Planta Medica

An International Journal of Natural Products and Medicinal Plant Research

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Stuttgart · New York
 Rüdigerstraße 14
 D-70469 Stuttgart
 Postfach 30 11 20
 D-70451 Stuttgart

Thieme Publishers
 333 Seventh Avenue
 New York, NY 10001, USA
 www.thieme.com

Reprint

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N-Terminal Amino Acid Sequences of Intact and Cleaved Forms of Mung Bean Nuclease

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Abstract

We report, for the first time, the N-terminal amino acid sequences of both intact and cleaved forms (fragments A and B) of Mung bean nuclease, purified from sprouts of *Vigna radiata* or purchased from Amersham Biosciences. The N-terminal sequence of Mung bean nuclease shows high similarity with the putative bifunctional nuclease from *Arabidopsis thaliana* (AC: AAM63596).

Key words

Mung bean nuclease · *Vigna radiata* · Fabaceae · ribosome-inactivating protein · Edman degradation

Abbreviations

MBN: Mung bean nuclease
PAGE: polyacrylamide gel electrophoresis
PVDF: poly(vinylidene difluoride)
SDS: sodium dodecyl sulfate

Phosphodiester bonds, such as those found in both DNA and RNA, are extraordinarily resistant to hydrolysis. For example, the half-life for hydrolysis of the simplest phosphodiester bond, dimethyl phosphate, in 1 M NaOH is approximately 15 years at 35 °C [1]. Nucleases are part of a larger group of enzymes, the phosphodiesterases, which accelerate the rate of DNA hydrolysis by factors exceeding 10 [2]. This impressive catalytic power is, to date, poorly characterized both quantitatively and mechanistically [3].

As previously reported, Mung bean nuclease (MBN) is a member of a class of plant nucleases possessing a pronounced specificity towards nucleic acid substrates lacking ordered structure [4]. MBN, originally isolated by Sung and Laskowski [5], is endowed with DNase, RNase and ω -mono-phosphatase activities [6]. Used in molecular biology, MBN selectively degrades single strand DNA or RNA to yield mono- or oligonucleotides with a phosphate group at the 5'-termini.

MBN activity shows a base preference for A > T(U) > C > G, except for ribo-poly U which is hydrolyzed faster than ribo-poly A. The nuclease is a glycoprotein. Its molecular weight, analysed by SDS-PAGE without reducing agents, was 39 kDa [6]. In the pres-

ence of reducing agents, about 70% of the enzyme molecules seem to have a nick in the chain, as they give rise to two polypeptides of 15 (A chain) and 25 kDa (B chain), covalently linked by disulfide(s) bond(s).

MBN is used as probe for the structural characterization of nucleic acids [7]. This enzyme utilizes zinc ions as cofactors [6], but the precise role of this metal in the hydrolytic mechanism is unclear. More recently, it has been found that in absence of Zn²⁺, MBN cleaves dsDNA as PD-L1 does (type 1 ribosome-inactivating protein from *Phytolacca dioica* L.) at specific regions of the pBR322 plasmid [8]. Despite its extensive use in molecular biology studies, its primary structure is not known nor where the nick on the polypeptide chain is located.

In this paper, we provide sequence data of MBN, which could be used to clone its gene and obtain enough protein for further structural and biological characterization.

Both commercial and laboratory purified MBN, analysed by SDS-PAGE, showed a band of 44 kDa in the absence of β -mercaptoethanol (● Fig. 1, lane 1) and two additional bands of 25 and 15 kDa in the presence of the reducing agent (● Fig. 1, lanes 2 and 3). A densitometric scan of the gel of ● Fig. 1 essentially confirmed the results obtained by Kowalski et al. (1976) [6], i.e., the presence of about 35% intact protein (band 1), while the remaining 65% was contained into bands 2 and 3 (● Fig. 1, lanes 1 and 3).

To obtain preliminary sequence data on MBN, the bands of lanes 1 and 3 (● Fig. 1) were transferred to PVDF membrane (Applied Biosystems) and directly subjected to automatic Edman degradation for N-terminal sequencing. Native, untreated MBN was also subjected directly to automatic Edman degradation.

The obtained sequence data are shown in ● Table 1. Native protein and unreduced band 1 of lane 1 (● Fig. 1) produced (up to residue ten) a main sequence and a secondary one. It was possible to distinguish the two sequences, as they were present with different yield and because one of the two was the sole sequence obtained from bands 1 and 3 of lane 3 (reduced and S-pyridyl-ethylated MBN). Band 2 of lane 3 gave only the secondary sequence. This experiment perfectly confirms more directly what was found by Kowalski et al. [6]. In addition, the results allowed us to position the proteolytic cleavage site of MBN molecule between X^L-Ser-Asn-Tyr-. This cleavage is very likely due to the presence of high proteolytic activities after germination of *Vigna radiata* sprouts [9], or even present in seeds, as found for other experimental systems (nicked form of PD-S2 from *Phytolacca dioica* seeds [10]; cleaved form of concanavalin A [11]).

Fig. 1 Analysis by SDS-PAGE of the MBN from *Vigna radiata* sprouts. M, marker proteins; lane 1, 0.5 µg of protein in absence of mercaptoethanol; lanes 2 and 3, 0.25 and 0.5 µg of protein in the presence of β -mercaptoethanol.

Peptide/protein	Sequence	Notes
MBN native	1-WGKDGHAIVX-10 1-SNYXLTEALL-10	64% 36%
SDS-PAGE – SH		
band 1 (44 kDa)	1-WGKDGHAIVX KIAQARLDTA-20 1-SNYXLTEALL FL-12	
SDS-PAGE + SH		
band 1 (44 kDa)	1-WGKDGHAIVX KIAQA-15	
band 2 (25 kDa)	1-SNYXLTEALL FLSHF-15	
band 3 (15 kDa)	1-WGKDGHAIVX KIAQA-15	
RP-HPLC		
peak 1	1-WGKDGHAIVC KIAQARLDTA ATEAV-25	
peak 2	1-SNYXLTEALL FLSHFQGDI-19	
peak 3	1-WGKDGHAIVC KIAQA-15	Intact MBN

Table 1 Amino acid sequences of bands of MBN in the presence or absence of β -mercaptoethanol (see ● Fig. 1) or after S-pyridylethylation (see ● Fig. 2). X, amino acid residue not assigned

To obtain further sequence information on the two chains of MBN, native protein was submitted to S-pyridylethylation. After RP-HPLC (● Fig. 2), peaks 1, 2 and 3 (identified as fragments A and B, and native MBN, respectively, by SDS-PAGE; data not shown), were subjected to Edman degradation, giving the expected amino acid sequences (● Table 1).

A search for sequence similarities, performed with the BLAST program, revealed that the N-terminal sequences of the A and B fragments of MBN share a high percentage of identity with a bifunctional nuclease from *Arabidopsis thaliana* (A.C. AAG52597), belonging to the S1/P1 nuclease family (EC: 3.1.30.1; ● Fig. 3). The first amino acid residue of the MBN sequence is Trp, as reported for other nucleases belonging to the family EC: 3.1.30.1 [7].

Moreover, the N-terminal sequence of the B fragment (25 kDa) shares a high percentage of identity with an internal region of the same nuclease, confirming that it originated from a post-translational cleavage of native MBN.

Further investigations on MBN primary structure are still in progress to extend our knowledge on the structure/function relationships of such nuclease. Unfortunately, the purification pro-

1	50
A. thaliana	MANQKGLHV V MMIITVWLL Y AAPNIHWGK EGHEIICKIA QTRLDETA AK
MBN	WGK DGHAIVCKIA QARLDTAATE
	*** : ** * : **** * : *** : * :
51	100
A. thaliana	AVKELLPE SA EGDLSLCL W ADRVKFYHW SPSLHYINT P DACSYQYN RD
MBN	AV
	**
101	150
A. thaliana	CKDESGEKGR CVAGAI NYT TQLLSYKTA A SSQSQYNLT E ALLFVSHF MG
MBN	†SNYXLTE ALLFLSHF QG
	* : * ** * **** : * * *
151	200
A. thaliana	DIHQPLHVS Y ASDKGGNT IE VHWYTRKAN L HHIWDSNIE TAEADLYNS A
MBN	DI
	**
201	250
A. thaliana	LEGMDALKK NITTEWADQ V KRWDCTKKT ACPDIYASE G IQAACDWAY K
MBN	
251	290
A. thaliana	GVTEGDTLED EYFYSRLPI V YQRLAQGGVR LAATLNRI FG
MBN	

Fig. 3 Alignment of the N-terminal sequences of the A and B fragments of MBN with putative bifunctional nuclease from *Arabidopsis thaliana* (AC: AAM63596). “*” identical, “:” conserved and “.” semi-conserved residues. Position 110 in MBN is proposed as glycosylated Asn for the presence for N-glycosylation site (N-X-[S or T] pattern). The proteolytic cleavage site of MBN (see text) is indicated by the arrow (†).

Fig. 2 RP-HPLC separation of S-pyridylethylated fragments of MBN. For peptide identification, see ● Table 1.

cedure yields only ~50 μ g of MBN/1 kg of sprouts and its market price is not always affordable [6]. Nevertheless, it should be emphasized that primary structure data, obtained in the present work, could be used as the starting point for molecular biology studies aimed at cloning the MBN gene. This strategy will provide the basis for obtaining the recombinant enzyme both for further structural and biological characterization and for potential commercial purposes.

Materials and Methods

Purification of MBN: Native MBN was purified from *Vigna radiata* L. Wilczek sprouts as described elsewhere [4] or purchased by Amersham Biosciences.

Protein electrophoresis: Homogeneity of isolated proteins was determined by SDS-PAGE [12] with a Mini-Protein II mini-gel apparatus (Bio-Rad), using 6% (w/v) stacking polyacrylamide gel and 15% (w/v) separation gel. Silver staining was used to highlight the protein bands.

Reduction, S-pyridylethylation and RP-HPLC purification of protein and peptides: MBN was reduced and S-pyridylethylated

with 4-vinylpyridine as previously reported [13]. Separation of protein and peptides by RP-HPLC was performed on a Waters Breeze instrument. A and B fragments of MBN after S-pyridylethylation were obtained using a Alltech C4 column (0.46 × 15 cm; 5 µm particle size) eluted with a linear gradient of solvent A (TFA 0.1%) and solvent B (acetonitrile + TFA 0.1%), from 5 to 65% of solvent B over 60 min at a flow rate of 0.2 mL/min.

N-terminal amino acid sequencing by Edman degradation: Native protein was separated by SDS-PAGE and after electrophoresis, protein was transferred to PVDF membrane (Applera) by electroblotting with the mini trans-blot cell (Bio-Rad), according to the manufacturer's instructions in 10 mM CAPS, pH 11.0, containing 10% methanol. PVDF membranes were then stained for 1 min with Coomassie Blue R-250, destained with the washing solution (50% methanol), dried and directly analysed by Edman degradation on a Procise Model 491C sequencer (Applied Biosystem Inc.).

For the amino acid sequence analysis by automated Edman degradation, native protein or peaks derived from S-pyridylethylation of MBN were dried, resuspended in 50% (v/v) acetonitrile, adsorbed on filter treated by Biobrene™ and analysed by Edman degradation on a Procise Model 491C sequencer as reported [13].

Alignment of MBN sequences: The search of sequence similarities was performed with the BLAST program available on-line (<http://www.ncbi.nlm.nih.gov/BLAST>). Sequences were aligned using the Clustal W software in the default set-up.

Acknowledgements

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This work was financially supported by grants (Ateneo 2006) from the Seconda Università di Napoli, Italy.

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received November 26, 2007

revised January 10, 2008

accepted February 24, 2008

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DOI 10.1055/s-2008-1034371

Planta Med 2008; 74: 588–590

© Georg Thieme Verlag KG Stuttgart · New York

Published online April 1, 2008

ISSN 0032-0943

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