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N-glycosylation of tomato nuclease TBN1 produced in *N. benthamiana* and its effect on the enzyme activity

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ABSTRACT

A unique analysis of an enzyme activity versus structure modification of the tomato nuclease R-TBN1 is presented. R-TBN1, the non-specific nuclease belonging to the S1-P1 nuclease family, was recombinantly produced in *N. benthamiana*. The native structure is posttranslationally modified by *N*-glycosylation at three sites. In this work, it was found that this nuclease is modified by high-mannose type *N*-glycosylation with a certain degree of macro- and microheterogeneity. To monitor the role of *N*-glycosylation in its activity, hypo- and hyperglycosylated nuclease mutants, R-TBN1 digested by α-mannosidase, and R-TBN1 deglycosylated by PNGase F were prepared. Deglycosylated R-TBN1 and mutant N94D/N112D were virtually inactive. Compared to R-TBN1 wt, both N94D and N112D mutants showed about 60% and 10% of the activity, respectively, while the N186D, D36S, and D36S/E104 N mutants were equally or even more active than R-TBN1 wt. The partial demannosylation of R-TBN1 did not affect the nuclease activity; moreover, a little shift in substrate specificity was observed. The results show two facts: 1) which sites must be occupied by a glycan for the proper folding and stability and 2) how *N. benthamiana* glycosylates the foreign nuclease. At the same time, the modifications can be interesting in designing the nuclease activity or specificity through its glycosylation.

1. Introduction

N-glycosylation is widely spread across eukaryotic organisms – yeasts, plants, and animals. Currently, it is known that *N*-glycosylation occurs in a limited way also in *Eubacteria* and *Archea* [1]. *N*-glycosylation is a posttranslational modification of proteins, starting in endoplasmic reticulum during protein translation and in many cases ending in Golgi apparatus, from where maturated *N*-glycoproteins are usually excreted outside the cell. Up to date, research efforts have led to the discovery of different types of *N*-glycans (oligomannose, complex, hybrid, poly-*N*-acetyllactosamine, etc.). Moreover, the general roles of the glycans (e.g., correct folding of glycoproteins, stabilization, and protection of protein surfaces, recognition by specific molecules) were widely described [1,2]. Especially, the *N*-glycosylation of plants was described in many publications (e.g. [3–5]). This posttranslational modification in plants is currently studied due to the rising number of recombinant therapeutic glycoproteins (mainly human) produced in plants (e.g., *Nicotiana benthamiana*) [6–8], and given the wide application field of these nucleases this research can be relevant also for development of methods for molecular biology or other biotechnological applications [9].

Plant non-specific endonucleases also belong among *N*-glycoproteins. They were described in several different processes that are crucial for plant organism, i.e., reproduction [10], senescence [11], hypersensitive response [12], response to salt stress [13], and in other crucial processes. These nucleases are widespread across the higher plant species including crops.

However, only little information exists about their mechanism of action in plants or their glycosylation, being the inseparable part of their overall structure. Some progress in the view of the potential action was reached with BFN1 nuclease from *A. thaliana*. Farage-Barhom and co-workers (2011) observed the localization of BFN1 around the fragmented nuclei upon programmed cell death during senescence of *A. thaliana* leaf [14]. Ko et al. [15] continued the work on *A. thaliana* nucleases by finding that ENDO2, the *Arabidopsis* homologue of BFN1, did not show any activity after its deglycosylation by PNGase F and subsequent analysis using in-gel activity assay [15]. In our previous study engaged in the characterization of three plant homologous

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recombinant nucleases (R-TBN1 from *S. lycopersicum*, R-HBN1 from *H. lupulus* and R-ABN1 from *A. brassica*), it was also found out that the enzymes lost their activity after total deglycosylation analyzed by in-gel activity assay [16]. Interestingly, bacterial nucleases of the same type exist, which entirely lack glycosylation, while still maintaining their molecular stability and activity [17]. Therefore glycosylation of these enzymes can be viewed as an additional level of stability and activity (and perhaps specificity) regulation evolved in eukaryotes.

In this work, we focus on how much the *in vitro* activity of R-TBN1 is influenced by different modifications of its glycosylation. This endonuclease, originated from tomato and produced in *N. benthamiana*, was previously described as an apoptotic enzyme with a broad substrate specificity, and moreover, anticancer activity [16,18]. The solution of its three-dimensional structure led to the explanation of its unique structural features and to the proposition of its catalytic mechanism [19]. However, we still do not know the contribution of the glycan chains to the activity and/or stability of the glycoprotein.

2. Material and methods

2.1. Material

Endoglycosidase PNGase F was purchased from Roche (cat. 11365185001). Endoglycosidases F1 and F2 were included in the Native Protein Deglycosylation Kit purchased from Sigma-Aldrich (cat. NDEGLY). Exoglycosidase α(1→2, 1→6)mannosidase from *Canavalia ensiformis* was purchased from Sigma-Aldrich (cat. M7257).

2.2. Preparation of R-TBN1 wt and its mutants

The mutants lacking one (N94D, N112D, N186D) or two (N94D/N112D) glycosylation sites and the mutants with additional glycosylation sites (D36S creates a new site at N34), D36S/E104 N) were prepared using site-directed mutagenesis (the position of the amino acids are numbered according to the mature protein [16]). The following table contains primer sequences possessing base substitution/s (bold, underlined), which were used for PCR reaction:

Enzyme variant	Results in	Primer sequence
TBN1 N94D	hypoglycosylation	5´ggtgcaattcag**g**actttactactcaactc3´
TBN1 N112D	hypoglycosylation	5´cgtcgatat**g**atatgacagaggctttgc3´
TBN1 N186D	hypoglycosylation	5´gacattgaagggg**g**acttcactgacggaatttgg3´
TBN1 D36S	hyperglycosylation	5´cggaatatgttaacggt**tct**ttatcggccc3´
TBN1 D36S/E104N	hyperglycosylation	5´ctctcattacaga**a**a**c**ggaacttctgatcgtcg3´

After annealing of two complementary primers containing the mutation site, KOD Hot Start polymerase was used for the synthesis of the new DNA strands. The combined hypoglycosylated mutant N94D/N112D was prepared subsequently from the mutant N94D using primers for the mutant N112D. A similar approach was used for the combined hyperglycosylated mutant D36S/E104 N. Mutated plasmids were cloned equally as the wild-type R-TBN1 [18]. All enzymes were recombinantly produced in *N. benthamiana* and purified by ammonium sulfate precipitation, ion-exchange chromatography and affinity chromatography (as described previously [16,18]).

2.3. Cleavage of R-TBN1 by specific endoglycosidases

Partly demannosylated R-TBN1 was prepared using α(1→2, 1→6) mannosidase from *Canavalia ensiformis*. The reaction mixture contained 8 μg of R-TBN1 wt, 1 μg of α-mannosidase in 100 mM acetate buffer, pH 4.6. The cleavage was carried out at 37 °C for 6 h.

For deglycosylation of R-TBN1 wt, 8 μg of the nuclease (in 50 mM Tris-HCl, pH 7.5) and 3 U of PNGase F (1U/μl) was mixed, and the cleavage took place at 37 °C for 3 h.

The cleavage by Endoglycosidases F1 and F2 was performed according to the manufacturer´s protocol.

2.4. Mass spectrometry analysis

Masses of intact and endoglycosidases-treated R-TBN1 were measured using an Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics). Measurements were carried out in the linear positive mode with 2,5-dihydroxybenzoic acid or sinapinic acid as matrix. Spectra were externally calibrated with Protein Calibration Standard II (Bruker Daltonics).

To infer the glycosylation pattern of R-TBN1, the sample of R-TBN1 was first separated by SDS-PAGE. The band containing R-TBN1 was cut into cubes, dithiothreitol solution was added to reduce disulfide bridges, and free cysteines were blocked with iodoacetamide solution. To ensure protein high sequence coverage and successful detection of all glycosylation sites, three different proteases were used for protein digestion – trypsin, chymotrypsin and endoprotease Asp-N. The solution of each protease was added to gel cubes and incubated at 4 °C for 20 min. Then the excess of a protease was removed, and the digestion ran at 37 °C for 4 h. Peptides were extracted from the gel cubes by sonication for 20 min. Released peptides were desalted and concentrated using ZipTip pipette tips with C18 stationary phase following the manufacturer´s instructions. A solution containing eluate was left to evaporate.

The MS/MS spectra of digested R-TBN1 were acquired using a UHPLC Dionex Ultimate3000 RSLC nano (Dionex, Germany) connected with mass spectrometer ESI-Q-TOF Maxis Impact (Bruker, Germany). Peptides were dissolved in 10 μl of a solution containing 3% of acetonitrile and 0.1% of formic acid. The sample was first loaded on a trap column (Acclaim PepMap 100 C18, 100 μm x 2 cm, particle size 5 μm, Dionex, Germany) and then the flow was directed to an analytical column (Acclaim PepMap RSLC C18, 75 μm x 150 mm, particle size 2 μm). Eluted peptides were directly introduced into an ESI ion source – Captive spray (Bruker Daltonics). MS/MS spectra were acquired in data-dependent analysis mode. During each round, up to five most intense precursors were selected for fragmentation. The precursors were selected in the range of 400–2200 *m/z* and spectra of fragments were recorded in the range of 50–2200 m/z. Peak lists from measured data were extracted with DataAnalysis 4.1 and uploaded to the data management system Proteinscape 3.1 (both by Bruker Daltonics). Proteins were identified by Mascot searching the human part of Swissprot database with an added sequence of TBN1. The Spectra Classifier within Proteinscape environment was used to identify the spectra tentatively corresponding to glycopeptides. The classification is based on the presence of the characteristic oxonium ions (e.g. m/z = 204.1 for HexNAc and m/z = 366.1 for HexHexNAc) and characteristic mass distances of m/z = 162.1 (Hex), 203.1 (HexNAc), etc. Successfully classified spectra were used for glycan identification using GlycoQuest engine (part of Proteinscape) with GlycomeDB as a database gathering known glycan structures. During the identification, the molecular weight (MW) of a peptide moiety of a given glycopeptide was determined too. The

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