS studies indicated low micro-molar (*ca* 3–8) binding affinities for the TIP peptide–carbohydrate complexes (data not shown). The development of independent quantification methods of affinities (ELISA, SPR) is being carried out at present in our laboratory.

The result from the mass spectrometric data that the sequential variant TIP peptide **5** with the alanine replacements APAGAAA exhibits strongly diminished, yet some residual affinity to chitobiose suggested that the hexapeptide sequence TPEGAE represents only a partial binding motif for the lectin-like activity of TIP peptides. Furthermore, the MS data indicate that cyclisation of the TIP peptides and formation of a loop structure do not appear to be strict prerequisites for obtaining affinity to carbohydrate ligands. With the assumption that the carbohydrate-binding motif comprises a relatively short amino acid sequence, cyclisation may not have a substantial impact on the binding, and hence affinities of linear and cyclic TIP peptides may be similar. In order to further characterise the essential binding affinity motif, partial TIP peptides were synthesised and their complex formation with chitobiose were determined by ESI-MS analysis. The ESI-FTICR spectrum of the shorter TIP sequence, TNF(105–111) (TPEGAE) indeed showed a clearly identifiable complex ion with chitobiose (Figure 3), together with the ions of the free peptide (*m/z* 602) and the monomer and dimer ions of the carbohydrate sequences. Studies on partial and modified TIP peptide–carbohydrate complexes using a combination of high-resolution mass spectrometry and NMR methods are presently being carried out to clarify molecular details of the peptide–ligand interaction.

Proteolytic Stability in Serum and Affinity Characterisation of the TIP Lectin-binding Domain

TIP peptides are rapidly inactivated after intravenous administration, which was thought to be caused by proteolytic degradation of the peptides in blood. In order to ascertain this assumption, stability studies using mass

DOI: 10.1002/psc

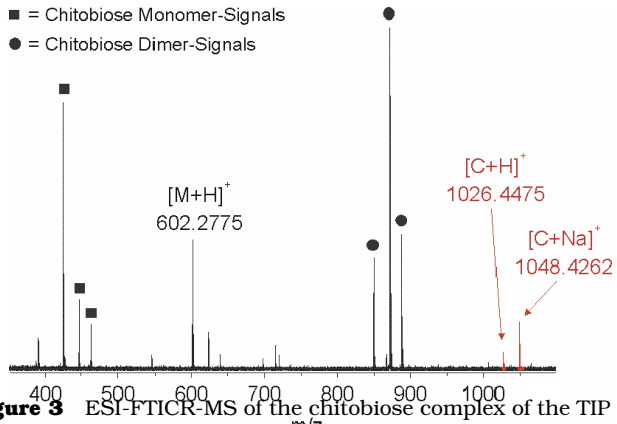


Figure 3 ESI-FTICR-MS of the chitobiose complex of the TIP partial peptide, TPEGAE (see Figure 2 and Table 2 for details of experimental conditions). This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

spectrometric analysis were performed upon spiking of human plasma with different TIP peptides. These studies revealed substantially increased stabilities of the cyclised TIP peptides against proteolytic degradation in plasma in comparison to the linear peptides. No significant proteolytic fragmentation of peptide **2** was observed by MALDI-MS after 2 h (Figure 4(a)). In contrast, MALDI-FTICR-MS of the linear peptide **3** (Figure 4(b)) showed a number of degradation products at the same reaction time, predominantly by *N*-terminal degradation (Table 3). A search for partial sequences of peptide **3** was performed using the GPMAW 6.02 program (Lighthouse Data) by subjecting all m/z values that were observed with a signal-to-noise ratio >3 to a partial sequence search, which provided unequivocal matching of partial sequences, obviating the determination of background spectra. This detailed database analysis revealed that: (i) all proteolytic fragments comprise the *C*-terminal part of the peptide sequence, suggesting that this part is protected from digestion, (ii) the acetamidomethyl-protecting group was not stable against degradation in plasma, and therefore this group appears not suitable for the design of biologically stable peptides [26], (iii) the removal of the acetamidomethyl group leads to subsequent oxidation of the TIP peptide. The resulting cyclic peptide, analogous to peptide **2**, was found to be stable against proteolytic fragmentation. A possible explanation for this result would be the efficient binding of the cyclic TIP peptide to proteins in plasma to inhibit their proteolytic cleavage.

Identification of Lectin-binding Glycoproteins in Plasma by Affinity Proteome Analysis Using TIP Peptides

The observations that TIP peptides: (i) are resistant to proteolytic digestion and (ii) show specific binding to carbohydrates prompted the assumption that

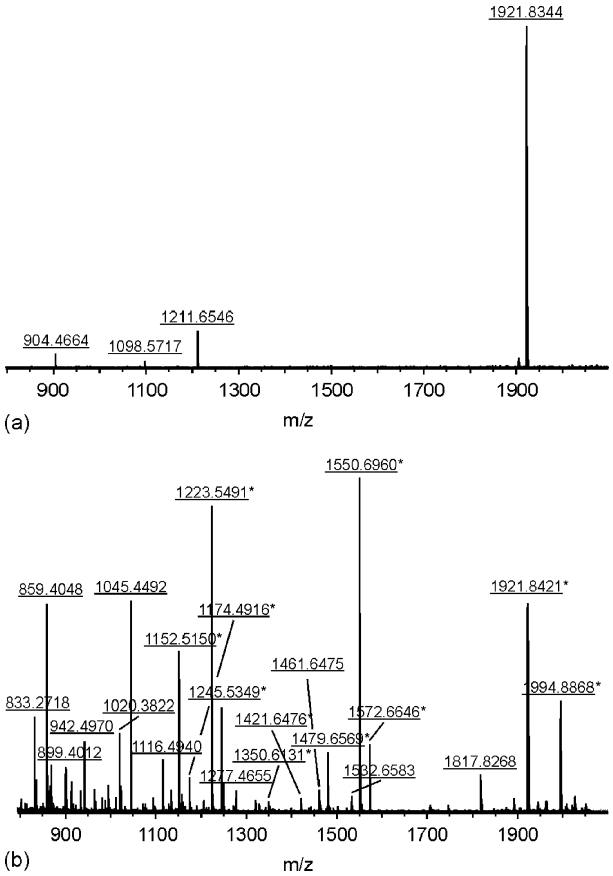


Figure 4 (a) MALDI-FTICR-MS of cyclic TIP peptide **2** after 2 h incubation in human plasma. (b) MALDI-FTICR-MS of the linear TIP peptide **3** after 2 h incubation in human plasma. Ions assigned to peptide fragments are indicated by an asterix (*), and the corresponding fragment masses are summarised in Table 3.

these peptides might exert affinity to specific plasma glycoproteins. To substantiate this assumption, affinity studies were first performed using a micro-column prepared with immobilised human plasma. Equimolar mixtures of different TIP peptides were applied on the column, and affinity-bound peptides were eluted and characterised by MALDI-MS. these experiments showed a significantly stronger binding to plasma proteins of the cyclic peptides compared to the linear TIP peptides (data not shown), which may be explained by a stabilising effect of the essential peptide conformation for the peptide–carbohydrate interaction upon cyclisation. This assumption is further supported by the much higher proteolytic stability in plasma found for the cyclic peptide.

Furthermore, identification of specific interaction proteins of the TIP peptide was obtained by mass spectrometric proteome analysis upon immobilisation of the cyclic peptide **2** to NHS-activated Sepharose. Human plasma was subjected to affinity isolation with the TIP peptide **2**, and the affinity-eluted proteins reduced with dithiothreitol (DTT) and separated by

DOI: 10.1002/psc

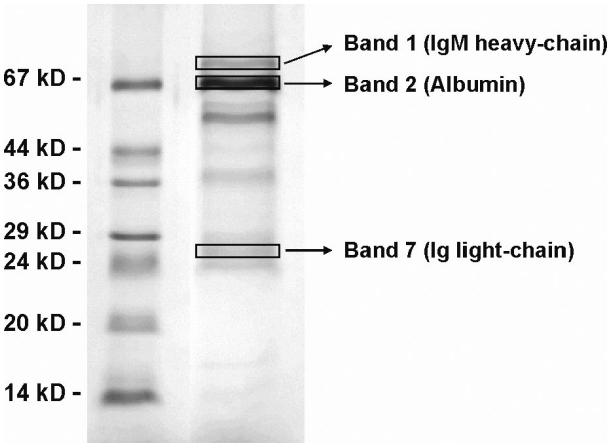
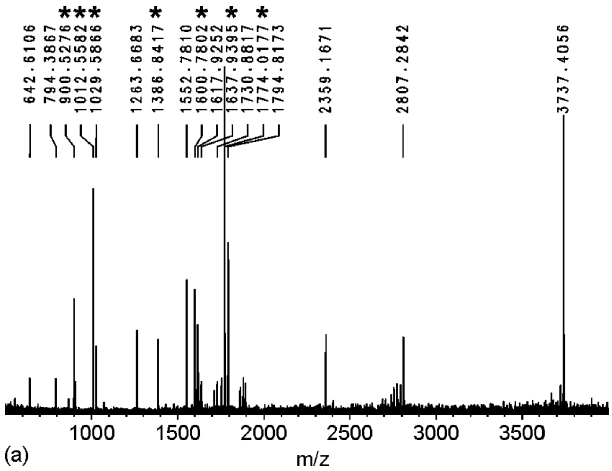


Figure 5 1D gel electrophoretic separation of human plasma proteins isolated by affinity chromatography with TIP peptide **2**. Protein bands identified as IgM, heavy chain (1), albumin (band 2) and Ig-light chain (band 7) are enclosed.

1D gel electrophoresis (Figure 5). Approximately ten distinct protein bands were manually excised and subjected to in-gel digestion with trypsin. Analysis of the proteolytic digest mixtures by MALDI-FTICR-MS and PMF database search provided the identification of protein interaction partners for TIP peptide, of which albumin (band 2) and the IgM heavy chain are noteworthy (Figure 6). Of the five different classes of human immunglobulins, only IgM was identified as a TIP interaction protein. This result is remarkable since IgM contains five different glycosylation sites each having a high content of oligomannose [27], which is in agreement with previous studies that suggest a specific interaction of TIP peptides with branched mannoses [6]. In contrast to IgM, albumin is synthesised *in vivo* without carbohydrates; however, glycosylated forms of the protein have been found in human plasma [28,29]. Since the glycosylation of albumin seems to have an influence on the binding properties, e.g. to fatty acids [30], it may be responsible for the interaction with TNF-TIP peptides.

CONCLUSIONS

TNF-α is a member of the cytokine family that stimulates acute phase reaction. Besides promoting inflammatory effects, it also possesses lectin-like properties. These properties are mediated by a 15-amino acid domain, called the TIP domain. Using electrospray ionisation mass spectrometry, we could show that synthetic peptides derived from the lectin-like domain of TNF-α are able to bind chitobiose, a disaccharide present in many glycan structures, but not cellobiose. The highest yield of peptide–carbohydrate complexes was observed with linear TIP peptides, confuting that a cyclisation of TIP peptides is essential for their carbohydrate affinity.



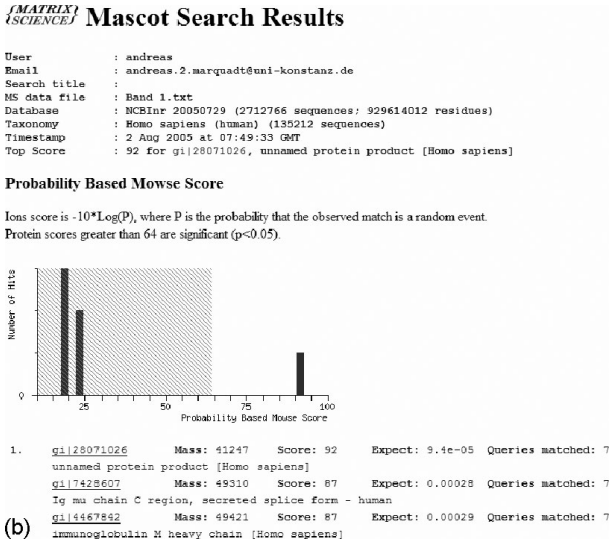


Figure 6 (a) MALDI-FTICR-MS of the in-gel tryptic digest mixture of band 1. Peptide fragments identified as IgM are denoted by asterisks. (b) Mascot data base identification of IgM heavy chain at a threshold mass determination accuracy<8 ppm.

Proteolytic stability experiments carried out in human plasma as well as in rat serum showed a much faster degradation of linear TIP peptides compared to cyclic TIP peptides, potentially caused by a stronger binding of cyclic TIP peptides to specific plasma glycoproteins. Mass spectrometry-based affinity methods were applied to identify human serum albumin and IgM as interaction partners for TIP peptides. The biological functions of these proteins with respect to their interaction with TIP peptides require further analysis.

Acknowledgements

We thank Dr Nikolay Youhnovski and Dr Xiaodan Tian for their expert assistance with FTICR-MS and affinity mass spectrometry procedures. This work has been supported by the Deutsche Forschungsgemeinschaft,

Bonn, Germany (Forschergruppe 'DNA- and Oligosaccharide chips – analysis of secondary gene products' (FOR 434)).

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