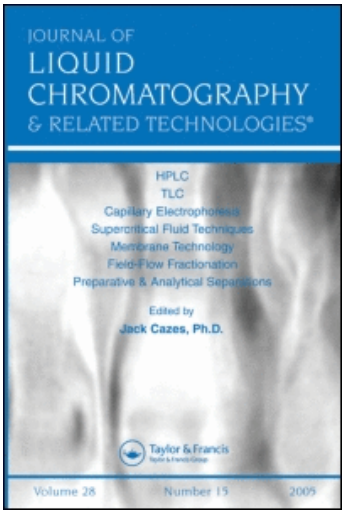

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Teffera, Yohannes and Bakhtiar, Ray(2005) 'Confirmation of the Binding Site of Cyclophilin-Cyclosporin Non-Covalent Complex Using Limited Proteolysis and Liquid Chromatography-Quadrupole-Time-of-Flight Mass Spectrometry', Journal of Liquid Chromatography & Related Technologies, 28: 10, 1497 — 1508
To link to this Article: DOI: 10.1081/JLC-200058337
URL: http://dx.doi.org/10.1081/JLC-200058337

Journal of Liquid Chromatography & Related Technologies®, 28: 1497–1508, 2005

ISSN 1082-6076 print/1520-572X online
DOI: 10.1081/JLC-200058337

Confirmation of the Binding Site of Cyclophilin-Cyclosporin Non-Covalent Complex Using Limited Proteolysis and Liquid Chromatography-Quadrupole-Time-of-Flight Mass Spectrometry

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Abstract: The limited proteolysis approach has been based on the fact that exposed regions of a protein can be subjected to time resolved cleavage with specific proteases. Although this is not a new concept, the ability to utilize modern mass spectrometry techniques in conjunction with limited proteolysis has gained prominence. Herein, we report the use of time-resolved limited proteolysis, liquid chromatography, and a hybrid quadrupole-time-of-flight mass spectrometry (LC-Q-ToF-MS), which offers sensitivity, high mass resolution, and mass accuracy, to confirm the binding pocket of human cyclophilin A—cyclosporin A (CyP:CsA) non-covalent complex. The hydrophobic binding pocket of human CyP is defined by 13 amino acid residues that are in contact of less than 4 Å with a segment of CsA. The effect of CsA binding on proteolytic susceptibility of CyP was evident. In the absence of CsA, a marker tryptic peptide resulting from the putative cleavage of lysines 91 and 119 was identified. This peptide contained five of the critical residues in the CyP binding pocket. Full protection against trypsin mediated cleavage at lysines 91 and 119 was achieved in the presence of CsA. The utility of this approach could be further realized in higher-throughput structural screening of libraries of ligands for CyP.

Keywords: Time of flight, Mass spectrometry, Trypsin, Active site, Cyclophilin

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INTRODUCTION

Cyclosporin A (CsA) is a cyclic peptide of 11 amino acid residues (Figure 1), which was originally isolated from the fungus *Tolypocladium inflatum gams.* CsA is one of the leading immunosuppressive agents, which has been prescribed to organ transplant patients. CsA mechanism of action is complex. Briefly, CsA binds to its intracellular receptor, cyclophilin (CyP), which forms a ternary non-covalent complex by binding to the calmodulin-stimulated protein phosphatase calcineurin (CN). Consequently, the CsA-CN-CyP complex inhibits de-phosphorylation of the nuclear factor of activated T-cells (NFAT), which results in prevention of interleukins production in T-cells. Hence, T-cell activation and proliferation are dramatically reduced, which lead to tolerability and long-term graft and patient survival.[1–3]

The limited proteolysis approach has been predicated on the basis that exposed regions of a protein can be subjected to time resolved cleavage with specific proteases.[4,5] Although this is not a new concept, the ability to utilize modern mass spectrometry techniques in concert with limited proteolysis has gained prominence.[6,7]

The broad acceptance of the liquid chromatography-mass spectrometry (LC-MS) in macromolecular analysis has its origin in introduction of electrospray ionization (ESI), for which Professor John B. Fenn shared the 2002 Chemistry Nobel Prize.[8] Herein, we report the use of time-resolved limited proteolysis, liquid chromatography, and a hybrid quadrupole-time-of-flight mass spectrometry (LC-Q-ToF-MS)[9] to confirm the specific interaction within the human CyP:CsA non-covalent complex.

EXPERIMENTAL

Materials

Sequencing grade trypsin, recombinant cyclophilin A (>95% SDS-PAGE, expressed in *E. Coli* buffered aqueous solution), cyclosporin A (95% purity), and erythromycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents were HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA, USA) and used without additional purification.

MVNPTVFFDIAVDGEPLGRVSFELFADKVPKTAENFRALSTGEKGFGYKGSCFHRIIPGF
MCQGGDFTRHNGTGGKSIYGEKFEDENFILKHTGPGILSMANAGPNTNGSQFFICTAKT
EWLDGKHVVFGKVKEGMNIVEAMERFGSRNGKTSKKITIADCGQLE

Figure 1. Amino acid sequence of recombinant human CyP A shown in standard single letter amino acid abbreviation.

Time Resolved Proteolysis Experiments

The time resolved limited proteolysis of CyP and CyP : CsA non-covalent complex was performed using sequencing grade trypsin in a 50:1 protein-to-enzyme ratio, respectively. Cyclophilin A (50 μg/mL) and CyP:CsA (1:2 and 1:4 molar ratios) were digested separately, but simultaneously, in a pH 7.4 buffer at 37°C using a temperature water bath. The CsA stock solution was prepared using 100% HPLC grade methanol. However, the final CsA methanol solution added to the pH 7.4 aqueous buffer did not exceed 0.013% (by volume). In addition to the incubation of CyP with trypsin, a solution of CyP:erythromycin (1:4 molar ratio, respectively) was also digested as a control. A 90 μL aliquot of each digest was sampled at time points: 0, 15, 30, 60, 90, 120, and 180 min. To quench trypsin activity, 30 μL of 1% trifluoroacetic acid (TFA) was added to each aliquot. The mixture was vortexed for 5 min and subjected to LC-MS analysis without further purification. All incubations were performed in duplicate.

Liquid Chromatography Mass Spectrometry

The LC-MS analysis was performed on Agilent 1100 series binary pumps (Palo Alto, California, USA) coupled to a Q-ToF II hybrid mass spectrometer from Micromass (Manchester, UK), operated in the electrospray positive mode (ESI+). Chromatographic separations were achieved using a Vydac (Hesperia, California, USA) RP-C8, 5 μm particle size, 300 Å, and 5 mm × 2.0 mm column. HPLC gradient was performed by 0.1% formic acid in water (A), and 95% acetonitrile:5% water:0.1% formic acid (B). The gradient consisted of 0–50% B in 40 min, 50–90% B in 10 min, and, subsequently held at 90% B for 10 min, followed by re-equilibration with 100% A for 15 min. The flow rate was 0.20 mL/min. The injection volume was 15 μL. The Agilent 1100 series standard autosampler tray was maintained at 10°C.

The Q-ToF II conditions were optimized for sensitivity including: ESI capillary = 3000V, source temperature = 120°C, desolvation temperature = 400°C, and a resolution of 7000 measured at m/z 609 as full width at half maximum (FWHM). For LC-MS analysis, the quadrupole mass filter was set to bandwidth bypass mode with all ions transmitted into the ToF analyzer with a scan range of m/z 400–2000 and one second integration time. All ToF measurements were recorded in continuum mode. For MS/MS fragmentation, the quadrupole was set up to pass precursor ions of selected m/z values to the hexapole collision cell (using argon as collision gas), and product ion spectra were acquired with the ToF analyzer. Nitrogen was used as the desolvation and nebulizing gas at flow rates of 400- and 50 L/hour, respectively. Leucine-enkephalin (1.0 μg/mL) was introduced at a flow rate of 5 μL/min to serve as an internal mass calibrant

(referred to as the "lock-mass"). The MS and MS/MS spectra were analyzed using Masslynx 4.0 software (Micromass, Manchester, UK).

RESULTS AND DISCUSSION

The human cyclophilin A (hCyP A), which possesses peptidyl-prolyl *cis-trans* isomerase activity, consists of 165 amino acids (ca. 18012 Da; Figure 1). The peptidyl-prolyl *cis-trans* isomerase activity of hCyP A is inhibited by CsA with an $IC_{50} = 25$ nM.[10] To this end, the CsA:CyP A complex inhibits CN with a $K_i = 336$ nM. CsA is one of the leading immunosuppressive agents (e.g., Sandimmune®), which has been prescribed to organ transplant patients. CsA has been successfully prescribed to patients that have undergone kidney, liver, or heart allogeneic transplantation.[11]

In the present model of CsA-CyP binary complex,[2,12–14] residues 1–3 and 9–11 of CsA (Figures 2 and 3) fit into a hydrophobic groove of CyP. The remaining distinct molecular region of CsA (residues 4–8), which is thought to interact with CN, is referred to as the "effector domain". The CyP contact residues with CsA are Arg-55, Phe-60, Met-61, Gln-63,

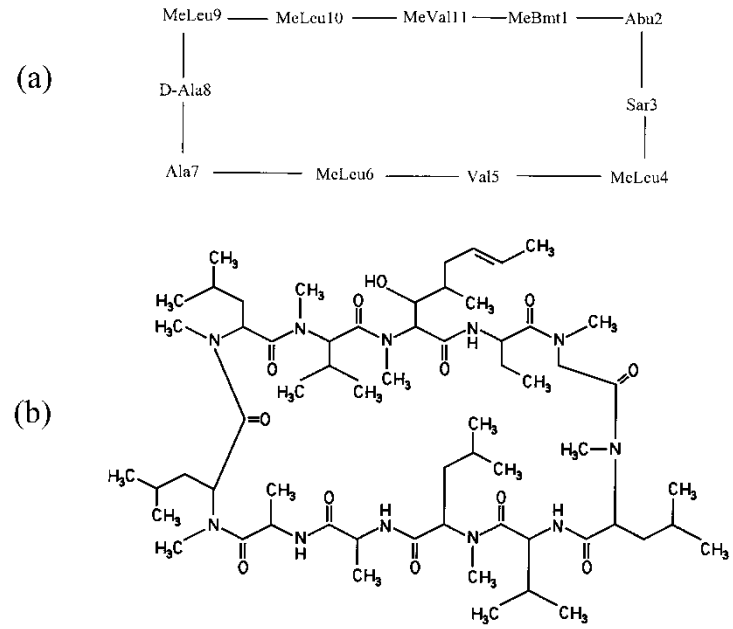

Figure 2. (a) Chemical structure of CsA using amino acid abbreviation. Non-standard symbols used: MeBmt, (4*R*)-4-[(*E*)-2-butenyl]-4, *N*-dimethyl-*L*-threonine; Abu, *L*-alpha-aminobutyric acid; MeLeu, *N*-methyl-*L*-leucine; MeVal, *N*-methyl-*L*-valine; Sar, sarcosine. (b) Expanded chemical structure of CsA.

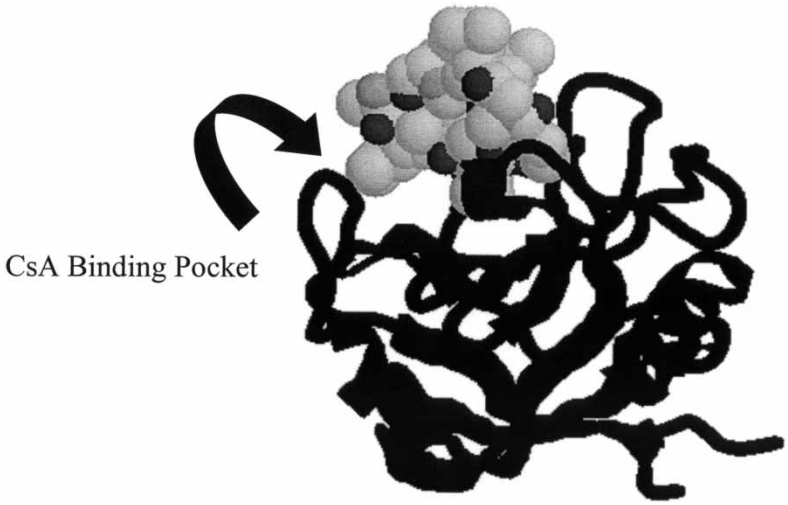

Figure 3. Molecular ribbon representation of CyP:CsA non-covalent complex taken from X-ray crystal structure of a monomeric complex at 2.1 Å resolution. The structure was obtained from RCSB Protein Data Bank (URL: www.rcsb.org). RCSB is a worldwide repository for the processing and distribution of 3-dimensional biological macromolecular structure data.

Gly-72, Ala-101, Asn-102, Ala-103, Gln-111, Phe-113, Trp-121, Leu-122, and His-126.[2,12–14] The CyP does not undergo significant conformational changes upon binding to CsA.[2] In contrast, extensive structural changes have been observed between the free and bound CsA.[12–14]

The proteolysis of native proteins depends upon their tertiary structure and accessibility of specific sites for enzymatic cleavage. Hence, proteolysis can be utilized to probe the exposed or flexible domains of a polypeptide. Trypsin, a serine protease, has been widely used in MS-based protein analysis, yielding specific cleavages after lysine and arginine amino acids (unless a proline residue is followed in the C-terminal direction).[15] Human CyP contains 14 lysine and 6 arginine amino acids (Figure 1). Table 1 summarizes the identities of tryptic peptides (commonly designated as T), which could result from enzymatic digestion of CyP using trypsin. The list of the tryptic peptides in Table 1 was simply generated by *in silico* digestion of the primary amino acid sequence of human CyP with designation of their location and expected molecular weight (MW).

A series of LC-MS signals were observed when a sample of CyP was subjected to time-resolved proteolysis (pH $\sim$ 7.4) and analyzed using LC-ESI-Q-ToF-MS. Figure 4 depicts a LC-MS total ion-chromatogram obtained from such an experiment, which contains signals mainly corresponding to the resulting tryptic peptides, as well as a signal attributed to the remaining undigested (intact) CyP A (retention time $\sim$ 31.8 min). The

Table 1. List of tryptic peptides which are expected to result from the digestion of recombinant human CyP by trypsin

Tryptic peptide	Location	Mass (Mono)	Mass (Avg.)	Sequence number
T1	1–19	2076.0350	2077.3702	MVNPTVFFDIAVDGEPLGR
T2	20–28	1054.5335	1055.1855	VSFELFADK
T3	29–31	342.2267	342.4356	VPK
T4	32–37	736.3504	736.7765	TAENFR
T5	38–44	704.3705	704.7729	ALSTGEK
T6	45–49	570.2802	570.6400	GFGYK
T7	50–55	705.3017	705.7898	GSCFHR
T8	56–69	1540.7167	1541.8018	IIPGFMCQGGDFTR
T9	70–76	669.3194	669.6903	HNGTGGK
T10	77–82	695.3490	695.7643	SIYGEK
T11	83–91	1153.5655	1154.2739	FEDENFILK
T12*	92–118	2733.3003	2735.0738	HTGPGILSMANAGPNTNGSQFFICTAK
T13	119–125	847.4076	847.9155	TEWLDGK
T14	126–131	685.3911	685.8176	HVVFGK
T15	132–133	245.1739	245.3200	VK
T16	134–144	1277.5744	1278.4635	EGMNIVEAMER
T17	145–148	465.2336	465.5056	FGSR
T18	149–151	317.1699	317.3430	NGK
T19	152–154	334.1852	334.3703	TSK
T20	155–155	146.1055	146.1884	K
T21	156–165	1061.5063	1062.2007	ITIADCGQLE

*T12 was used to confirm the CyP : CsA binding site.

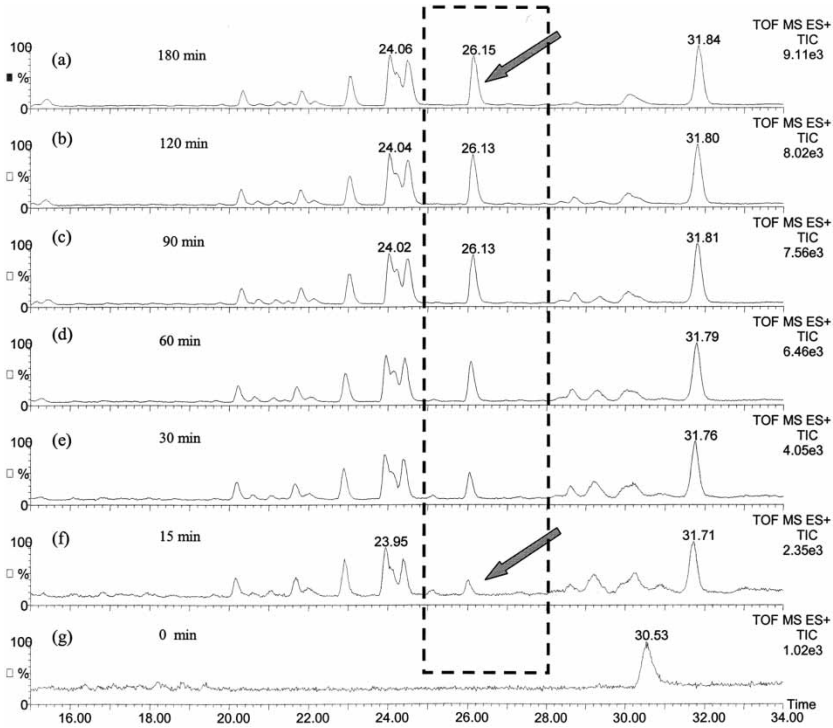

Figure 4. Total ion-chromatograms obtained from the time-variations of the signal intensities of limited trypsin digest of recombinant human CyP A. The tryptic peptide, T12 (see Table 1) at retention time of ~26.1 min, progressively increased in intensity at longer incubation intervals.

progression of the proteolytic digestion was monitored by quenching an aliquot of the incubation at specific time points using TFA followed by LC-MS analysis. The sampling ($n = 2$) time points were selected at 0 min (control), 15, 30, 60, 90, 120, and 180 min. It was important to avoid any denaturation step during the incubation process since it would preclude useful information regarding the native tertiary structure. A notable observation in Figure 4 was the time dependent increase in relative ion abundance of a signal at 26.1 min. Distinct variations in the apparent proteolytic rate constants for a given protein substrate could be, in part, rationalized by initial lower rates of digestion, at relatively less accessible substrate sites, ensued by faster rates as protease accesses buried hydrophobic grooves at longer incubation times.[7]

Figures 5 and 6 depict the peptide map obtained under the same experimental conditions (i.e., using the same batch of trypsin enzyme) as Figure 4 (*vide supra*), with the exception that respective 2:1 and 4:1 molar ratios of CsA:CyP were incubated at 37°C overnight (ca. 12 hours) and subjected to

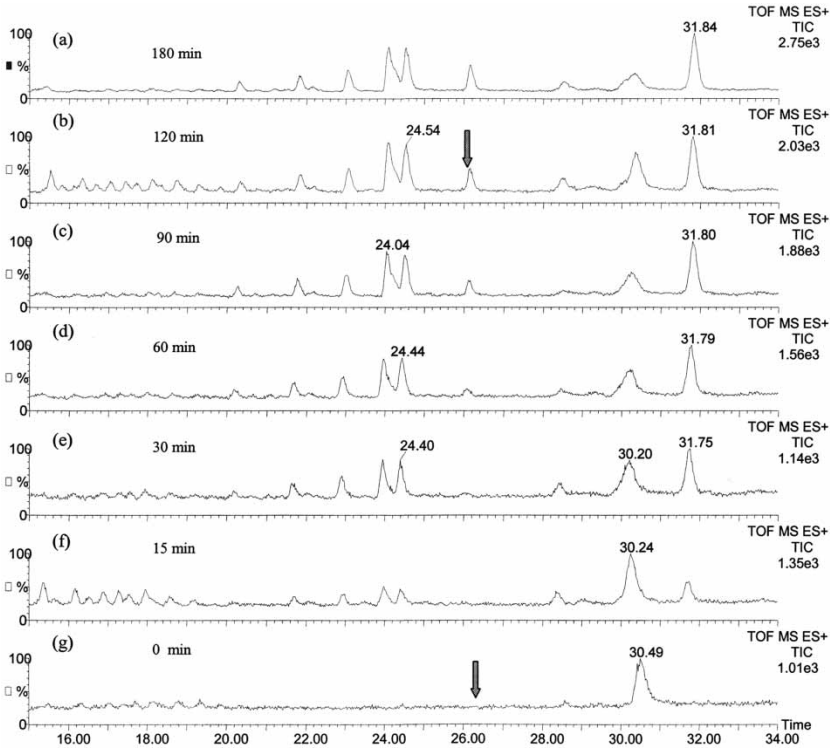

Figure 5. Total ion-chromatograms obtained from the time-variations of the signal intensities of limited trypsin digest of CyP:CsA at a molar ratio of 1:2, respectively. In contrast to data depicted in Figure 4, the intensity of the tryptic peptide, T12, dramatically decreased.

LC-MS analysis. The dramatic reduction in the signal at 26.1 min suggested that the presence of CsA had profoundly influenced the susceptibility of a segment of CyP to limited trypsin digest. The two putative cleavage sites at Lys-91 and Lys-119 led to a tryptic peptide (T12) with a predominant triply charged signal at m/z 912.4. Shown in Figure 7, is the expanded high resolution electrospray spectrum of the m/z 912.4 from the extracted ion chromatogram at retention time of $\sim$26.1 min. The spacing between each isotopic peak was about 0.34, confirming its triply charged identity.

Figure 8 depicts a series of extracted ion-chromatograms (corresponding to the putative T12), which were obtained from the parallel incubations at respective 1:4, 1:2, and 1:0 (control) molar ratios of CyP:CsA. In addition to the control experiment, an incubation using CyP:erythromycin at a respective 1:4 molar ratio was examined. Since, erythromycin, an antibiotic with no structural resemblance to CsA, has not been known to be a ligand for CyP, an analogous observation to the control experiment was observed (Figure 8c).

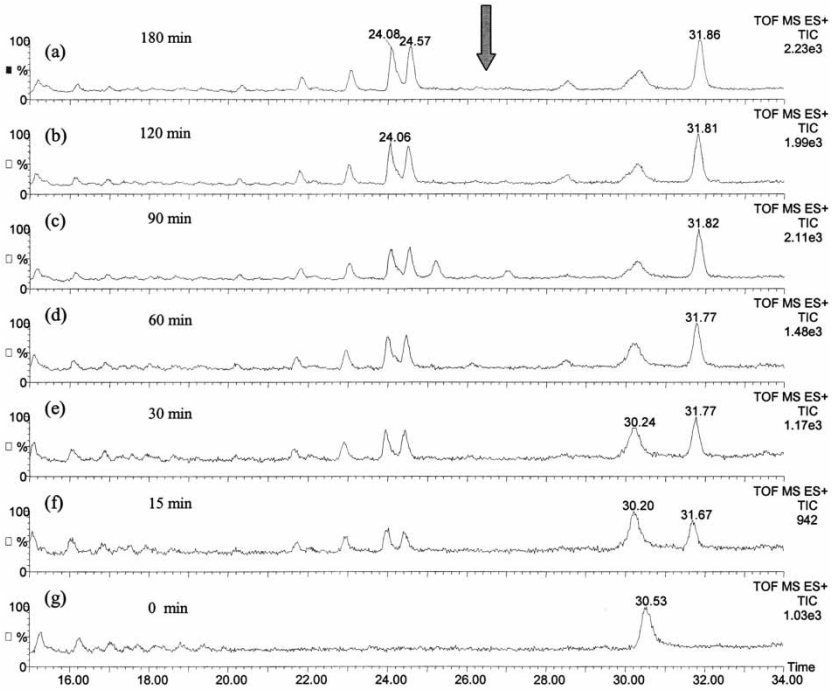

Figure 6. Same experiment as depicted in Figure 5, except that a CyP:CsA molar ratio of 1:4 was used, respectively. A pronounced inhibition for the formation of T12 was observed.

The latter observation provided additional compelling evidence that the observed cleavage of T12 (Table 1) was not the result of non-specific interactions. As shown in Figure 8, subsequent to a 2 hr incubation period, the extracted ion chromatograms for the 1:4 molar ratio of CyP:CsA completely blocked the formation of T12. However, a dramatic increase in T12 abundance was noted for the control and erythromycin experiments, lending support that specific binding of CsA to CyP inhibited the trypsin's ability to cleave the

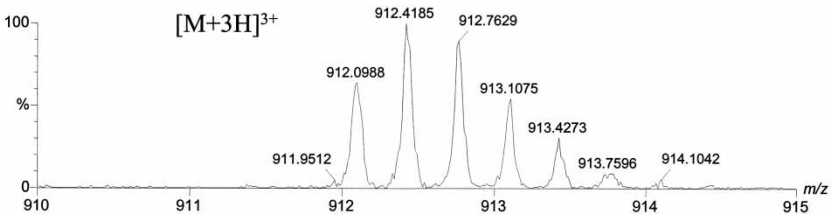

Figure 7. Expanded LC-ESI-Q-ToF spectrum of the predominant triply charged T12.

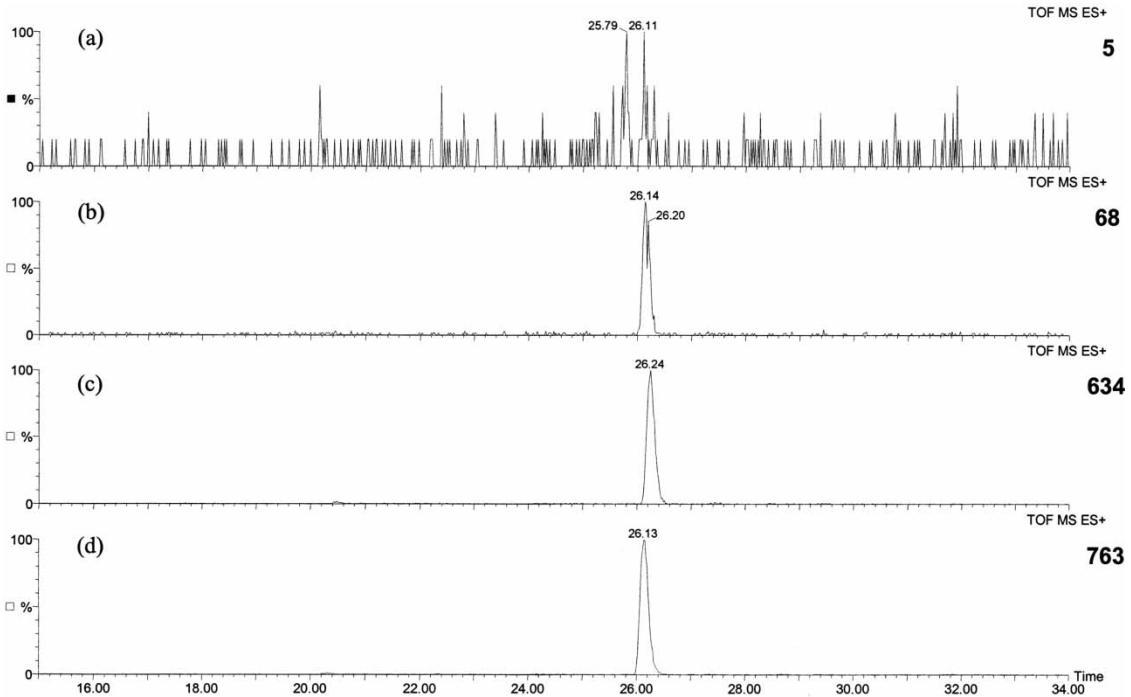

Figure 8. Extracted ion-chromatograms obtained for T12 subsequent to a 2 hr incubation: (a) Respective 1:4 molar ratio of CyP:CsA; (b) Respective 1:2 molar ratio of CyP:CsA; (c) Respective 1:4 molar ratio of CyP:erythromycin; and (d) the control experiment where only CyP was digested with trypsin. The upper right corner read-outs correspond to the Q-ToF detector counts.

lysine residues at positions 91 and 118. This is in accord to the NMR[12] and X-crystallography[13] reports where CyP residues Arg-55, Phe-60, Met-61, Gln-63, Gly-72, Ala-101, Asn-102, Ala-103, Gln-111, Phe-113, Trp-121, Leu-122, and His-126 were in contact of less than 4 Å with MeBmt-1, Abu-2, Sar-3, MeLeu-10, and MeVal-11 of CsA. To this regard, peptide T12 (spanning from residues 92–118) contains 5 of the above critical residues at positions 101, 102, 103, 111, and 113 (*vide supra*). An increase in the abundance of T12 was suggestive of the accessibility of trypsin to the CyP binding pocket in the absence of CsA.

In summary, we have demonstrated that one of the pathways of limited tryptic proteolysis of recombinant human CyP was altered by its specific non-covalent interaction with CsA. Nearly full protection to trypsin mediated cleavage at Lys-91 and Lys-119 was detected in the presence of varying concentrations of CsA. The relative abundance of a marker peptide (T12), inferred from accurate mass measurement, containing five binding pocket amino acid residues in conjunction with time-course analysis, led to a compelling argument in support of confirmation of specific CyP:CsA non-covalent interaction. To our knowledge, this is the first example of a time-resolved proteolysis/accurate mass measurement of CyP:CsA complex. This relatively simple approach can be utilized in the absence of high-resolution X-ray crystallography or NMR data, in a rapid fashion, to screen and qualitatively evaluate the binding domain of CyP ligands. To this end, the proteolytic susceptibility of CyP and/or structural homologs can be monitored by modification of ligands using a relatively small amount of sample.

ACKNOWLEDGMENT

We are grateful to Dr. John Mehl for his support of this project and helpful technical discussions.

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Received December 29, 2004
Accepted January 20, 2005
Manuscript 6559