Molecular characterization of a *Penicillium chrysogenum* exo-1,5-α-L-arabinanase that is structurally distinct from other arabinan-degrading enzymes[1]

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Abstract The nucleotide sequence of the *abnx* cDNA gene, which encodes an exo-arabinanase (Abnx) of *Penicillium chrysogenum* 31B, was determined. Abnx was found to be structurally distinct from known arabinan-degrading enzymes based on its amino acid sequence and a hydrophobic cluster analysis. The protein in the protein database with the highest similarity to Abnx was the *Neurospora crassa* conserved hypothetical protein. The *abnx* cDNA gene product expressed in *Escherichia coli* catalyzed the release of arabinobiose from α-1,5-L-arabinan. The activity of the recombinant Abnx towards a series of arabino-oligosaccharides, as expressed by k_{cat}/K_m value, was greatest with arabinohexaose.
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Key words: Exo-arabinanase; Arabinobiose; Nucleotide sequence; Expression; *Penicillium chrysogenum*

1. Introduction

Several enzymes that degrade arabinose-containing polymers of plant cell walls have been purified from various microorganisms (for a review, see [1]). These polymers include branched L-arabinan, a highly branched α-1,5-L-arabinan possessing 1,2- or 1,3-α-linked L-arabinofuranosyl residues as side chains, and arabinoxylan, a β-1,4-D-xylan to which α-L-arabinofuranosyl residues are attached at position 2 or 3. Although Beldman et al. [1] proposed classifying arabinan-degrading enzymes into six types depending on their substrate specificity and mode of action, most of the enzymes previously isolated belong to three types: endo-1,5-α-L-arabinanase (EC 3.2.1.99)

and two kinds of α-L-arabinofuranosidase (EC 3.2.1.55), which are distinguished by their activity towards polymers.

The *Penicillium chrysogenum* 31B strain produced at least five different arabinan-degrading enzymes in the culture filtrate [2–4]. In order to elucidate the functions of the enzymes in the degradation of plant cell walls, we have started to purify all five enzymes and isolate the corresponding genes. One of the enzymes, an exo-arabinanase termed Abnx, has been reported to catalyze the release of dimeric arabinose from the non-reducing end of substrates consisting of α-1,5-L-arabinofuranose units with both high and low molecular mass [2]. Abnx is the first enzyme to be shown to have this mode of action. McKie et al. [5] reported that *Pseudomonas fluorescens* subsp. *cellulosa* produced an arabinanase with endo and exo modes, which mainly releases arabinotriose from linear α-1,5-L-arabinan. The deduced amino acid sequence of the *Pseudomonas* arabinanase showed greatest identity to that of the *Aspergillus niger* endo-arabinanase, suggesting that the former enzyme belongs to endo-type arabinanases [5]. So far, the nucleotide sequence of the gene encoding an exo-acting arabinanase has not been determined. This paper reports the nucleotide sequence for the gene that encodes Abnx of *P. chrysogenum* 31B (*abnx*). We also report characterization of the recombinant Abnx expressed in *Escherichia coli*.

2. Materials and methods

2.1. Chemicals and reagents
Debranched arabinan and α-1,5-L-arabino-oligosaccharides with different degrees of polymerization (DPs) were obtained from Megazyme International Ireland. All other chemicals were from Wako Pure Chemical Industries (Osaka, Japan) unless otherwise stated.

2.2. Strain, plasmid, media, and growth conditions
E. coli DH5α and the plasmids, pCR II-TOPO (Invitrogen) and pMAL-c2X (New England BioLabs), were used for cloning, sequencing, and overexpression of the polymerase chain reaction (PCR) products. Transformants were grown in LB medium consisting of 1% peptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.0, supplemented with ampicillin (50 μg/ml).

2.3. DNA manipulations and nucleotide sequencing
General procedures for DNA manipulations were done as described by Maniatis et al. [6]. Nucleotide sequences were determined by the dideoxy chain termination method of Sanger et al. [7] with the DYEnamic ET Terminator Cycle sequencing kit (Amersham Biosciences)

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Abbreviations: CDS, coding sequence; CLM-PCR, cassette ligation-mediated polymerase chain reaction; DP, degree of polymerization; GH, glycosyl hydrolase; HCA, hydrophobic cluster analysis; HPAEC, high-performance anion-exchange chromatography; MBP, maltose binding protein

on an automated DNA sequencer (model 373 Stretch; Applied Biosystems).

2.4. Primers and PCR conditions

The primers used in this study are shown in Fig. 1. The C1 and C2 primers used for cassette ligation-mediated (CLM) PCR were obtained from Takara Bio (Shiga, Japan). The PCR reactions (0.1 ml) contained 0.5 U of *TaKaRa LA Taq* DNA polymerase (Takara), 0.2 mM of dNTPs, 50 ng of the template, 0.5 mM each of primers, and $1 \times GC$ buffer supplied by the manufacturer. Typically, 25 cycles of PCR were conducted at the following temperatures: 94°C for 1 min, 40–60°C for 2 min, and 72°C for 2 min.

2.5. N-terminal and internal amino acid sequences of Abnx

To determine the N-terminus of Abnx, the deglycosylated protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using a 10% gel by the method of Laemmli [8] and electrophoretically transferred onto a polyvinylidene difluoride membrane.

For internal sequence determination, the deglycosylated Abnx was proteolytically digested by lysylendopeptidase in a polyacrylamide gel and the peptides were separated with reversed-phase high-performance liquid chromatography, as previously described [9]. The N-terminus of the protein and the N-terminal sequences of the peptides were determined by automated Edman degradation using a gas phase sequencer.

2.6. Total RNA extraction and cDNA synthesis

One hundred milliliters of medium, consisting of 0.2% NH_4NO_3, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4$, 0.1% peptone, and 1% sugar beet extract, pH 5.0, was inoculated with pre-cultured *P. chrysogenum* 31B and incubated at 30°C for 13 days under static conditions in a 200 ml Erlenmeyer flask. Total RNA was prepared from the mycelia using an RNeasy Mini Kit (Qiagen). Single-stranded cDNA was synthesized from total RNA using random hexamer primers and a reverse transcriptase (SuperScript II; Invitrogen).

2.7. Enzyme assay

A typical assay for enzyme activity was performed with debranched arabinan as the substrate. The reaction mixture contained 20 μl 2% debranched arabinan in 20 mM Na-acetate buffer, pH 5.0, and 3 μl enzyme solution, and was incubated at 37°C for 30 min. The mixture was boiled for 5 min to inactivate the enzyme, and 180 μl 25 μg/ml D-galacturonic acid was added as an internal standard. The release of arabinobiose was quantified by high-performance anion-exchange chromatography (HPAEC) using a Dionex DXc-500 system with a CarboPac PA-1 column (4×250 mm; Dionex). Sugars were eluted at a flow rate of 1 ml/min with 0.1 M NaOH for 5 min followed by a linear gradient from 0 to 0.45 M Na-acetate in 0.1 M NaOH for 30 min. The effluent was monitored with pulsed amperometric detection.

3. Results and discussion

3.1. Partial amino acid sequences of Abnx

The purified Abnx was obtained from the culture filtrate of *P. chrysogenum* 31B by the method of Sakamoto and Thibault [2]. The molecular mass of Abnx was reduced from 47 to 42 kDa by treatment with endoglycosidase H, demonstrating that the enzyme was a glycoprotein. After removal of carbohydrates linked to Abnx with endoglycosidase H, the N-terminal amino acid sequence of the enzyme was determined to be SSPTSLTXVTIFSPPSDYIVPRTLY, where X indicates an undetermined residue. To analyze the partial internal amino acid sequence of Abnx, the deglycosylated enzyme was treated with lysylendopeptidase. The sequences of the resultant two main fragments, F73 and F84, were NWGPVVEDVTYP-TYTDRPGM and TWNEISRVHDTVXGY, respectively.

3.2. Sequence and characterization of the gene encoding Abnx

The region between the N-terminus of the enzyme and the F73 peptide was PCR-amplified using the genomic DNA of
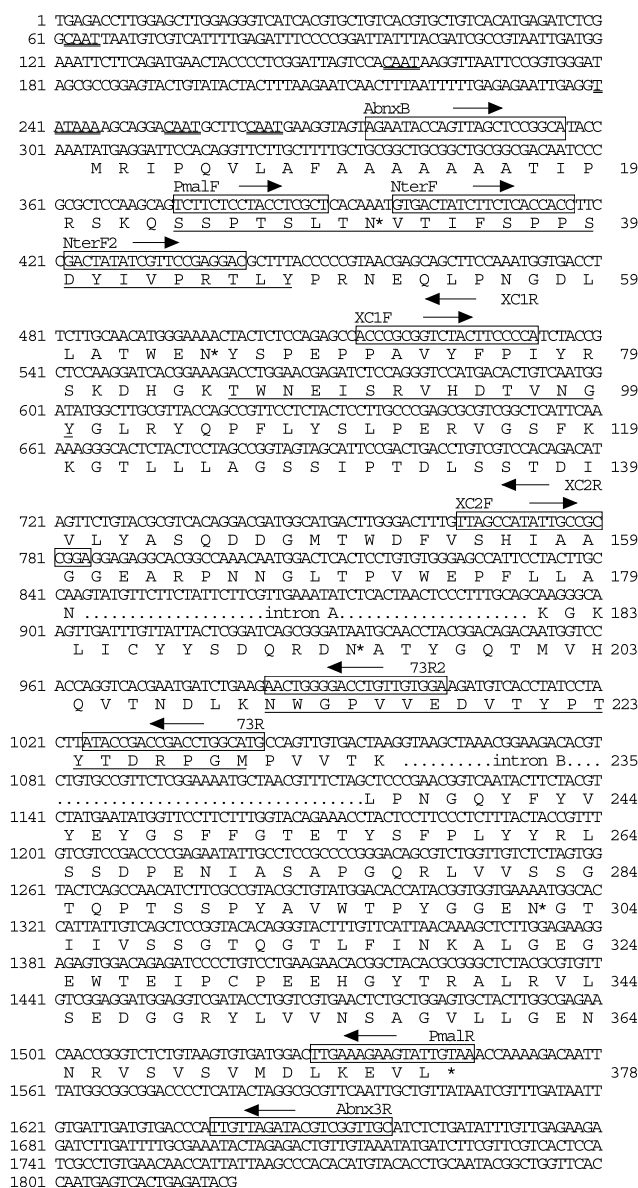

Fig. 1. Nucleotide sequence of *abnx* from *P. chrysogenum* 31B. The deduced amino acid sequence is shown beneath the nucleotide sequence. The N-terminal amino acid sequence of the mature *P. chrysogenum* Abnx and proteolytic fragments, determined by Edman degradation, are underlined. TATAAA and CAAT boxes are double underlined. Potential *N*-glycosylation sites are indicated by asterisks. The primer sequences are boxed and their directions are shown by arrows.

P. chrysogenum 31B as the template. The primary and secondary rounds of PCR were performed using two sets of primers, NterF (5′-GTIACIATHTTYWSICCNCC)/73R (5′-CATNC-CIGGICKRTCIGTRT) and NterF2 (5′-GAYTAYATHG-TICCIMGIAC)/73R2 (5′-TCIACIACIGGICCCCARTT). A PCR product of 0.6 kb, termed NT-1, was obtained. Subsequently, the 5′ region of NT-1 was amplified from a *Pst*I cassette-ligated genomic DNA using two sets of primers (C1/XC2R in the primary amplification and C2/XC1R in the secondary round of PCR) by CLM-PCR as described previously [10]. The amplified fragment (ca. 1.2 kb) was sequenced and was found to have the putative ATG start codon of the *abnx* gene. In order to obtain the 3′ region of NT-1,

CLM-PCR was performed using two sets of primers (C1/XC1F in the primary amplification and C2/XC2F in the secondary amplification of PCR) and a *Hind*III cassette-ligated genomic DNA as the template. A fragment of 1.2 kb was obtained and sequenced. Together, the 5′ and 3′ sequences yielded a 1695 bp sequence from the putative ATG start codon of the *abnx* gene. A BLAST search of the protein sequence database revealed high similarity between the deduced amino acid sequences of *abnx* and a gene encoding a *Neurospora crassa* conserved hypothetical protein (GenBank accession number AL355929 and protein_id no. CAB91342.2). Based on this result, we speculated that the coding sequence (CDS) of *abnx* starts with an ATG codon at nucleotide 305 and stops with a TAA termination codon at nucleotide 1544. The CDS of the *abnx* gene and the flanking regions are shown in Fig. 1.

To clone the full-length *abnx* cDNA, two primers (AbnxB and Abnx3R) were synthesized based on the sequencing data of genomic DNA. The two primers were used for PCR amplification using the cDNA that was reverse-transcribed from total RNA of *P. chrysogenum* 31B as the template. Sequencing the amplified fragment (ca. 1.1 kb) demonstrated that the CDS was interrupted by two introns, A and B, having lengths of 49 and 56 bp, respectively (Fig. 1). In both introns, the fungal consensus 5′ and 3′ splicing sites GTPuNGPy and PyAG were conserved [11]. Furthermore, the sequences CACTAAC and TGCTAAC were found in introns A and B, respectively, and were identical to the fungal intron internal consensus sequences NPuCTPuAC [12] and PyGCTAAC [13]. The CDS of the *abnx* gene contained 1134 bp which encodes a protein of 378 amino acids with a calculated molecular mass of 41 636 Da and a calculated isoelectric point of 5.11. The calculated mass was in good agreement with that of the deglycosylated Abnx. The amino acid sequences determined by Edman degradation of polypeptides were found at positions 24–48 (Abnx), 86–100 (F84), and 211–230 (F73) in the deduced amino acid sequence. Four asparagine residues at amino acid positions 31, 65, 194, and 302 were identified as potential *N*-glycosylation sites. Analysis of the amino acid sequence using the signal-peptide-prediction program signalP version 1.1 (http://www.cbs.dtu.dk/services/SignalP/) indicated that the precursor of Abnx might be cleaved at Ala-Thr at positions 16 and 17. The mature Abnx starts with Ser-24 and it is probable that the N-terminal extension of the Abnx precursor contains a signal peptide and a pro-peptide. In the 5′ upstream region, a putative TATAAA box is present at nucleotide 240 and four CAAT boxes are present at nucleotides 62, 157, 253, and 262, whereas CT-rich sequences, which are known fungal promoter elements, were not found.

3.3. Expression of the abnx gene in E. coli

In order to obtain the region of the mature Abnx, PCR was performed using the two primers, PmalF and PmalR, and the cloned *abnx* cDNA as the template. PmalF and PmalR primers have *Bam*HI and *Hind*III sites, respectively, at their 5′ ends. The amplified fragment (ca. 1.1 kb) was digested with *Bam*HI and *Hind*III, and ligated to the restriction enzyme sites of the pMAL-c2X vector, forming a new plasmid termed pMAL-AX. Because the PCR product was inserted into a pMAL-c2X downstream of the *malE* gene, which encodes maltose binding protein (MBP), the recombinant Abnx expressed from pMAL-AX was expected to be a MBP fusion

Abnx. For production of the recombinant Abnx, *E. coli* DH5α transformed with the plasmid pMAL-AX was grown in 1 l LB medium containing ampicillin (50 µg/ml) and isopropyl-β-thiogalactopyranoside (1 mM) as the inducer. The recombinant protein was then purified from the cell-free extract using an amylose resin column (New England BioLabs). The extract was dissolved in a buffer consisting of 20 mM Tris–HCl, pH 7.4, 200 mM NaCl, and 1 mM EDTA and put on the column equilibrated with the above buffer. The bound proteins were eluted by the same buffer containing 10 mM maltose. This procedure yielded 75 mg of MBP fusion Abnx from 1 l of the transformant culture medium.

3.4. Characteristics of the recombinant MBP fusion Abnx

HPAEC analysis of the reaction products of debranched arabinan with the recombinant Abnx revealed that the protein released only arabinobiose from the substrates (data not shown). The temperature and pH dependence of the activity of the recombinant Abnx (Fig. 2) were similar to those of the *P. chrysogenum* Abnx. The stabilities of the enzymes were found to be similar under the same temperature conditions. The recombinant Abnx was stable in the pH range 4–10 although the reason for the activation of the enzyme by the treatment at pH 10 is not known (Fig. 2b). The stable pH range of the recombinant enzyme was substantially more alkaline than that of the native Abnx (pH 3–8). This difference seemed to be explained by the presence of the MBP linked to Abnx.

The kinetic parameters of the recombinant Abnx towards arabino-oligosaccharides with different DPs (DP2 to DP7) were examined at pH 5.0 and 37°C. Table 1 shows that k_{cat}/K_m increased with increasing DP, reaching the highest value at DP6.

In order to study the action pattern of the recombinant Abnx, product formation at different reaction times was monitored with HPAEC using α-1,5-L-arabinoheptaose as the substrate. Based on the mode of action of Abnx [2], the substrate α-1,5-L-arabinoheptaose would be expected to yield arabinopentaose, arabinotriose, arabinobiose, and arabinose. During the early stage of the enzyme reaction, arabinobiose and arabinopentaose, but not arabinotriose, were formed. During later stages of the reaction, arabinobiose and arabinotriose accumulated and a small amount of arabinose was de-
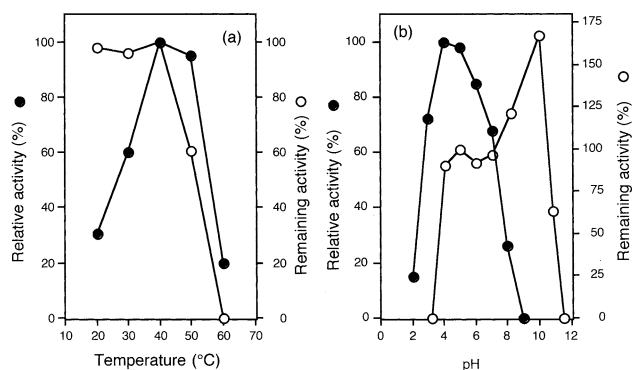

Fig. 2. Effects of temperature (a) and pH (b) on activity (closed circles) and stability (open circles) of the recombinant MBP fusion Abnx. All experiments were carried out under the same conditions described previously [3] except that debranched arabinan was used as the substrate for the enzyme assay.

Table 1
Kinetic parameters of the recombinant Abnx towards arabino-oligo-saccharides with different DPs

Substrate	K_m (mM)	k_{cat}/K_m (s^{-1}. mM^{-1})
DP3	1.01	3.01
DP4	0.85	11.53
DP5	0.63	17.80
DP6	0.33	39.22
DP7	0.36	32.62

In a typical assay, 0.168 µg of the enzyme was incubated with various concentrations of arabino-oligosaccharide as the substrate. Concentration of the recombinant Abnx was calculated as the molecular mass of 82 305. Content of arabino-oligosaccharides used for the substrates was determined by the phenol/sulfuric acid method [14]. The data were analyzed by Lineweaver–Burk plots using a least-squares linear regression.

tected (Fig. 3). Generally enzymes degrade substrates in either multi-chain or single-chain attack mode. The data obtained here suggest that Abnx acts in a multi-chain attack mode and that arabinotriose is more resistant to the enzyme than larger oligomers as demonstrated in Table 1.

3.5. Amino acid sequence alignment

Arabinan-degrading enzymes have been mainly grouped into four glycosyl hydrolase (GH) families based on their amino acid sequence (GH 43, GH 51, GH 54, and GH 62) ([15–17], and http://afmb.cnrs-mrs.fr/CAZY/index.html). Four *A. niger* enzymes that are examples of each of these respective families are endo-1,5-α-L-arabinanase (*abnA*) [18], α-L-arabinofuranosidase (*abfA*) [19], α-L-arabinofuranosidase (*abfB*) [20], and 1,4-β-D-arabinoxylan arabinofuranohydrolase (*axhA*) [21]. However, the deduced amino acid sequence of Abnx has no similarity to the deduced sequences of enzymes belonging to these GH families. Furthermore, no similarity was found in the sequence of the *P. fluorescens* arabinanase with endo and exo modes [5]. Using the FASTA program, the deduced protein sequence of the *abnx* gene showed moderately high homologies with the deduced sequences of a *N. crassa* conserved hypothetical protein (381 amino acids; 39% identity in a 372 amino acid overlap) and a *Streptomyces coelicolor* putative bifunctional protein (GenBank accession number AL359779 and protein_id no. CAB95280.1; 555 amino acids; 30% identity in a 322 amino acid overlap).

Although the identities of these amino acid sequences were low, comparisons of primary structures of proteins are generally not a reliable way to detect structural similarities. Therefore, we performed a hydrophobic cluster analysis (HCA) to more precisely classify Abnx. HCA allows detection of conserved secondary structure elements in proteins which show low sequence identity [22,23]. Abnx was compared with the mentioned four *A. niger* arabinan-degrading enzymes, *N. crassa* hypothetical protein, and *S. coelicolor* bifunctional protein using HCA. The secondary structures were also predicted by PHDsec on the Internet (http://cubic.bioc.columbia.edu/pp/). No structural similarity was found between Abnx and the four *A. niger* enzymes (data not shown), demonstrating that Abnx is distinct from known arabinan-degrading enzymes in terms of protein structure. In contrast, HCA revealed that Abnx was considerably similar to *N. crassa* hypothetical protein throughout the protein (Fig. 4). This result strongly suggests that the *N. crassa* protein is a member of GHs, and perhaps has the same function as Abnx. Abnx also

showed moderate similarity to the N-terminal domain of *S. coelicolor* bifunctional protein by HCA. The bifunctional protein has been suggested to be a secreted sugar binding protein and a sugar hydrolase (http://www.sanger.ac.uk/Projects/S_coelicolor/), although experimental evidence that it has GH activity has not been proven. The data obtained here support the hypothesis that the protein belongs to GHs.

To further characterize Abnx, the similarity of module sequences was analyzed using the 'Protein families database of alignments and HMMs' (http://www.sanger.ac.uk/Software/Pfam/search.shtml). This analysis showed that a bacterial neuraminidase repeat sequence, called an Asp box (Pfam accession number PF02012), is present at positions 78–89 and 142–153 of Abnx. The repeats are found in a variety of GHs as well as in other proteins including bacterial ribonucleases and sulfite oxidases [24]. Like the Asp boxes of other proteins, those of Abnx have the sequence SXDXGXTW with five conserved amino acids [25].
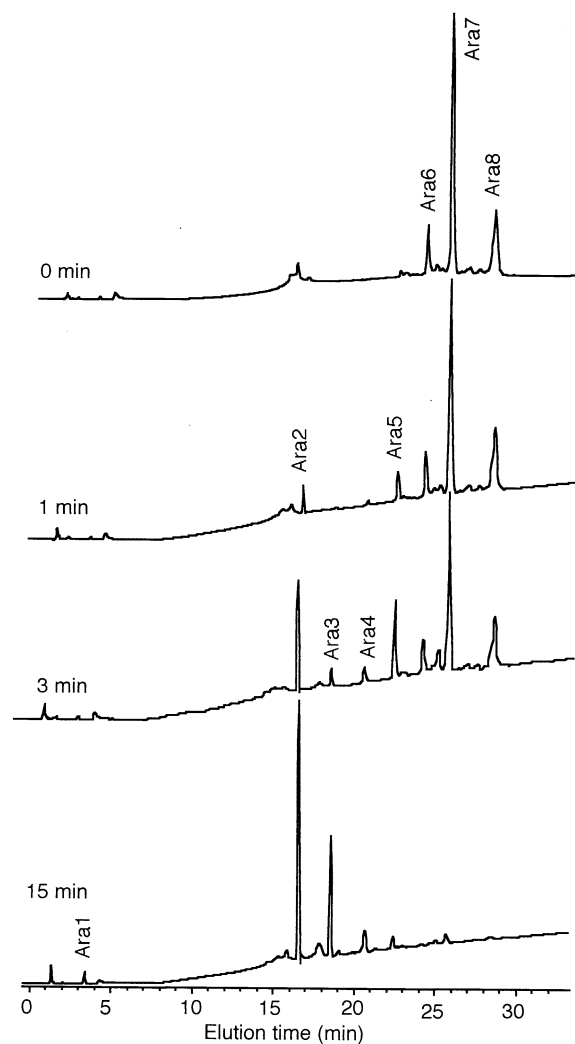

Fig. 3. Analysis of the enzymatic products of α-1,5-L-arabinoheptaose with the recombinant MBP fusion Abnx. A reaction mixture containing 7 µg of the enzyme and 100 µl of 5 mM arabinoheptaose in 20 mM Na-acetate buffer, pH 5.0, was incubated at 37°C for the times shown. Aliquots were taken at intervals and inactivated by boiling for 5 min, followed by analysis of the reaction products with HPAEC. Ara1 to Ara8 represent arabinose to arabinooctaose, respectively.
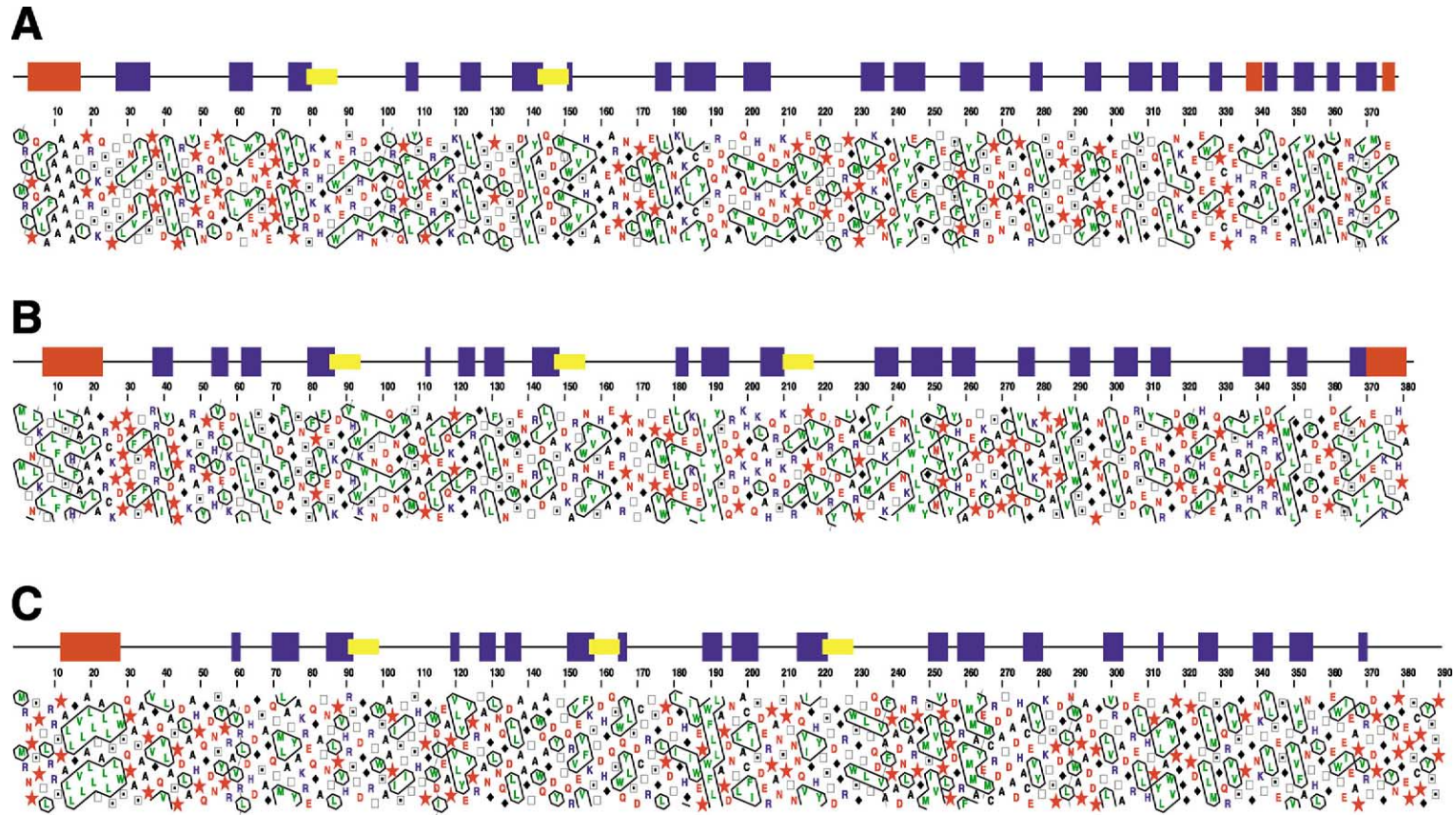
Fig. 4. Comparison of secondary structures and HCA plots of Abnx (A), *N. crassa* conserved hypothetical protein (B), and N-terminal region of *S. coelicolor* putative bifunctional protein (C). The HCA plots can be obtained from the drawcha server on the Internet (http://smi.snv.jussieu.fr/hca/hca-form.html). Clusters of hydrophobic amino acids are circled. Glycine, proline, serine, and threonine are represented by diamonds, stars, squares with dots, and open squares, respectively. Predictions of the secondary structures are based on both hydrophobic cluster shapes and the result obtained with the PHDsec secondary structure prediction program. In the secondary structures, α-helices, β-strands, and Asp boxes are indicated in red, blue, and yellow, respectively.

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