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X-ray sequence ambiguities of *Sclerotium rolfsii* lectin resolved by mass spectrometry

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Summary. X-ray crystallography, although a powerful technique for determining the three-dimensional structure of proteins, poses inherent problems in assigning the primary structure in residues Asp/Asn and Glu/Gln since these cannot be distinguished decisively in the electron density maps. In our recently published X-ray crystal structure of the *Sclerotium rolfsii* lectin (SRL) at 1.1 Å resolution, amino acid sequence was initially deduced from the electron density map and residues Asp/Asn and Glu/Gln were assigned by considering their hydrogen bonding potential within their structural neighborhood. Attempts to verify the sequence by Edman sequencing were not successful as the N terminus of the protein was blocked. Mass spectrometry was applied to verify and resolve the ambiguities in the SRL X-ray crystal structure deduced sequence. From the Matrix assisted laser desorption/ionization time-of-flight-mass spectrometry (MALDI TOF-MS) and liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis of tryptic and chymotryptic peptides of SRL, we could confirm and correct the sequence at five locations with respect to Asp/Asn and Glu/Gln. Analysis data also confirmed the positions of Leu/Ile, Gln/Lys residues and the sequence covering 118 of the total 141 residues accounting to 83.68% of the earlier deduced sequence of SRL.

Keywords: *Sclerotium rolfsii* lectin – Amino acid sequence – Mass spectrometry – Protein crystal structure

Introduction

Lectins are a unique class of proteins, which exhibit specific and reversible non-covalent binding to glycoconjugates, either free in solution or on cell surfaces. Unlike the carbohydrate-binding enzymes, lectins do not alter the covalent structure of glycosyl ligands upon binding. The unique specificity of lectins towards complex carbohydrate moieties has made them valuable molecular tools for investigating the distribution and functions of glycoconjugates on the cell surface and hence they are aptly described as ''deciphers of glycocode'' (Nilsson, 2003).

Although lectins are widely distributed in plants, animals and microorganisms, the vast majority of the literature is mostly restricted to plant and animal lectins (Drickamer and Taylor, 1993; Sharon and Lis, 2004). It is only in recent years that fungal lectins received greater attention and there are now several reports on their purification and characterization (Guillot and Konska, 1997; Wang et al., 1998). Many of these lectins are reported to have interesting biological activities such as antiproliferative (Yu et al., 1993; Wang et al., 1995), antitumor (Wang et al., 1996, 1997, 2000), immunomodulatory (Ko et al., 1995; Hsu et al., 1997; She et al., 1998), and insecticidal activities (Trigueros et al., 2003), hence they may have wider pharmacological and biotechnological applications.

In the past four years crystal structures of nine fungal lectins have been reported (Paaveenthan et al., 2003; Wimmerova et al., 2003; Brick et al., 2004; Walser et al., 2004; Ban et al., 2005; Carrizo et al., 2005; Mancheno et al., 2005; Cioci et al., 2006; Leonidas et al., 2007), which reflects the gathering impetus in this area. All these fungal lectins have a unique protein fold that differs from any of the previously described lectin folds and have sequence homology to an extent of 30–60%. Meanwhile, various bioinformatical approaches have been developed, providing useful information for studying the structures and functions of lectin (see, e.g., (Chou, 1995, 1996,

2004; Chou and Heinrikson, 1997). Based on similarities in carbohydrate binding property, sequence homology and crystal structure, these proteins are grouped into different families (Rosen et al., 1996; Cooper et al., 1997; Kruger et al., 2002; Brick et al., 2004; Carrizo et al., 2005; Imberty et al., 2005) indicating their diversity.

The primary structure of the majority of the fungal lectins has been deduced either from the cDNA sequence (Fukumori et al., 1990; Crenshaw et al., 1995; Rosen et al., 1996; Cooper et al., 1997; Kruger et al., 2002; Trigueros et al., 2003; Yang et al., 2005; Cioci et al., 2006; Van Damme et al., 2007) or the high-resolution X-ray crystal structure (Paaveenthan et al., 2003; Carrizo et al., 2005; Cioci et al., 2006; Leonidas et al., 2007). The lectin isolated from the edible mushroom *Agaricus bisporus* (ABL) has a remarkable anti-proliferative activity on colon epithelial cells. Recently, the crystal structure of ABL has been determined at 2.5 Å resolution (Carrizo et al., 2005) and the amino acid sequence derived from the high quality electron density map revealed serious differences with the earlier reported cDNA sequence (Crenshaw et al., 1995). Similar discrepancies were also observed for *Psathyrella velutina* lectin (Cioci et al., 2006) and *Erythrina cristagalli* lectin (Svensson et al., 2002). Such discrepancies in assigning the amino acid sequence were also reported for other proteins (Keefe et al., 1992; Kilby et al., 1995) highlighting the strength of X-ray crystallography in revealing errors in the amino-acid sequence. Although high resolution X-ray crystallography can be a powerful tool for deriving the amino acid sequence information of protein, it has inherent limitations in differentiating Asp/Asn and Glu/Gln. Also the X-ray sequence of a protein is usually of insufficient accuracy due to problems in defining disordered residues on the protein's molecular surface or at the two termini. Under such circumstances mass spectrometry could be an ideal tool of choice for resolving these discrepancies (Lunin et al., 2004; Hou et al., 2007).

Sclerotium rolfsii, a soil borne plant pathogenic fungus, secretes a developmental-stage specific lectin (SRL), which is purified from the sclerotial bodies and shows a pH-dependent oligomerization (Swamy et al., 2001, 2004). SRL displays strong binding to di-saccharide Gal $\beta 1 \rightarrow 3$ GalNAc-α- (TF, Thomsen Friedenreich antigen) (Wu et al., 2001) similar to *Agaricus bisporus* lectin (Presant and Kornfeld, 1972) and *Xerocomus chrysenteron* lectin (Trigueros et al., 2003).

Recently, we reported the crystal structure of SRL in its free form at 1.1 Å resolution and its complex with N-acetyl-D-galactosamine and N-acetyl-D-glucosamine at 2.0 Å and 1.7 Å resolution respectively (Leonidas et al., 2007). Findings reveal that SRL shares common structural topology, glycan specificity, and two conserved carbohydrate-binding sites with ABL and XCL. However, unlike ABL and XCL, which are homotetramers, SRL exist as homodimer. Indeed, the amino acid sequence of SRL shows high similarity with ABL (64%) and XCL (58%). Though the crystal structure data obtained for free SRL are of the highest resolution data for any protein of this family, ambiguities arose in the assignment of the amino acid sequence and in particular in the differentiation between Asp/Asn and Glu/Gln. In order to verify and resolve ambiguities in the X-ray deduced amino acid sequence of SRL, we adopted mass spectrometry, since our efforts to sequence by chemical methods were not successful due to the acetylated N-terminus. Here we describe MALDI-TOF-MS and ESI-MS/MS (Zu et al., 2007) analysis for determining primary sequence of SRL and present the corrected sequence.

Materials and methods

Materials

Sclerotium rolfsii lectin (SRL) was purified from the sclerotial bodies as described earlier (Swamy et al., 2001). Trypsin (bovine, TPCK-treated), chymotrypsin (bovine, TLCK-treated), 2,5-dihydroxybenzoic acid (DHB, 98% pure), were purchased from Sigma-Aldrich. All other chemicals used were of analytical grade and the aqueous solutions were prepared using deionized water from Milli-Q Ultrapure Water System (Bedford, MA) and filtered through 0.22 µm nylon filters from Fischer Scientific (Pittsburgh, PA, USA).

Sample preparation for MALDI-TOF-MS and LC-ESI-MS/MS

Purified SRL was subjected to electrophoresis on SDS PAGE (12%) and the protein band appeared after staining the gel with Coomassie blue was excised. The lectin band in the excised gel was subjected to in-gel tryptic/chymotryptic digestion as described by Rosenfeld et al. (1992) with some modifications. Briefly, the excised gel was sliced to small pieces, transferred to sterile siliconized eppendorff and destained by repeated washing with 50 mM NH_4HCO_3, dehydrated using acetonitrile and finally dried by speed-vac. Enzymatic digestion was carried out by incubating the dried gel pieces with trypsin or chymotrypsin (100 µg in 1 ml of 25 mM NH_4HCO_3 containing 0.5 mM $CaCl_2$, pH 8.0) initially for 1 h on ice. Further incubation was carried out overnight at 37 °C after adding additional 40 µl of $NH_4HCO_3/CaCl_2$, buffer. Digested samples were centrifuged briefly to collect the supernatants and the settled gels were extracted either with 5% formic acid in 50% acetonitrile (for ESI-MS/MS) or 5% trifluroacetic acid in 50% acetonitrile (for MALDI-TOF-MS). The extraction step was repeated 3–4 times using fresh extraction solution and all the wash supernatants (tryptic or chymotryptic digests) were pooled and concentrated to 50 µl by speed-vac and used for MS analysis.

Mass spectrometry

MALDI-TOF-MS analysis was carried out with *Ultraflex* TOF/TOF (Bruker Daltonics, Bremen, Germany) mass spectrometer, equipped with

nitrogen laser (337 nm). The enzyme digest samples were mixed with equal volume of saturated matrix solution (2,5-dihydroxybenzoic acid in 50% acetonitrile/H_2O with 0.1% trifluroacetic acid). This mixture (1 µl) was deposited on the probe plate and dried by stream of air and the spectra were recorded in the reflectron positive ion mode using Bruker Daltonics FLEX control software and the spectral data were processed by Bruker Daltonics FLEX analysis software. A standard peptide mixture (P.N: 206195, Bruker peptide calibration standard) was used for external calibration.

HPLC-ESI-MS/MS

On-line HPLC separation of tryptic and chymotryptic digests of SRL was carried out on HP1100 (Agilent) at a flow rate of 0.150 ml/min. The solvent system consisting sol. A; 0.1% formic acid in water and sol. B; acetonitrile in 0.1% formic acid was used. Tryptic peptides were separated on a C8 reverse-phase column (4.6 × 150 mm; ZORBAX RX-C8, 5 µm, Agilent) using a linear gradient of sol. B 20–95% achieved in 45 min. The chymotryptic peptides were fractionated on 4.6 × 250 mm, C18 reverse-phase column (Phenomenex, NAXSIL, 5 µm CTB) with a linear gradient from 5% solvent B to 95% solvent B in 75 min. ESI-MS/MS data were obtained using an *Esquire 3000 plus* mass spectrometer (Bruker Daltonics, Germany) consisting of two octopoles followed by an ion trap. Nitrogen and helium were used as nebulizer and collision gas for collision induced dissociation (CID) experiments respectively. Fragmentation data were acquired over a range of 50–2800 m/z in positive ion mode and analyzed using Esquire data analysis software (version 3.1). Acquired MS/MS spectra were interpreted manually.

Results and discussion

The amino acid sequence of SRL determined earlier from near-atomic resolution (1.1 Å) electron density maps, where all amino acids are clearly defined within the electron density (Leonidas et al., 2007), is shown in Fig. 1. Ambiguities in residue assignment between Asp–Asn, and Glu–Gln were resolved with X-ray crystallography by examining double difference and difference electron density sigmaA maps and their hydrogen-bonding pattern with neighbouring residues. This was also facilitated by the existence of two SRL molecules in the asymmetric unit resulting to different neighbouring environment. However, residues 14, 113, 123, and 133 are at the surface of the protein in both molecules of the non-crystallographic dimer and their side chain atoms are only involved in hydrogen bond interactions with water molecules, which can act both as donors and acceptors. Hence, the chemical microenvironment of these residues was not helpful in resolving the ambiguity in their identity since both Asp or Asn and Glu or Gln could satisfy the electron density map and their hydrogen-bonding pattern. In order to verify and resolve the ambiguities in X-ray derived amino acid sequence of SRL, we sequenced the SRL by MALDI-MS and LC-ESI-MS/MS (tandem mass spectrometry), as chemical and cDNA sequence are not available. The expected proteolytic peptide and fragmentation ion masses of X-ray crystallographic derived amino acid sequence of SRL were calculated using programs http://delphi.phys.univ_tours.fr/pyrolysi/cutter.html and http://hodgkin.mbu.iisc.ernet.in/~pfia/index.htm.

From the X-ray crystallographic analysis it was clear that the N-terminal of SRL is blocked which further confirmed our failure to sequence intact SRL by Edman degradation. Hence we decided to determine the sequence from the tryptic and chymotryptic in-gel digests by mass spectrometry. The purified protein was subjected to in-gel digestion with trypsin and chymotrypsin and the digests were used for mass spectrometric analysis.

Peptide mapping of tryptic digest of SRL by MALDI-TOF-MS and sequencing of tryptic peptides by LC-ESI MS/MS

LC-ESI MS/MS

Unfractionated tryptic digest after the in-gel digestion of SRL was subjected to MALDI-TOF-MS to characterize the peptide mass pattern. MALDI-TOF-MS has been used as the preferable method because of its robustness in complex samples, tolerance to many biological buffers, salts and the prevalence of single charged peaks. The expected, observed protonated monoisotopic masses of tryptic peptides obtained by MALDI-TOF-MS and their positions in the final sequence are shown in Table 1. Of the total 15 tryptic peptides expected, 12 peptides were identified in MALDI-TOF-MS. Mass peaks corresponding to designated peptides T4, T5, and T8 were not detected, probably because of suppression of ions in the mixture or they were not recovered from the gel. Efforts to obtain sequence information by further subjecting the assigned tryptic peptides to fragmentation analysis were not successful due to poor fragmentation. Despite the fact that the spectrum showed considerable number of fully resolved peaks (Fig. 2), the approach of peptide mass mapping gave no positive hit.

Hence, the tryptic digest of SRL was subjected to online LC-ESI-MS/MS and the reconstructed total ion cur-

Ac-TYKITVRVYQTNPNAFFHPVEKTVWKYANGGTWTITDDQHVLTMGGSGTSG
TLRFHADNGESFTATFGVHNYKRWCDIVTNLAADETGMVINQQYYSQKNREEA
RERQLSNYEVKNAKGRNFEIVYTEAEGNDLHANLIIG**-COOH**

Fig. 1. Amino acid sequence of SRL deduced from X-ray crystallographic studies at 1.1 Å resolution

Table 1. Peptide fragments obtained from a tryptic digest of the SRL MALDI-TOF-MS

Tryptic peptide	Amino acid no.[a]	Expected (Da) $(M+H)^{+b}$	Observed (Da) $(M+H)^{+}$	Dev. (Da)
T1	1–3	453.00	453.31	+0.31
T2	4–7	488.32	488.15	+0.17
T3	8–22	1790.89	1790.69	+0.2
T4	23–26	533.31	–	–
T5	27–54	2896.35	–	–
T6	55–73	2141.97	2141.77	−0.20
T7	74–74	175.12	174.69	+0.43
T8	75–99	2890.34	–	–
T9	100–101	289.16	289.81	−0.65
T10	102–105	504.24	504.05	+0.19
T11	106–107	304.16	303.86	+0.30
T12	108–115	980.51	980.42	+0.09
T13	116–118	332.19	331.89	+0.30
T14	119–120	232.14	231.61	+0.53
T15	121–141	2332.15	2331.13	+1.02

[a] Assignments were made by comparing the observed peptide masses with the expected masses of the SRL derived from X-ray crystallographic data
[b] Expected mass calculated from monoisotopic weights
– Not observed

rent (TIC) chromatogram is presented in Fig. 3. Each of the peaks is labeled according to the corresponding tryptic peptides. Expected, observed protonated masses, deduced sequences of tryptic peptides from their CID and their positions in the final sequence are presented in Table 2.

Peptides, T1, T2, T3, T4, T5, T8, T10, T11, and T13 were successfully sequenced based on their fragmentation spectrum by CID. Because of the poor quality CID spectra sequences of peptides T6, T7, T9, and T12 were confirmed solely from their molecular masses. High molecular weight peptides, having m/z 2892.31 and 2896.35 were not observed in the LC-ESI-MS indicating probably the protein part constituting these peptides was resistant to tryptic digestion or they could not be extracted from the gel.

Observed m/z values of sequence specific ions from the CID mass spectral analysis for peptides T1, T2, T3, T4, T5, T8 T10, T11, and T13 are presented in Table 3. Fragmentation ions of protonated peptides produced by CID are labeled according to the standard nomenclature proposed by Biemann (1990). Observed monoisotopic masses of the sequence defining ion series in each spectrum are shown directly above and below the respective sequence.

Fragmentation ions of low mass (<1/3 of the peptide mass) were not observed in the CID spectra, essentially because of the inherent limitation of the ion trap mass

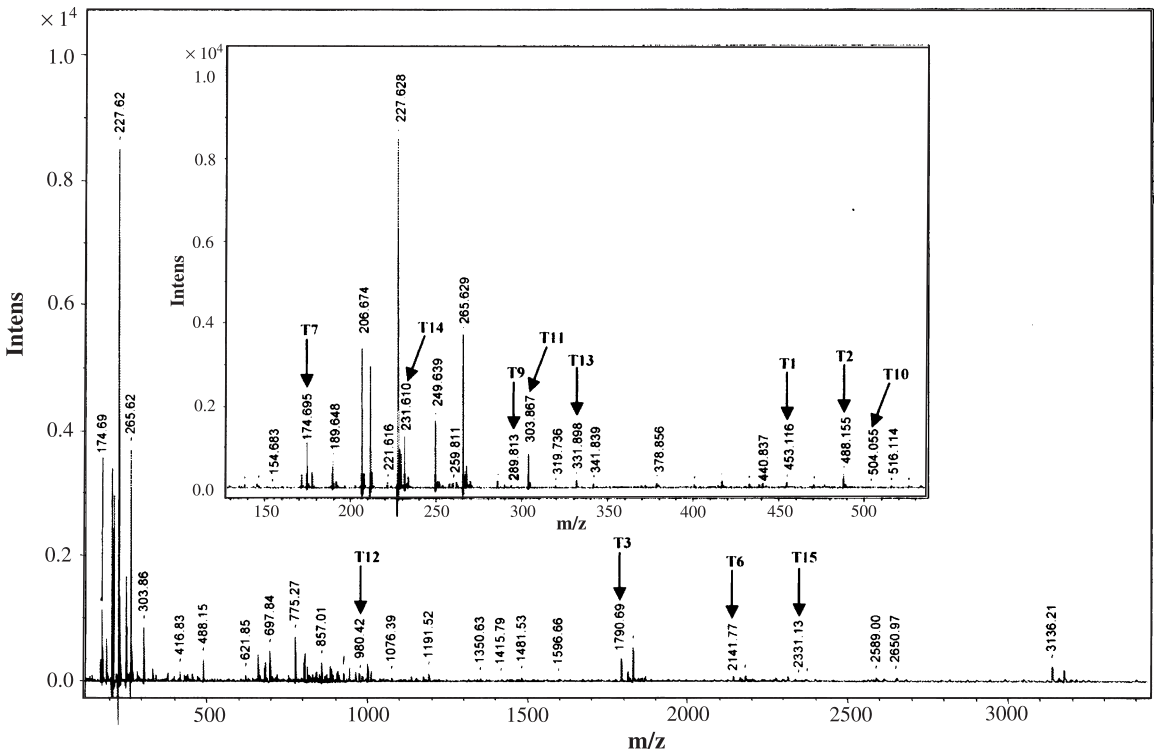

Fig. 2. Positive ion reflectron MALDI-TOF spectra of in-gel tryptic digest of the SRL. The samples were prepared, using dried droplet method, by mixing the sample with 2,5-dihydroxybenzoic acid as matrix. Peaks arising from the tryptic digest are labeled according to the sequence position in the protein. The inset illustrates the expanded spectrum of low mass region

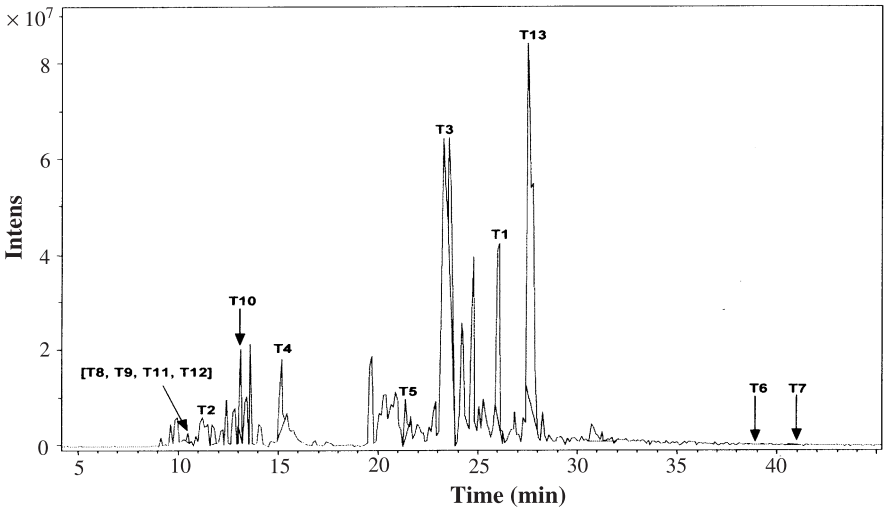

Fig. 3. Total ion current (TIC) chromatogram of tryptic digests of SRL

Table 2. Tryptic peptides of SRL detected by LC-ESI-MS/MS

Peptide no.	Amino acid no.[a]	Expected $(M+H)^{+b}$ (Da)	Observed $(M+H)^{+}$ (Da)	Charge state	Ret. time (min)	Error (ppm)	Peptide sequence
T1	1–3	453.30[n]	453.3	+1	26.1	0.00	Ac-TYK[c]
T2	4–7	488.32	488.3	+1	11.8	40.95	ITVR[c]
T3	8–22	1790.89	1791.6 $(896.3)^{2+}$	+2	23.8	150.70*	VYQTNPDAFFH PVEK[c]
T4	23–26	533.31	533.3	+1	15.2	18.75	TVWK[c]
T5	55–73	2141.97	2142.0 $(1071.5)^{2+}$	+2, +3, +4	21.5	14.00	FHADNGESFTAT FGVHNYK[c]
T6	74–74	175.12	175.10	+1	39.0	114.22	R[m]
T7	100–101	289.16	289.1	+1	41.0	207.54	NR[m]
T8	102–105	504.24	504.2	+1	10.5	79.33	EEAR[c]
T9	106–107	304.16	304.2	+1	10.6	131.49	ER[m]
T10	108–115	980.51	979.5 $(490.3)^{2+}$	+1, +2	13.4	20.41*	QLSNYQVK[c]
T11	116–118	332.19	332.2	+1	10.5	30.10	NAK[c]
T12	119–120	232.14	232.1	+1	10.6	172.33	GR[m]
T13	121–141	2332.15	2330.20 $(1165.6)^{2+}$	+2, +3	27.5	8.58*	NFQIVYTE AEGNNLHANLIIG[c]

[a] Assignments were made by comparing observed peptide masses with a theoretical digest of the SRL derived from the X-ray crystallographic data
[b] Expected mass calculated from monoisotopic molecular weights
[n] Acetylation at the N terminus
[c] Sequence deduced from CID spectrum and the corrected amino acids are indicated in bold
* After correction of amino acid
[m] Sequences confirmed solely on molecular mass

analyzer. Observed protonated masses of tryptic peptides having m/z 1791.6, 979.5, and 2330.2 corresponding to sequence residues 8–22, 108–115, and 121–141 were different from their calculated masses by 396 ppm, 1031 ppm, and 772 ppm respectively (Table 2).

In order to account for these observed differences, changes were made with the residues N (14) → D, E (113) → Q, E (123) → Q, and D (133) → N. With these changes made in each of the peptide, the error reduced to 150 ppm, 20 ppm, and 8.5 ppm for peptides m/z 1791.6, 979.5 and 2330.2 respectively. The replacements at these positions were further confirmed from their CID spectral data, which yielded numerous sequence-specific daughter ions (Table 3. (G), (F), and (I)). Also the observed mass accuracy for all the tryptic peptides lie within $\pm < 0.3$ Dalton threshold limit required to differentiate unambiguously the Asn/Asp or Gln/Glu, which differ by 1 Dalton (Biemann, 1992). The CID spectrum of the peptide having

Table 3. CID mass spectral data for tryptic peptides. The m/z values (found) for particular ion series are listed by rows

(A) $(M+H)^+$ = m/z 453.3				(B) $(M+H)^+$ = m/z 332.2				(C) $(M+H)^+$ = m/z 488.3				(D) $(M+H)^+$ = m/z 533.3			
a				a	158.1	287.1		a			442.6	a	173.1		
b			435.3	b	115.2	185.9	315.1	b	215.1	314.2	470.3	b	201.1	387.1	515.2
c				c				c				c			
Ac-The – Tyr - Lys				Asn - Ala – Lys				Ile - Thr – Val - Arg				Thr - Val - Trp - Lys			
x	336.3			x				x		300.0	201.0	x			173.1
y	310.4	147.2		y	218.0	147.0		y	375.2	274.1	175.1	y	432.2	333.2	147.0
z	293.4			z	201.0	130.1		z	358.2	257.1	158.0	z		316.1	

(E) $(M+H)^+$ = m/z 504.2				(F) $(M+2H)^{2+}$ = m/z 490.3						
a			458.8	a	214.2	301.2	415.5	578.3	706.3	
b			486.2	b	242.0	329.1		606.1	734.4	833.5	961.6
c	276.1			c			460.2			
Glu - Glu –Ala - Arg				Gln - Leu – Ser - Asn - Tyr - Gln - Val - Lys						
x				x				562.2		
y	375.2	246.1	175.0	y	851.3	738.4	651.3	537.3	374.2	246.1	147.1
z	357.0	229.1	157.9	z		721.2	634.4	520.4	357.2	

(G) $(M+2H)^{2+}$ = m/z 896.3														
a	235.0	363.7	464.2	578.1			861.4							
b	263.1	391.2	492.1	606.2	703.4			1036.4	1183.6	1320.6	1417.8	1516.7	1645.7	
c			509.2		720.2	835.3							1662.6	
Val - Tyr – Gln - Thr - Asn - Pro - Asp - Ala - Phe - Phe - His - Pro - Val - Glu - Lys.														
x						1115.5		929.6	782.4	635.3	498.5			
y			1401.7	1300.7	1186.6	1089.6	974.5	903.5	756.4	609.3	472.2	375.2		
z		1512.7	1384.6	1283.8	1169.5	1072.5	957.5	886.3	739.4	592.4		259.0		

(H) $(M+2H)^{2+}$ = m/z 1071.5																			
a							743.4	830.3	977.2		1149.7				1553.7				
b	285.1	356.2	471.2	585.3	642.2	771.3	858.3	1005.3	1106.5	1177.5	1278.5	1425.6	1482.7	1581.6	1718.8				
c	302.1		488.1		659.2			875.2							1735.7				
Phe - His – Ala - Asp - Asn - Gly - Glu - Ser - Phe - Thr - Ala - Thr - Phe - Gly - Val - His - Asn - Tyr - Lys																			
x					1583.8	1526.5		1310.8		1062.5	991.4		743.3	686.2			336.3		
y		1857.9	1786.8	1671.7	1557.7	1500.6	1371.6	1284.7	1137.6	1036.5	965.5	864.4	717.4	660.3	561.3	424.2	310.2		
z		1840.8	1769.9	1654.8	1540.6	1483.5	1354.6	1267.7	1120.4	1019.4	948.5	847.5	700.1	643.3	544.3	407.3	293.2		

(I) $(M+2H)^{2+}$ = m/z 1165.6																					
a						737.5			1038.2					1565.5	1702.8						
b			390.0	503.3	602.5	765.3	866.6		1066.1	1195.1	1252.2	1366.4	1480.6	1593.5	1730.5						
c							883.7														
Asn - Phe – Gln - Ile - Val - Tyr - Thr - Glu - Ala - Glu - Gly - Asn - Asn - Leu - His - Ala - Asn - Leu - Ile - Ile - Gly																					
x							1591.7	1490.5				1104.1	990.2	876.0	763.5						
y						1728.6	1565.5	1464.6						850.3	737.5	600.1	529.2	415.0			
z						1711.7		1447.4		1195.1	1118.5	1061.4	947.8								

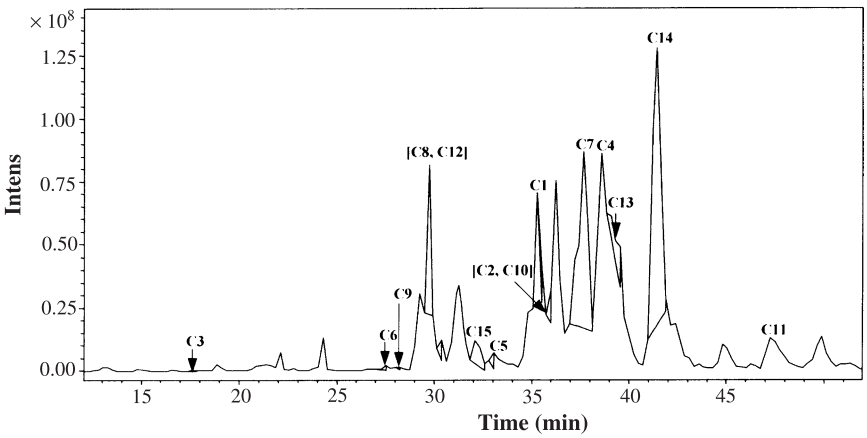

Fig. 4. Total ion current (TIC) chromatogram of chymotryptic digest of SRL

Table 4. Peptide fragments observed from a chymotryptic digest of SRL in LC-ESI-MS/MS

Peptide no.	Amino acid no.[a]	Expected $(M+H)^{+b}$ (Da)	Observed $(M+H)^{+}$ (Da)	Charge state	Ret. time (min)	Error (ppm)	Peptide sequence
C1	3–9	878.55	878.5	+1	35.2	56.91	KITVRVY[c]
C2	18–25	995.53	996.2 $(439.7)^{2+}$	+1, +2	35.9	672.55	HPVEKTVW[c]
C3	26–27	310.18	310.1	+1	17.7	257.98	KY[c]
C4	34–42	1041.52	1041.3 $(521.3)^{2+}$	+1, +2	37.7	211.27	TITDDQHVL[c]
C5	45–53	736.35	736.3	+1	32.9	67.90	GGSGTSGTL[c]
C6	54–55	322.19	322.1	+1	27.4	279.41	RF[c]
C7	64–67	439.22	439.1	+1	37.0	273.28	TATF[c]
C8	68–72	589.27	589.2	+1	29.8	118.805	GVHNY[c]
C9	73–75	489.29	489.2	+1	28.3	183.97	KRW[c]
C10	90–95	764.39	764.3	+1	35.6	117.75	VINQQY[c]
C11	97–104	961.47	962.3	+1	47.7	155.87*	SEKNREEA[m]
C12	110–112	383.16	383.0	+1	30.0	417.75	SNY[c]
C13	123–126	523.28	522.2	+1	39.3	172.3*	QIVY[c]
C14	127–141	1566.78	1566.2 $(783.6)^{2+}$	+1, +2, +3	41.4	261.78*	TEAEGNNLHAN LIIG[c]
C15	139–141	302.21	302.1	+1	32.0	364.11	IIG[c]

[a] Assignments were made by comparing observed peptide masses with a theoretical digest of the SRL derived from X-ray crystallographic data
[b] Expected mass calculated from monoisotopic molecular weights
[c] Sequence obtained from CID spectra and the corrected amino acids are indicated in bold
* After correction of amino acid
[m] Sequence confirmed based on molecular mass

m/z 453.3, representing the acetylated N-terminus peptide, shows mass of b3 ion shifted by 42 Da accounting for N terminal acetylated threonine (Table 3(A)). This is in agreement with our earlier observation that SRL is not amenable to Edman degradation, attempted during the earlier phase of its characterization.

The results of sequencing tryptic peptides by MALDI-TOF-MS and LC-ESI MS/MS indicated errors in the X ray sequence, i.e., N → D at residue 14, E → Q at residue 113, E → Q at residue 123, and D → N at residue 133. Apart from these corrections the results also confirmed the crystallographically-deduced sequence for residues 1–26, 55–74, 100–141. However, amino acid residues 27–54, and 75–99 could not be resolved from the tandem mass spectrometry data of tryptic peptides although these two large stretches accounting to a total of 53 residues are well defined in the X-ray crystal structure (Leonidas et al., 2007). It may be concluded that these peptides could not be extracted from the gel or retained on the reverse phase column thoughout the LC-ESI-MS/MS analysis. Such difficulties have been reported previously and the sequence for the peptide was confirmed solely based on the well defined X-ray data (Lunin et al., 2004).

Table 5. CID mass spectral data for chymotryptic peptides. The m/z values (found) for particular ion series are listed by rows

(A) $(M+H)^+$ = m/z 878.5

a			315.2	414.3	570.5	669.4	
b	129.0	242.1	343.2	442.2	598.4	697.4	
c			360.3	459.4		714.4	
	Lys	- Ile -	Thr	- Val -	Arg	- Val -	Tyr
x			663.6		463.4		
y		750.3	637.4	536.2	437.2	281.0	
z		733.5	620.3	519.2	420.1	264.1	

(B) $(M+H)^+$ = m/z 996.2

a						664.3	763.8	
b		234.9		463.1	591.3	692.4	791.4	
c			351.0	480.1	607.8			
	His	- Pro -	Val	- Glu -	Lys	- Thr -	Val	- Trp
x				688.4	559.5	431.9		
y		858.3	761.3	662.3	533.4		303.8	204.8
z			744.3	645.3	516.9	388.5		187.9

(C) $(M+H)^+$ = m/z 310.10

a			
b		129.0	
c		147.0	
	Lys	-	Tyr
x			
y			
c			

(D) $(M+H)^+$ = m/z 1041.3

a		186.9				783.4	882.4		
b		215.0	316.2	431.1	546.1	673.9	811.3	910.4	1023.2
c					691.3	828.4			
	Thr	- Ile -	Thr	- Asp -	Asp	- Gln -	His	- Val -	Leu
x									
y			827.5	726.6	611.2	496.1	368.2		
z			810.5	708.9	479.1				

(E) $(M+H)^+$ = m/z 736.3

a					476.1		
b			360.8	447.3	504.3	605.2	
c			377.3	464.0	521.1	622.1	
	Gly	- Gly -	Ser	- Gly -	Thr	- Ser -	Gly	- Thr -	Lys
x					504.3				
y		622.2	535.1	478.2	377.3				
z		605.2	518.3	461.2	360.8				

(F) $(M+H)^+$ = m/z 322.1

a			
b	157		
c			
	Arg	-	Phe
x			
y			
z			

(G) $(M+H)^+$ = m/z 439.1

a		246.0	
b		173.0	273.9
c			
	Thr	- Ala -	Thr - Phe
x			
y		267.0	165.9
z		249.9	149.9

(H) $(M+H)^+$ = m/z 589.2

a			380.0	
b		293.9	408.1	
c				
	Gly	- Val -	His	- Asn -
x				
y		532.3	433.1	296.0
z		515.0	416.0	278.9

(I) $(M+H)^+$ = m/z 489.2

a			
b		285.1	
c		302.1	
	Lys	- Arg -	Trp
x		387.8	
y		361.1	
z		344.0	

(J) $(M+H)^+$ = m/z 764.3

a							555.3
b		213.1	327.0	455.2	583.2		
c			343.9	472.2			
	Val	- Ile -	Asn	- Gln -	Gln	-	Tyr
x				336.3			
y			552.1	438.1	309.9		
z		648.2	535.1	421.2	293.9		

(K) $(M+H)^+$ = m/z 383.0

a		173.8	337.0	
b		201.9	365.0	
c				
	Ser	- Asn -	Tyr	
x				
y		295.9		
z		278.9	164.8	

(L) $(M+H)^+$ = m/z 302.10

a	86.2	199.0	256.9	
b		227.0		
c				
	Ile	- Ile -	Gly	
x		102.1		
y		189.0		
z				

(M) $(M+H)^+$ = m/z 522.2

a		214.4	313.3	
b		242.0	341.1	504.2
c				
	Gln	- Ile -	Val	- Tyr
x		307.4		
y		281.0	181.9	
z				164.8

(N) $(M+2H)^{2+}$ = m/z 783.6

a						460.9	574.2	688.4							
b				301.8				716.3	829.1	963.3	1057.4	1171.2	1264.3	1377.5	
c						505.1									
	Thr	- Glu -	Ala	- Glu -	Gly	- Asn -	Asn	- Leu -	His	- Ala -	Asn	- Leu -	Ile	- Ile -	Gly
x							1104.2		876.3		626.2				
y				1264.3	1135.4	1078.9	964.0	850.3	737.3	600.0	529.6	415.3	302.1	188.9	
z				1247.1	1117.8	1061.3			720.3	583.2		398.8	284.9		

Sequencing of chymotryptic peptides of SRL by LC-ESI MS/MS

In order to obtain the sequence for these two stretches (residues; 27–54 and 75–99) that were not resolved from the tryptic peptides as well as to provide the necessary overlap to establish the order of the sequenced tryptic peptides, the SRL was subjected to chymotryptic digestion and the resulting peptides were analyzed by LC-ESI-MS/MS.

The reconstructed total ion current (TIC) chromatogram of the chymotryptic digest of SRL is shown in Fig. 4. Each of the peaks is labeled according to the corresponding chymotryptic peptides. Expected, observed protonated masses, deduced sequences of chymotryptic peptides from their CID and their positions in the final sequence are presented in Table 4.

Observed m/z values of sequence specific ions from the CID mass spectral analysis for chymotryptic peptides (C1–C10 and C12–C15) are presented in Table 5. Monoisotopic masses obtained for the sequence defining ion series in each of the spectrum are shown directly above and below the respective sequence. The data presented in Table 5 showed that the observed protonated monoisotopic masses of chymotryptic peptides of m/z 962.30 (residues 97–104), 522.20 (residues 123–126), and 1566.2 (residues 127–141) were differing from their theoretical masses by 862 ppm, 2068 ppm, and 370 respectively. While accounting for these differences corrections were made with regard to Asn/Asp and Gln/Glu. Replacement of Q → E at residue 98, E → Q at residue 123 and D → N at residue 133, lead to greatly reduced error by 155.87 ppm for m/z 962.3, 172.3 ppm for m/z 522.20 and 261 ppm for 1566.2. Replacements at these positions were justified and further confirmed from their CID spectral data (Table 5 (M) and (N)), which yielded numerous sequence-specific daughter ions (see Table 5). The mass accuracy of the chymotryptic peptides are with-

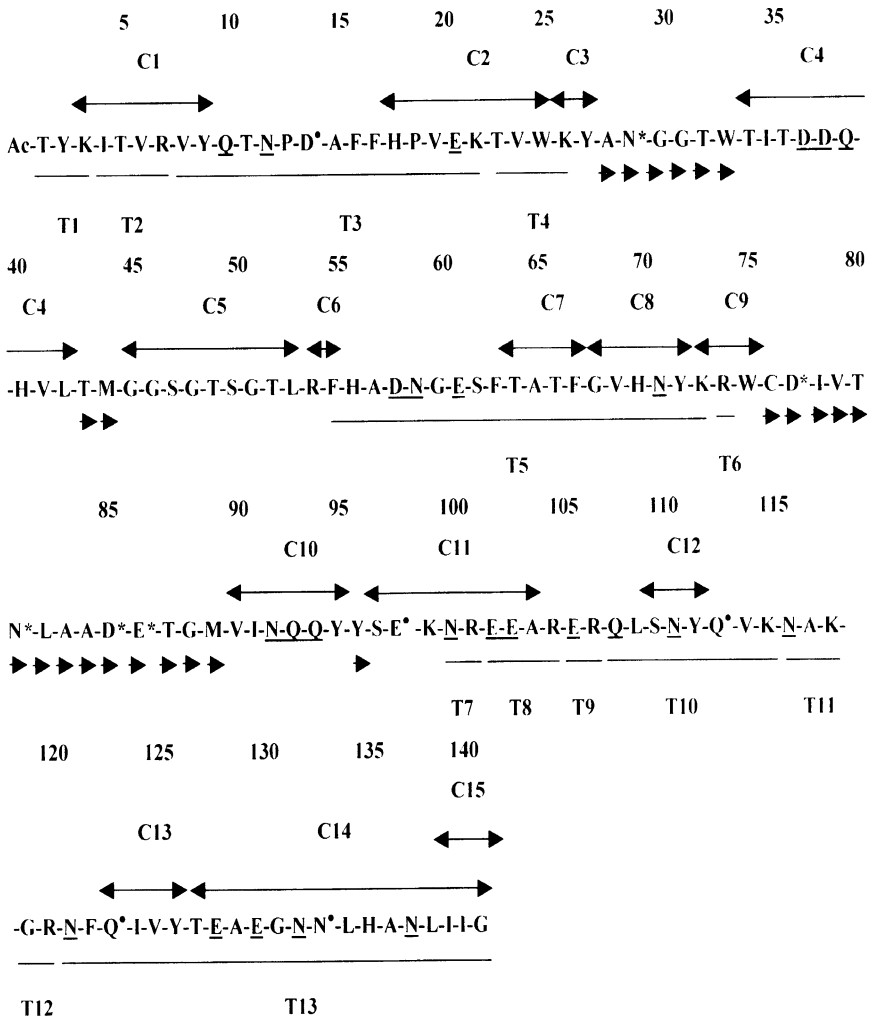

Fig. 5. Amino acid sequence of SRL determined by tandem mass spectrometry. Peptides are identified by letters that indicate the enzyme used for preparation: *T* indicates tryptic peptides (—) and *C* chymotryptic peptides (↔). Asterisks indicate ambiguities resolved by X-ray crystallography. Arrows (▶) indicate amino acid residues identified by X-ray crystallography. • Corrected amino acids; X confirmed based on fragmentation ions

in the admissible limit of $\pm < 0.3$ Dalton except for peptides C2 and C14, for which the mass accuracy value was found to be 0.67 and 0.41 Daltons respectively and could not be explained. However the sequences for these two peptides C2 (18–25) and C14 (127–141) were established from the sequence data of tryptic peptides T3 (8–22), T4 (23–26), and T13 (121–141) obtained by LC-ESI-MS/MS analysis.

Differentiation of lysine and glutamine

Since the isobaric amino acids lysine and glutamine, which differ in mass by 0.04u, are difficult to distinguish by low energy CID analysis, trypsin cleavage specificity was considered for assigning K-3, Q-10, K-22, K-26, Q-39, K-73, Q-93, Q-94, K-99, Q-108, K-115, K-118 (Fig. 5). However, Q-113 and Q-123 were assigned based on the accuracy of monoisotopic masses of both precursor ion as well as sequence specific daughter ions observed in the CID spectra of tryptic peptide of m/z 979.30 and, 2330.20 also chymotryptic peptide having m/z 522.2.

Differentiation of leucine and isoleucine

Leucine and isoleucine having identical mass cannot be differentiated by low energy CID, hence advantage of chymotrypsin cleavage specificity was taken for differentiating these two amino acids. Based on the chymotrypsin specificity and CID spectrum of chymotryptic peptides, we were able to assign unambiguously I-4, I-35, L-42, L-53, I-91, L-109, I-124, L-138, I-139, and I-140. However, I-78, L-82, and L-134 could not be assigned, as the chymotryptic peptides containing these amino acids were not observed in LC-ESI-MS/MS. Considering the sequence homology of SRL with other fungal lectins (Leonidas et al., 2007), L-134 was assigned. The primary structure of SRL deduced from the data discussed above is shown in Fig. 5. Lines and arrow lines show tryptic and chymotryptic peptides, respectively.

The CID mass spectral data of chymotryptic peptides C1 (3–9), C2 (18–25), and C3 (26–27) and the tryptic peptides T1 (1–3), T2 (4–7) T3 (8–22), and T4 (23–26), provided overlapping amino acid sequence information and to deduce the sequence 1–27 and read Ac-TYKITVRVYQTNPDAFFHPVEKTVWKY. Similarly, the collision-activated dissociation mass spectra of C6 (54–55), C7 (64–67), C8 (68–72), and C9 (73–75) gave complementary sequence information obtained from the MS/MS spectral data of T5 (55–73), which provided sequence from 54–75; RFHADNGESFTATFGVHNYKRW. The sequence for positions 97–105 (SEKNREEAR) was deduced from the complementary CID spectral data obtained from T8 (102–105) and C11 (97–104). The MS/MS spectral data of C12 (110–112) provided overlapping amino acid sequence information of T10 (108–115) where as no complementary chymotryptic peptide was found for T11 (116–118), however based on CID spectral data of C12, T10, and T11, we could assign the amino acid sequence QLSNYQVKNAK (108–118) unambiguously. The C-terminal amino acid sequence NFQIVYTEAEGNNLHANLIIG was confirmed based on the MS/MS spectrum of C13 (123–126), C14 (127–141), and C15 (139–141) as they gave overlapping amino acid sequence information of T13 (121–141).

The combined Collision induced dissociation mass spectral data of tryptic and chymotryptic peptides was key to verify and resolve the ambiguities between Asn/Asp and Gln/Glu, and enabled us to assign the amino acid sequences corresponding to the positions 1–27 (N-terminus), 54–75, 97–106, 102–105, 108–118, and 121–141 (C-terminus) of the protein. The sequence TITDDQHVL (34–42) and GGSGTSGTL (45–53) deduced solely from the MS/MS spectral data of C4 and C5 as no corresponding complementary tryptic peptides were observed from tandem mass spectrometry.

Peptides corresponding to (Fig. 5) 28–33 (ANGGTW), 43–45 (TM) and 76–89, (CDIVTNLAADETGM) (Fig. 5), were not detected neither in the tryptic nor in the chymotryptic digests, by LC-ESI-MS/MS. However these sequences were confirmed from the high electron density maps obtained at 1.1 Å resolution. Residues N-29, D-77, N-81, D-85, and Q-86 were indeed assigned based on their differences in the *B*-factors and potential formation of hydrogen bonds (Leonidas et al., 2007). From these results of MS/MS spectral analysis we could assign the residues D 14, E 98, Q 113, Q 123, and N 137 unambiguously. Further the analysis data involving 13 tryptic and 15 chymotryptic peptides covered 118 residues of the total 141 residues accounted for 83.68% of the SRL sequence that was deduced by X-ray crystallography at near atomic resolution (1.1 Å).

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