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Original Communication

A novel member of the dipeptidyl peptidase III family from *Armillariella tabescens*

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ABSTRACT

Dipeptidyl peptidases III (DPPIII) are zinc-dependent peptidases and constitute the M49 family of metalloproteases. These enzymes are presumed to play a general role in peptide catabolism in the cell cytoplasm and may also have more specific roles in mammalian physiology. Here, we describe the recombinant production and characterization of DPPIII from the ringless honey mushroom *Armillariella tabescens* (*Arta*DPPIII). The purified protein possessed all characteristic features expected of an enzyme of the M49 family, such as cleavage of dipeptidyl-2-naphthylamides and inhibition by the peptide tynorphin. Previous reports that the enzyme is capable of oxidizing aflatoxin B_1 and thus acts as a detoxifying enzyme could not be reproduced. In accordance with its enzymatic properties, the amino acid sequence of *Arta*DPPIII exhibits all characteristics established for this protein family and lacks any features that could rationalize additional enzymatic activities, such as the putative aflatoxin oxidase activity. Thus, in conclusion, *Arta*DPPIII is a canonical member of the M49 family of metalloproteases and earlier claims that the enzyme carries novel and very unusual oxidative activities are not supported by our study.

KEYWORDS: aflatoxin, dipeptidyl peptidase, homology model, metallopeptidase, oxidase, phylogeny, protein structure, zinc.

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ABBREVIATIONS

AFO, aflatoxin oxidase; AFB_1, aflatoxin B_1; *Arta*DPPIII, dipeptidyl peptidase III from *Armillariella tabescens*; PAGE, polyacrylamide gel electrophoresis.

INTRODUCTION

Dipeptidyl peptidases III cleave dipeptides from the N-terminus of peptide substrates. With the exception of archaea, the enzyme was found in all kingdoms of life, *i.e.* prokaryotes, fungi, plants and animals [1]. Despite the ubiquitous presence of the enzyme in nature, it appears to carry out various functions ranging from catabolic metabolism to more complex physiological roles in plants and vertebrates [2-4]. The enzyme requires a bivalent cation such as zinc or cobalt in the active site to activate a water molecule for the nucleophilic attack on the carbonyl carbon of the scissile peptide bond. The metal ion is typically coordinated by two histidines and a glutamate side chain in the first coordination shell with a water molecule acting as the fourth ligand [5]. This water molecule closely interacts with another glutamate residue that is part of the characteristic active site motif HEXXXH, which is similar to the HEXXH motif found in other peptidases [6]. Replacement of this glutamate residue leads to complete inactivation of the enzyme and enabled the crystallization and structure elucidation of protein-substrate complexes [7, 8]. Dipeptidyl peptidase is composed of two domains, which form an open crevice to accommodate small peptides possessing between 4 and 12 amino acids.

The structure determination of DPPIII from the yeast *Saccharomyces cerevisiae* [5], *Homo sapiens* [7], and *Bacteroides thetaiotaomicron* [9] has demonstrated that the overall topology of the enzyme is highly conserved in nature. Thus, dipeptidyl peptidase III is a well-defined protein, both in terms of structure and the metal-dependent active site machinery required for peptide bond hydrolysis. In the light of this detailed knowledge, it was very surprising that a DPPIII homolog from the mushroom *Armillariella tabescens* was reported to oxidize aflatoxin B_1 (AFB_1), *i.e.* to possess an oxidase activity [10]. The ability to oxidize AFB_1 clearly appears to be related to the enzyme class of oxidoreductases [11] and thus we wondered whether the DPPIII homolog from *Armillariella tabescens* indeed exhibits biochemical and structural properties that are in accordance with an oxidoreductase rather than a hydrolase activity. Toward this end, we have expressed the putative *dppIII*-gene from *Armillariella tabescens* in *Escherichia coli*, purified the generated protein and investigated its enzymatic properties. Here, we demonstrate that the DPPIII homolog from *Armillariella tabescens* entirely behaves like a canonical DPPIII and lacks the oxidative activity previously ascribed to the protein.

MATERIALS AND METHODS

Protein expression and purification

The gene for *Arta*DPPIII was obtained from Thermo Fisher Scientific and was optimized for expression in *E. coli*. The gene was cloned into plasmid pLATE31 using primers *Arta*DPPIII_LICF, AGA AGGAGATATAACTATGGCAACCACCACCG, and *Arta*DPPIII_LICR, GTGGTGGTGATGGT GATGGCCCAGACGACGTTCAATAAAGC, according to the instructions of the aLICator LIC Cloning and Expression Kit 3 (Thermo Fisher Scientific). Amino acid sequence of the gene product is identical to the UniProt entry B0S4Q0, with a His-tag added to the C-terminus as a GHHHHHHG sequence. The variant E444A was prepared using mutagenesis primers *Arta*DPPIII_E444A_F, GCA GGTTGCAAACCATGCGCTGCTGGGTCATG GTAGC, and *Arta*DPPIII_E444A_R, GCTACCA TGACCCAGCAGCGCATGGTTTGCAACCTGC, as described in the manual for QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). Plasmids were sequenced (Macrogen) to confirm the correct sequence of the full-length gene and transformed into *E. coli* strain BL21-CodonPlus-(DE3)-RIL (Agilent Technologies) for expression. Bacteria were cultured in Luria-Bertani medium supplemented with 100 μg/ml ampicillin and 10 μM $ZnSO_4$. Expression was induced at OD_{600} = 0.6 with 0.25 mM IPTG (isopropyl β-D-1-thiogalactopyranoside, Thermo Fisher Scientific) and continued for 20 h at 18 °C, 130 rpm. Cells were harvested by centrifugation and the pellets stored at -20 °C.

The wild-type and variant protein were purified by affinity chromatography on Ni-NTA (Qiagen). Cells were resuspended in lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, pH 8.0) and disrupted using lysozyme and sonication. The lysate was cleared by centrifugation and filtration, and then applied to the column. Lysis buffer containing 20 mM imidazole was used to wash the column and the target protein was eluted with the same buffer containing 300 mM imidazole. Fractions were pooled and desalted on a PD-10 column (GE Healthcare). The purified protein was stored in 20 mM Tris-HCl at -80 °C.

Peptidase assay

Peptidase assays were performed using dipeptidyl-2-naphthylamides as substrates. Upon cleavage, 2-naphthylamine is produced. For substrate screening we used a colorimetric assay [12] in 1 mL reaction with 40 μM substrates at 37 °C, and for kinetic measurements a fluorometric assay [8] in 3 mL reaction at 25 °C. After the initial activity screening for optimum conditions, we used 50 mM Tris-HCl buffer pH 8.0 for all assays and kinetic measurements. The kinetic parameters were determined from initial rates by nonlinear regression in GraphPad Prism 5 (GraphPad Software).

Aflatoxin oxidase assay

The assay for aflatoxin oxidase activity was modified from Cao *et al.* [13]. The enzyme reaction was scaled down from 1 ml to 100 μl, keeping the buffer and $CuCl_2$ concentration the same. The amount of AFB_1 (Sigma-Aldrich) and purified protein was 50-500 ng and 10-100 μg per reaction, respectively. The reactions were incubated for 30 min or 24 h at 30 °C. Silica gel plates were developed in ether or chloroform: acetone (88:12, v/v).

Bioinformatic analysis

To obtain a homology model of the *Arta*DPPIII structure the amino acid sequence was submitted to the Phyre2 server [14]. To compare the amino acid sequence of *Arta*DPPIII to other characterized members of the M49 metallopeptidase family, sequences were retrieved from UniProt: Q9NY33 *Homo sapiens*, Q08225 *Saccharomyces cerevisiae*, Q8A6N1 *Bacteroides thetaiotaomicron*, Q7MX92 *Porphyromonas gingivalis*, A9TLP4 *Physcomitrella patens*, O55096 *Rattus norvegicus*, Q9VHR8 *Drosophila melanogaster*, Q557H1 *Dictyostelium discoideum*, and B0S4Q0 *Armillariella tabescens*. Sequences corresponding to other domains (NUDIX in *P. patens* and Armadillo-like fold in *P. gingivalis*) were removed before analysis. The phylogenetic tree was constructed in MEGA 7 software [15] employing the maximum likelihood method based on the JTT matrix-based model.

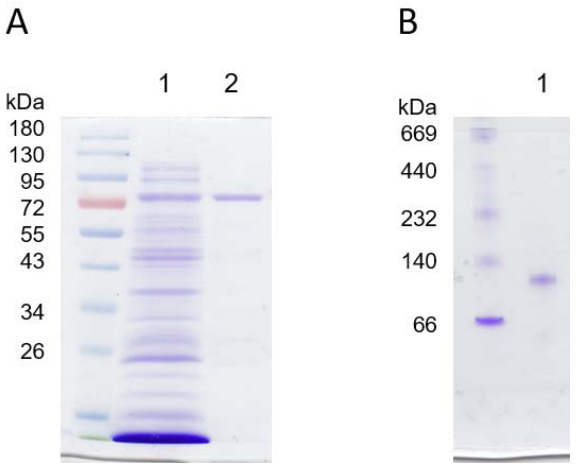

Figure 1. PAGE analysis of purified *Arta*DPPIII. Panel A: SDS-PAGE of the bacterial lysate (lane 1) and purified *Arta*DPPIII (lane 2). Panel B: Native PAGE of purified *Arta*DPPIII (lane 1).

RESULTS AND DISCUSSION

Wild-type *Arta*DPPIII and the E444A variant were successfully produced in *E. coli* BL21 host cells under similar conditions as previously used for other DPPsIII [5, 7]. Purification using Ni-NTA affinity chromatography produced electrophoretically homogeneous protein with a mobility expected for a protein of 78 kDa molecular mass (Figure 1, panel A). In addition, native PAGE showed a band corresponding to a molecular mass of ca. 78 kDa indicating that the protein is present as a monomer in the solution (Figure 1, panel B).

The purified *Arta*DPPIII protein was tested for activity with a series of dipeptidyl-2-naphthylamides clearly demonstrating that the protein possesses hydrolytic activity with Arg_2-2NA being the best substrate (Figure 2, panel A). The presence of $CoCl_2$ in the assay increased the activity ca. three-fold. On the other hand, (2*S*,3*S*)-1,4-bis(sulfanyl)butane-2,3-diol had an adverse effect on the enzymatic activity. Taken together, these features were previously reported for other members of this family and support our hypothesis that *Arta*DPPIII is a typical representative. A more detailed kinetic analysis using Arg_2-2NA as the substrate revealed a Michaelis-Menten parameter of 5.4 ± 0.2 µM, a k_{cat} of 2.16 ± 0.02 s^{-1} and an enzyme efficiency (k_{cat}/K_M) of $4*10^5$ M^{-1} s^{-1} (Figure 2, panel B). *Arta*DPPIII was inhibited competitively by tynorphin exhibiting an inhibition constant of 18 ± 2 nM (Figure 2, panels C and D). In contrast to wild-type *Arta*DPPIII, the E444A variant showed no hydrolytic activity toward Arg_2-2NA. Even at higher concentration of the variant protein and prolonged incubation (> 60 min) no hydrolysis of the substrate could be detected. In summary, all our experimental results confirm that *Arta*DPPIII is a typical representative in terms of the overall properties as well as the kinetic parameters determined for the wild-type enzyme and thus *Arta*DPPIII clearly behaves very similar to the yeast and human enzymes. This is also corroborated by the total lack of activity of the E444A variant. In previous studies, this residue was found to be essential for the peptidase activity and thus indicates that *Arta*DPPIII operates by the same enzymatic mechanism as other members of the M49 family.

Recently, it was claimed that *Arta*DPPIII (termed AFO) oxidizes aflatoxin B_1 (AFB_1) [10]. We have attempted to reproduce this finding but we did not observe any change in the mobility of AFB_1 using thin layer chromatography as previously reported by Cao and coworkers [13]. In an additional experiment, we produced *Arta*DPPIII in the absence of zinc salts in the medium to rule out any adverse effects from the zinc on the putative oxidase activity. This procedure led to reduced dipeptidyl peptidase activity; however, as before

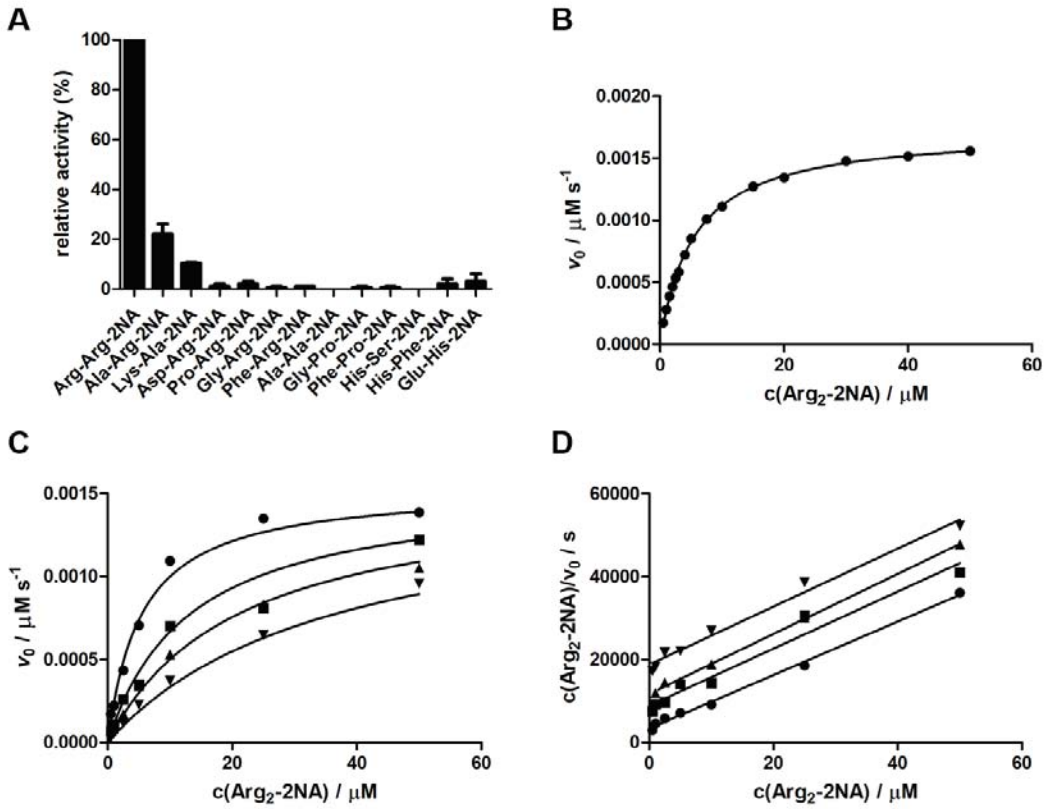

Figure 2. *Arta*DPPIII shows peptidase activity. A) Substrate specificity screen. B) Kinetic analysis of Arg_2-2NA hydrolysis by *Arta*DPPIII. C) Tynorphin inhibition of Arg_2-2NA hydrolysis by *Arta*DPPIII: ● 0 nM, ■ 25 nM, ▲50 nM, and ▼100 nM tynorphin. D) Hanes-Woolf plot of tynorphin inhibition.

no oxidase activity was observed based on thin layer chromatography even after prolonged (> 24 h) incubation (Figure 3).

This prompted us to compare the amino acid sequence of *Arta*DPPIII with other members of the family and to generate a homology model for the protein. In our reconstruction of the phylogenetic relationship among characterized DPPsIII, *Arta*DPPIII grouped with other eukaryotic DPPsIII with the exception of plant DPPsIII. The position in the phylogenetic tree reveals that *Arta*DPPIII is an orthologue of the yeast and human DPPIII exhibiting 36% and 38% identity, respectively (Figure 4). Equally important, the sequence similarity covers the entire length of the sequence (Figure 5). Thus, *Arta*DPPIII does not have an additional domain that could be held responsible for the reported oxidase activity. In keeping with this view, DPPsIII found in the plant kingdom carry an additional enzymatic activity, *i.e.* a pyrophosphohydrolase

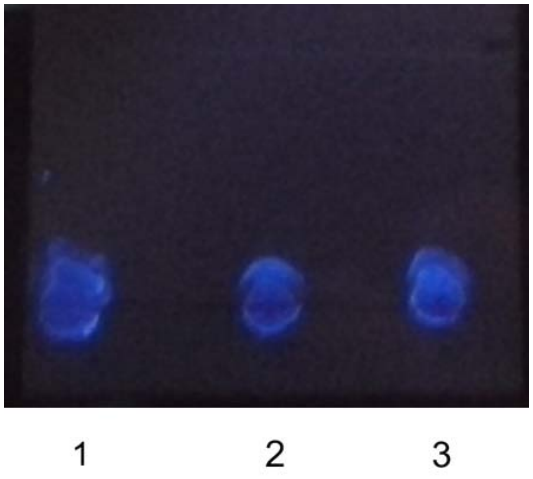

Figure 3. Aflatoxin B_1 shows no change of mobility after 24 hours of incubation with *Arta*DPPIII. (Lane 1: reaction mixture without enzyme, lane 2: reaction with *Arta*DPPIII expressed without additional zinc in the expression culture, lane 3: reaction with *Arta*DPPIII expressed with zinc added to expression culture.)

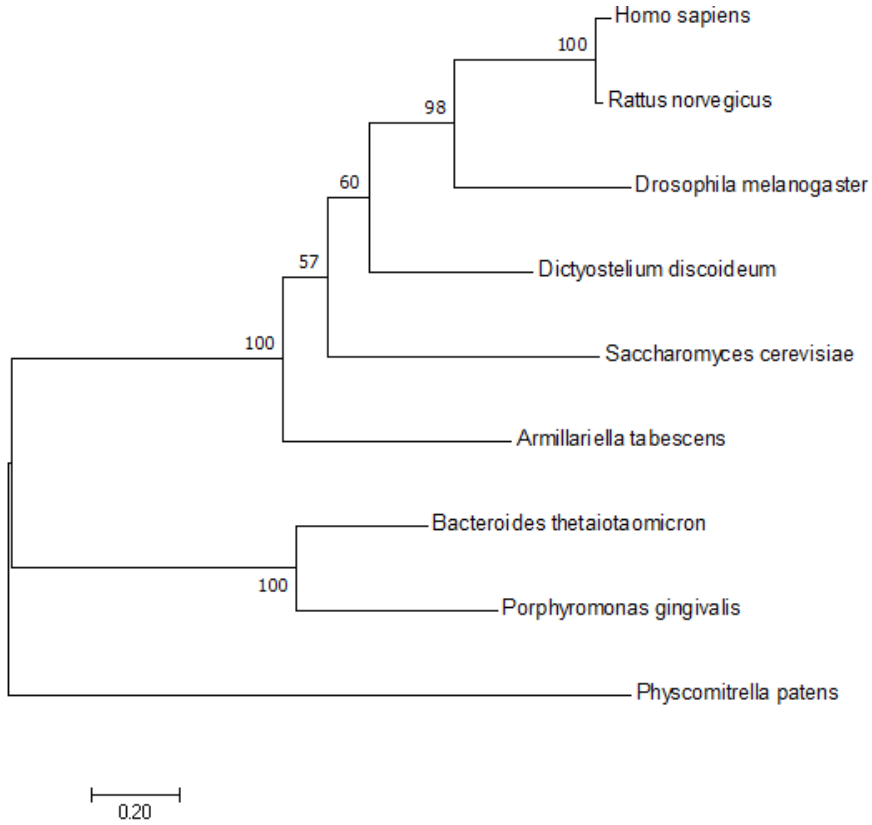

Figure 4. Phylogenetic tree of characterized DPPsIII inferred by using the Maximum Likelihood method, with boostrap values on branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

activity that is linked to a canonical NUDIX domain comprising approximately 200 amino acids at the N-terminus of the protein [3]. Similarly, the DPPIII from *Porphyromonas gingivalis* harbors an additional C-terminal domain comprising ca. 240 amino acids, which presumably adopts an Armadillo-like fold [16]. Although the exact function of this domain is still unknown, it supports the general concept that additional functions depend on the presence of distinct protein domains. In this context, it is also important to note that oxidoreductase activities generally require a redox cofactor, for example a metal ion, nicotinamide or a dithiol-disulfide group, for the handling of electrons that is necessary during the redox reaction. The absence of such a group in *Arta*DPPIII argues strongly against a potential oxidase activity of the enzyme.

Furthermore, the homology model obtained using the yeast and human DPPIII structure, confirmed the two-domain architecture of *Arta*DPPIII in keeping with the observed properties of the enzyme as a peptidase. Since *Arta*DPPIII also features the conserved HEXXXH motif, the amino acids required for metal-binding are also present, again arguing strongly that the enzyme has a canonical and fully functional peptidase activity, as evidenced by the results of our kinetic experiments (Figure 6).

CONCLUSION

Our biochemical study has unambiguously demonstrated that *Arta*DPPIII is a classical member of the metal-dependent peptidase family M49. Further analysis of the amino acid sequence has shown strong similarity to other eukaryotic DPPsIII, in particular the yeast and human enzyme. Multiple sequence alignment and homology modeling support our claim that *Arta*DPPIII has no additional domains that may potentially

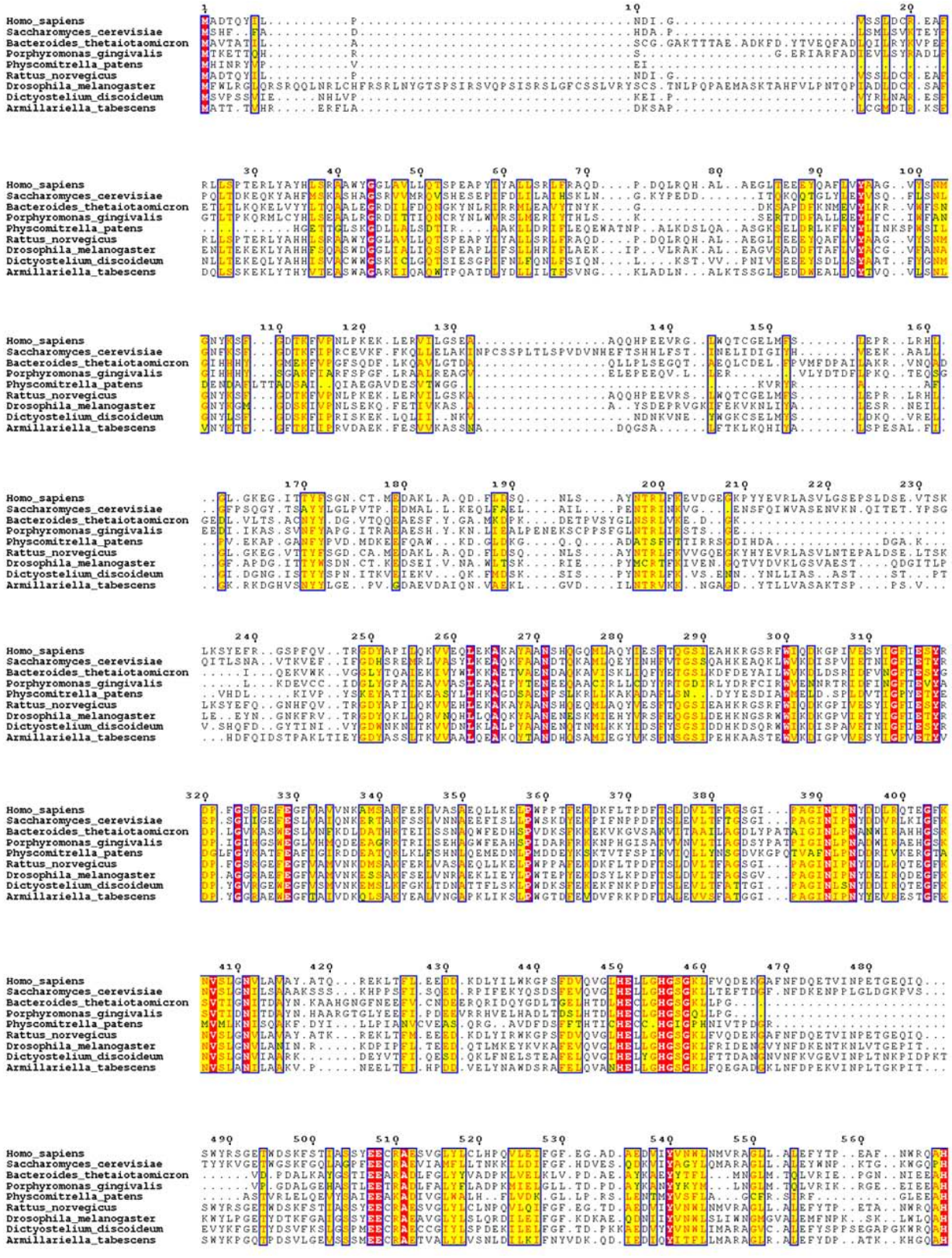
Homo_sapiens
Saccharomyces_cerevisiae
Bacteroides_thetaiotaomicron
Porphyromonas_gingivalis
Physcomitrella_patens
Rattus_norvegicus
Drosophila_melanogaster
Dictyostelium_discoideum
Armillariella_tabescens

Figure 5

Figure 5 continued..

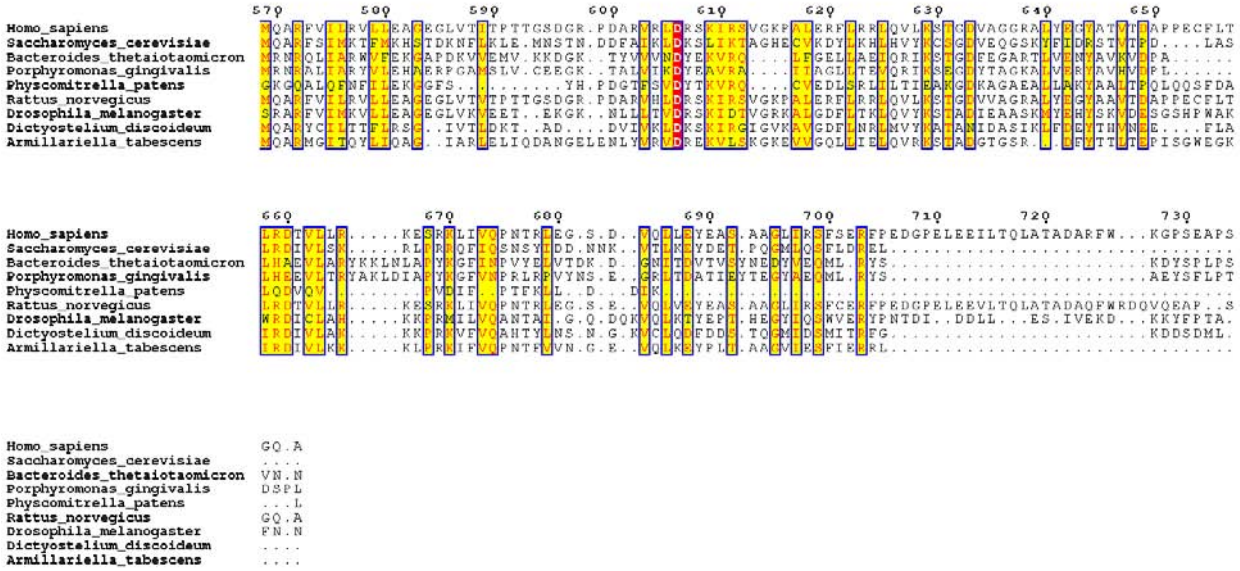

Figure 5. Alignment of characterized dipeptidyl peptidases III. 100% conserved residues are highlighted in red and 70% conserved in yellow. Numbers denote the length of the total alignment.

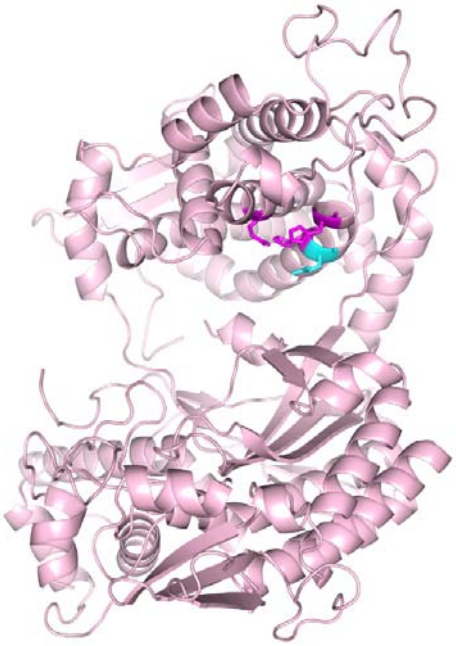

Figure 6. Homology model of *Arta*DPPIII based on yeast DPPIII structure (PDB code 3csk) as the highest scoring template. Active site residues are shown in stick representation, with zinc-binding residues (H443, H448, and E501) in magenta and catalytic glutamate (E444) in cyan. Image was generated using PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

confer additional properties. In accordance with these findings, we could not detect any aflatoxin oxidase activity. Therefore, we conclude that the previously reported aflatoxin oxidase activity is not supported by our study.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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