



Tryptic Mapping Based Structural Insights of Endo-1,4- β -Xylanase from *Thermomyces lanuginosus* VAPS-24

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Abstract An endo-1,4- β -xylanase, XynA, from *Thermomyces lanuginosus* VAPS-24, was purified to homogeneity and exhibited a molecular mass of approximately 20 kDa. The protein sequence of XynA was found to be similar to those of other *Thermomyces lanuginosus* derived xylanases and, as a result, could be used as a model enzyme for understanding the protein structure–activity relationship and facilitating protein engineering to design enzyme variants with desirable properties. Therefore, this xylanase will be an attractive candidate for applications in the bio-fuel and fine chemical industries for the degradation of xylans in steam pre-treated biomass.

Keywords Endo-1,4- β -xylanase · Tryptic mapping · *Thermomyces lanuginosus* · Protein sequence · Xylanase structure

The application of microbial enzymes in industry has increased due to the global demand for green technology [1, 2]. *Thermomyces lanuginosus* xylanases are excellent examples of microbial enzymes that have found widespread applications in industry [3]. The thermophilic fungus, *T. lanuginosus*, is well-known to produce excellent

levels of cellulase-free thermostable xylanases [3]. Although generally categorized into numerous glycoside hydrolase (GH) families (5, 7, 8, 10, 11, 30, 43, 62 and 98), most industrially relevant xylanases generally belong to GH families 10 and 11 [4]. The structure of the xylanase from *T. lanuginosus* has been reported to closely resemble those of other GH11 xylanases [5]. GH11 enzymes have been found to have a β -jelly-roll fold and an extended N-terminal region consisting of 11 amino acids. A number of protein engineering approaches have been employed to improve the thermostability of enzymes [6].

A crude xylanase (XynA) concentrate sample from *T. lanuginosus* VAPS-24 (Gene Accession Number: KU366607) was kindly provided by the Enzyme Technology and Protein Bioinformatics Laboratory, Department of Microbiology, Maharshi Dayanand University, Rohtak, Haryana, India. Details regarding the isolation and identification of the VAPS24 strain, and production of the xylanase, XynA, are detailed in Kumar et al. 2017 [7]. XynA was then purified via ultra-filtration using Amicon[®] Ultra filters as described previously [7]. The specific activity of the purified XynA was 49.96 IU/mg protein, where one IU was defined as the release of 1 μ mol of reducing sugar xylose equivalent from wheat-flour arabinoxylan per min at 50 °C. An ultra-filtration purified XynA sample was run on 12% SDS-PAGE as described previously [8] and the corresponding protein band was digested with trypsin to identify XynA xylanase as per the method proposed by Shevchenko and co-workers [9]. Digestion of the XynA enzyme was performed using 5–50 μ l of trypsin (10 ng/ μ l) (depending on the size of the protein band) overnight at 37 °C. Digests were identified using a Dionex Ultimate 3000 RSLC system attached to an AB Sciex6600 TipleTOF mass spectrometer after re-suspension in 20 μ l of 2% (v/v) acetonitrile/0.2% (v/v) formic acid solution.

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Table 1 Protein purification table for the purification of *T. lanuginosus* VAPS-24 xylanase, XynA

Fraction	Volume (ml)	Total protein (mg)	Activity (U/ml)	Total activity (U)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude	12	16.65	17.69	212.28	12.73	1	100
Ultra-filtrate	14	4.24	14.99	209.83	49.96	3.92	98.84

The Ultra-filtrate fraction was composed of the > 10 kDa and < 30 kDa cut-off fractions

Initially, de-salting of the peptides was performed, followed by separation on an Acclaim PepMap C18 RSLC column. Elution of the peptide was performed using a 4–60% gradient of B (A: 0.1% (v/v) formic acid; B: 80% (v/v) acetonitrile/0.1% (v/v) formic acid) at a flow-rate of 8 μ l/min over 15 min. An electrospray voltage of 5.5 kV was standardized for the emitter.

Peaks (Bioinformatics Solutions) were used for evaluation of the obtained MS/MS spectra with a conventional database comprising sequences of Eurotiales (Uniprot Swissprot), in addition to several sequences from commonly contaminating proteins as described previously [10]. The identification of proteins was permitted if they could be formed at larger than 99.0% probability and contained a minimum of 1 identified peptide (at 95% and greater). The peptide and proteins false discovery rate (FDR) cut-offs were set to 0.1 and 1%, respectively.

The *T. lanuginosus* VAPS-24 xylanase, XynA, could be purified to a 98.84% yield with a purification fold of 3.92 and a specific activity of 49.96 IU/mg on wheat-flour arabinoxylan (see Table 1). The ultrafiltration fraction after purification constituted the enzyme solution used in all subsequent enzyme assays. SDS-PAGE analysis of the purified enzyme displayed a single band with an estimated molecular mass of 20 kDa (Fig. 1). The single band of the XynA enzyme on the SDS-PAGE was then excised and subjected to tryptic digestion and evaluated using a Dionex Ultimate 3000 RSLC system attached to an AB Sciex6600 TripleTOF mass spectrometer.

Peaks (Bioinformatics Solutions) database search on the produced peptides produced matches against a xylanase from the *T. lanuginosus* SS-8 strain. Amino acid sequence coverage showed that the xylanase, XynA, produced by the *T. lanuginosus* VAPS-24 strain compares well with the xylanase from strain *T. lanuginosus* SS-8 (UniProt ID O43097) over a query coverage of 77% (Fig. 2a, b). XynA showed significant matches with two peptides, AGLNVNGDHYQIVATEGYFSSGYAR (2853.3115 MH⁺) and VNAPSIDGTQTFDQYWSVR (2184.035 MH⁺), identified as GH family 11 putative domains (Fig. 2c). The two peptides had ion score values of 76.16 and 77.96, respectively (Fig. 2c). The ion score is defined as $-10 \log(P)$, where P is the probability that an observed

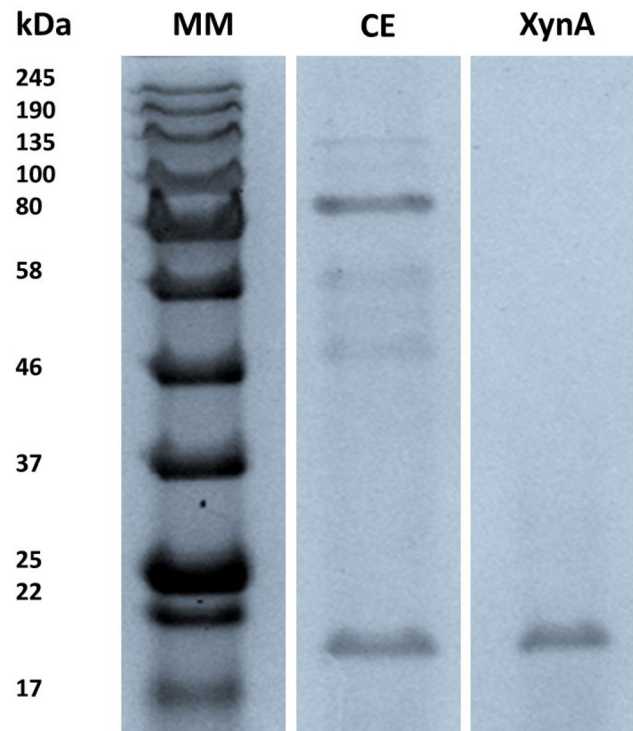


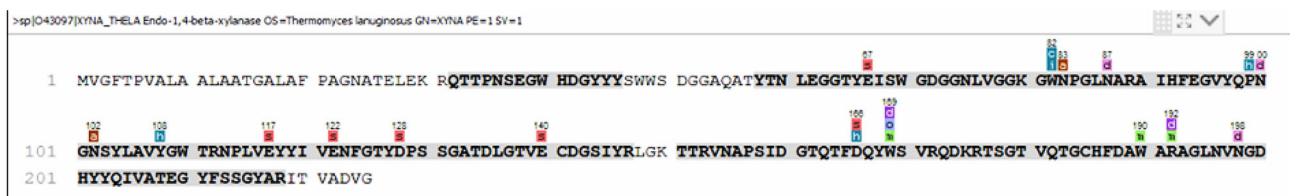
Fig. 1 SDS-PAGE analysis of the ultra-filtration purified XynA from *T. lanuginosus* VAPS-24. Lane 1: (MM) molecular weight standard, lane 2: (CE) crude extract of XynA and lane 3: XynA purified by ultra-filtration

match is a random event and individual ions scores of > 34 indicate identity or extensive homology ($P < 0.05$) [11]. However, seventeen post-translational modifications (PTMs) were identified on some amino acids in the XynA sequence which were not present on the SS-8 homolog, with three of these modifications occurring in the two GH family 11 putative domains and one of these PTMs (sulfonation) occurring on one of the two glutamic acid residues (residue E-117, Fig. 2B) of xylanases reported to be involved in enzymatic activity as noted by Wakarchuk and co-workers [12]. Upon purification from the growth media by ultra-filtration, the molecular mass of the *T. lanuginosus* VAPS-24 xylanase, XynA, was determined to be 20 kDa using SDS-PAGE (Fig. 1). The molecular weight of the xylanase was similar to that of XynSS8 from a *T. lanuginosus* SS-8 strain obtained by nanoLC ESI MS (21.3 kDa)

A

Accession	Score (...)	-10lgP	Coverage	#Peptides	#Unique	PTM	Avg. Mass	Description
O43097 XYN_A_THELA	99.2	350.69		61	53		24356	Endo-1,4-beta-xylanase OS=Thermomyces lanuginosus GN=XYN_A PE=1 SV=1
TRYP_PIG	99.2	277.80		23	23		24409	sp TRYP_PIG
K1C10_HUMAN	99.2	276.98		21	16		59519	sp K1C10_HUMAN
K2C1_HUMAN	99.2	254.35		18	15		65886	sp K2C1_HUMAN
K2ZE_HUMAN	99.2	248.95		17	14		65865	sp K2ZE_HUMAN
K1C9_HUMAN	99.2	222.28		12	11		62129	sp K1C9_HUMAN
P81536 XYN_A_BYSSP	98.8	161.69		10	2		20947	Endo-1,4-beta-xylanase OS=Byssoschlamys spectabilis PE=1 SV=1
REF_HEVBR	99.1	143.87		5	5		14722	sp REF_HEVBR
CAS1_BOVIN	98.4	126.56		3	3		24529	sp CAS1_BOVIN

B



C

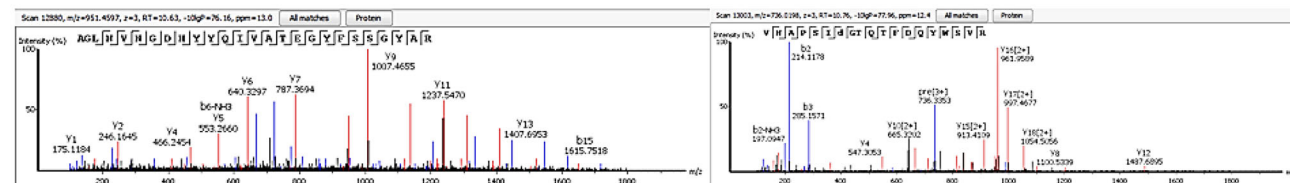


Fig. 2 Amino acid sequence identification of *T. lanuginosus* VAPS-24 XynA by tryptic mapping and MS/MS. **a** Protein match hit for *T. lanuginosus* VAPS-24 XynA against SS-8 xylanase in Peaks database showing 77% identity, **b** protein coverage view of *T. lanuginosus* VAPS-24 XynA against *T. lanuginosus* SS-8 xylanase sequence with

matched peptides shown in bold (shaded grey), while PTMs are shown above amino acid residues as coloured letters and **c** MS/MS spectra for the two putative GH11 family domains found in *T. lanuginosus* VAPS-24 XynA

[13]. The amino acid sequence of XynA was then determined using tryptic mapping. Tryptic mapping illustrated that XynA is highly similar to XynSS8 from a *T. lanuginosus* SS-8 strain, however, numerous PTMs were present on XynA which seemed to be absent on XynSS8 (Fig. 2). Similar to our work, Silva and co-workers reported differences in MS/MS fragmentation of their *Emericella nidulans* xylanase compared to that reported for other *Emericella nidulans* isolates—this was thought to be as a result of different isoforms of the enzyme from this fungal species, as the enzyme was produced from the same fungus, but grown under different conditions to those reported previously [14]. As a result, there were differences in PTMs and/or amino acid sequences of these isoforms. From this data, we confirmed that XynA from strain VAPS24 is a xylanase belonging to GH family 11 and an isoform of the xylanase produced by other strains of *Thermomyces lanuginosus* species, and as a result, the enzyme can be used as a representative model of *Thermomyces* species derived xylanases. Having this model representative protein structure can aid in numerous studies, such as docking and molecular dynamics, to better understand enzyme characteristics (i.e. catalytic

mechanism or inhibition) and in facilitating protein engineering approaches to design variants with desirable properties (i.e. improved catalytic efficiency or thermostability).

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